Evolution, species boundaries, and biogeography of *Spiranthes* (Orchidaceae): Uncoiling Ames's "most perplexing orchid genus in our flora"

By

Matthew Charles Pace

A dissertation submitted in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy

(Botany)

at the

UNIVERSITY OF WISCONSIN–MADISON

2015

Date of final oral examination: 5/22/2015

The dissertation is approved by the following members of the Final Oral Committee:
Dr. Kenneth Cameron, Professor, Botany
Dr. Johanne Brunet, Associate Professor, Entomology
Dr. Eve Emshwiller, Associate Professor, Botany
Dr. Thomas Givnish, Professor, Botany
Dr. Kenneth Sytsma, Professor, Botany

Dedicated to the memory of my parents, Marilyn and Charlie,
both of whom died before I began this manuscript

&

To my partner Shawn, whose love, support, patience, and friendship,
were instrumental in my completing it

Acknowledgements

A dissertation is a profoundly personal yet collaborative product. With this in mind, I wish to acknowledge those who, without their assistance and guidance, my research would have been greatly diminished. I gratefully acknowledge my graduate committee –Dr. Kenneth Cameron, Dr. Johanne Brunet, Dr. Eve Emshwiller, Dr. Thomas Givnish, and Dr. Kenneth Sytsma– in addition to Dr. David Baum, for their invaluable, careful, and thought provoking assistance and advice throughout my time at the University of Wisconsin – Madison. In particular, I thank my advisor, Dr. Kenneth Cameron for accepting me into the Department of Botany, suggesting *Spiranthes* as a research topic, and for providing stipend support for several years through Teaching Assistantships, and NSF funded Dimensions of Biodiversity Research Assistantships.

This project would not have been possible without the generous financial support of many sources: The Wisconsin State Herbarium (WIS); the Davis Research Grant, University of Wisconsin – Madison; the Bayard Long Award, Philadelphia Botanical Club; the John Thomson Award, Botanical Club of Wisconsin; Native Plant Society of New Mexico; the Graduate Student Research Grant, Society of Herbarium Curators; the Graduate Student Research Grant, American Society of Plant Taxonomists; and the Fred Case Grant, Native Orchid Conference. In particular, I would like to thank the donor behind the Flora Aeterna Award of the University of Wisconsin – Madison Botany Department: her generous support (both financially and in terms of inspiring holistic scholars) was simply critical to my field research across North America.

As *Spiranthes* is found across the globe, my research rests on the many shoulders of those who generously collected or sent me material for inclusion in my research, or issued me permits to conduct fieldwork: the Citizen Scientists of the Native Orchid Conference, Renne Boronka (Carnegie Museum of Natural History), Edwin Bridges (Avon Park Air Force Base), Daniel Carter, Alan Cressler, April Daniels (Mark Twain National Forest); Debbie DeBruler (Ouachita National Forest), Lucy Dueck, Jim Fowler, Malcolm Hodges (The Nature Conservancy), Dr. Tsan-Piao Lin (National Taiwan University), Tara Littlefield (KY Natural Heritage Program), Chris Ludwig (VA Natural Heritage), Steve Orzell (Avon Park Air Force Base), Joyce Reddoch, Larry Richardson (Florida Panther National Wildlife Refuge), Daniela Roth (NM State Forestry Division), Dr. Gerardo Salazar (UNAM), Dr. Scott Stewart, Nancy VanAlstine (VA DCR).

I would like to thank Dr. Ricardo Kriebel –post-doc in the Cameron and Sytsma labs– for sharing his incredible knowledge and expertise, which changed the course of my dissertation. Additionally, I thank my fellow graduate students in the Department of Botany for your thought provoking discussions, friendship, and support, in particular Dr. Rafael Arevalo-Burbano, Chloe Drummond, Giovanny Giraldo, Richard Hilleary, Steve Hunter, Daijiang Li, James McDaniel, Kristin Michels, Jeffrey Rose, Alison Scott, Brian Sidoti, Dr. Daniel Spalink, John Zaborsky, and Alejandro Zuluaga.

Thank you, to Dr. Barbara Thiers, Dr. Patricia Holmgren, Dr. Noel Holmgren, Dr. Robert Naczi, Dr. Karl Niklas, and Robert Dirig for your ceaseless support. You inspire me to be the best scientist I can, and I appreciate beyond measure the confidence that you place in me.

Many thanks to Mark Wetter, Senior Academic Curator at WIS, and the collections managers and directors of the following herbaria for the monumental task of coordinating loans of ca. 8000 herbarium specimens: AMES, BH, CHICO, CLEM, F, FLAS, FSU, JEPS/UC, LSU, MO, NY, NYS, RSA, SEL, UARK, UMEX, US, USF, and WILLI. Thank you to the UW Madison Botany Greenhouse staff –Dr. Mo Fayyaz, Cara Streekstra, James Adams, and Patricia Wirth– for caring for my living collections of *Spiranthes*, and to Sarah Friedrich for producing truly excellent illustrations to aide in the description of new species.

I would like to thank Norm Hirschy for translation of protologues from German into English; Jimmy Shilling and Darrell Baker for providing lodging while I conducted field work in DE and NJ, and Andrew Hemme (Longwood Gardens) for assistance in collecting plants in DE.

Shawn Martella was instrumental in locating many populations of *Spiranthes* throughout IN, IL, NJ, NM, NY, and WI. The impact of his unyielding support during my time in graduate school is immeasurable.

Lastly, I thank my parents, Marilyn and Charlie Pace. My father died just before my acceptance to the Department of Botany, and my mother died while I was in my third year of study. Thank you for your love and support. In particular, thank you for teaching me to grow marigolds and tomatoes when I was a young child, thereby planting the seed of botany within me, a seed which has culminated in the production of this manuscript.

Thank you.

***Abstract*—**The orchid genus *Spiranthes* is simultaneously a challenging but exciting group in which to study evolutionary biology and biogeography. Unusually for the family, *Spiranthes* is distributed across the globe, most species-rich in North America, a pollinator generalist with many species of bumble bees, and displays 'weedy' tendencies such as a requirement of frequent habitat disturbance for persistence. The genus is composed of ca. 35 species, with the majority of them placed within four species complexes. Species within these complexes display high levels of between-species morphological uniformity in addition to within-species morphological variability. The general attitude held by many botanists toward the genus is summed up by a quote from O. Ames: "*Spiranthes* is the most perplexing orchid genus in our flora. It is the least understood … It is a genus that repays intensive observation in the field and prolonged contemplation in the herbarium." In the present work, we present molecular phylogenetic, morphological, and biogeographic data which help to disentangle species boundaries within, and the evolutionary biogeographic history of, this confounding genus. Our molecular phylogenetic hypothesis is the most expansive to date for *Spiranthes* in terms of the number of samples and in-group species sampled, thereby providing important context for addressing evolutionary relationships, character evolution, and biogeographic history. Within the *Spiranthes cernua* species complex, we resolve the 117 year question regarding the identity of *Spiranthes odorata* and *Spiranthes triloba*, describe two new cryptic species from Central Florida and the Appalachian Highlands, and address species boundaries and questions of hybridization regarding *Spiranthes cernua*. Within the Western North American Cordillera species complex, we explore questions regarding the role of hybridization as a source of speciation and morphological diversity, with a subsequent re-alignment in the identity of some species. Our dated biogeographic analyses are the first to address the geographic and temporal origins, and migratory expansion, of this global genus. Our research finds that the North American Geologic Coastal Plain has been a major center of evolution for *Spiranthes*, and that it has served as a center of diversity from where multiple lineages have independently dispersed or migrated, including the Appalachian Highlands and Interior Lowlands of North America, the Caribbean, Eurasia, and Central American and the Sierra Madre Occidental of Mexico.

BIOLOGICAL INTRODUCTION TO THE GENUS *SPIRANTHES* (ORCHIDACEAE)

Spiranthes –the ladies' tresses– is a genus of primarily white-flowered, north temperate, terrestrial Spiranthoid orchids within the *Spiranthes* clade of the subtribe Spiranthinae (Orchidoideae: Cranichideae) (Salazar et al. 2003; 2009). Here, we provide a biological and ecological introduction to the genus.

Geographic distribution & biogeographic affinities. Worldwide, there are approximately 34 species of *Spiranthes*: 26 in North America, 2 in Mexico & Central America, 1 in the Caribbean, 2 in Eurasia & North Africa, and ca. 1–5 spp. across East Asia, Australasia, and western Oceania. *Spiranthes* is one of only a handful of orchid genera to display a near circumglobal distribution, and it is the only non-endemic orchid genus to be most species-rich in North America. A majority of North American species are restricted to the continent, however *S. romanzoffiana* is also found in Ireland and Scotland. *Spiranthes torta* is a primarily Caribbean species, but reaches its northern limit in Florida. Only *S. graminea* and *S. nebulorum* are Mesoamerican, restricted to the Sierra Madre Occidental of Mexico, and the central mountains of Honduras and Nicaragua. Within North America, there is a subdivision of the genus: a group of species distributed throughout the Western Cordillera systems, and an Eastern North American group that is found east of the Rocky Mountains. The species that comprise these subgroupings often have overlapping distributions, and many species frequently co-occur, or occur in contiguous habitats. Many species are found in multiple North American physiographic divisions, and a few species are especially widely distributed: e.g., *S. romanzoffiana* is found from the Arctic Circle to southern Arizona, whereas *S. sinensis* s.l. is distributed from southern Siberia to Tasmania. On the opposite end of the distributional spectrum are a few species

endemic to only a handful of sites: *S. igniorchis* in central Florida (Chapter 2), and *S. delitescens* in southern Arizona. (See Chapter 6 for an analysis of *Spiranthes*' biogeographic history.)

Ecology. Despite the wide geographic distribution of genus, the general habitat preferences of most *Spiranthes* are quite similar: short-statured, graminoid, frequently disturbed, damp, and open and sunny. Some species appear to display an affinity for alkaline soils, however this is not a requirement, and some species are labile in their pH tolerance. *Spiranthes casei* may be the most restrictive in terms of pH, growing in strongly acidic *Vaccinium* L. and *Pteridium* Gled. ex Scop. barrens; alternatively, *S. lucida* is limited to areas underlaid with limestone bedrock or with calcareous fens. Soil chemistry and the availability of nitrogen and phosphorus have been shown to be important factors relating to the distribution of S. *spiralis* (Hill et al. 2004; Sinker et al. 1985; Willems 2006). It is unclear if similar nutrient availability impacts the distribution or habitat affinities of other *Spiranthes*. Although some species do display affinities for certain pH limits, many species –e.g., *S. cernua* s.s., *S. lacera*, *S. romanzoffiana*, *S. spiralis*, *S. vernalis*– occur in acidic, neutral, and calcareous soils. It may be that rather than strict pH affinity, certain soil conditions limit the possibility of woody plant encroachment. Additionally, other abiotic and biotic stresses/disturbance agents (e.g., fire, grazing) typically occur in the same site, and there may be a complex relationship between different types of stresses that lead to the right habitat conditions to be present for a given species of *Spiranthes*. Research focused on the mycorrhizal associations which may be facilitating or limiting these ecological distributions is mostly lacking and is clearly needed.

Spiranthes are small plants which are easily overtopped by taller, often woody, taxa. As such, disturbances are needed to maintain viable populations. Research into the ecologically-related population dynamics of most species is wanting; however it appears that populations of

Spiranthes can go through cycles of non-flowering vegetative growth or subterranean dormancy for many years in-between periods of flowering (Jacquemyn et al. 2007, Reddoch & Reddoch 2009; Wells 1967; Pace pers. observ.). For many species –including *S. longilabris*, *S. magnicamporum*, and *S. triloba*– fire appears to be the most important abiotic disturbance force that enables reproduction and persistence on the wider landscape. Other species –such as *S. cernua* s.s., *S. praecox*, *S. sinensis* s.l., and *S. vernalis*– react favorably to fire, but also respond well to general disturbances (including anthropogenic disturbances) such as flooding, soil disturbance, mowing, and grazing. *Spiranthes cernua* s.s. is particularly interesting as many known extant populations of this species along the Coastal Plain are found in mowed roadside or power-line right-of-ways, where individual stems can number in the hundreds (Pace pers. observ.). It is unknown, but human mediated disturbances may have partially replaced natural disturbances such as fire and/or large mammal movement (e.g., the extirpation of *Bison bison* L. across most Eastern North America, and the extinction of the Eastern North American *Cervus canadensis* Erxleben subsp. *canadensis*). Similarly, British populations of *S. spiralis* appear to require grazing by rabbits, sheep, and/or goats to maintain persistence in many locations (Jacquemyn & Hutchings 2010).

Pollinator relationships and potential hybridization. *Spiranthes* is pollinated by bees: *Apis mellifera* and *Megachile* spp. in Asia, and *Bombus* spp. in Europe and North America (Catling 1982, 1983; Sheviak 1982). Various Halictidae also pollinate specific species in both the Old and New World; *S. lucida* is the only species in the genus to be solely pollinated by Halictidae and has a unique column morphology that corresponds to this pollination relationship. The pollinator relationships of *Spiranthes* are incompletely known; many species –e.g., *S. longilabris*, *S. porrifolia*, *S. praecox*, *S. tuberosa*– have no known pollinator. Unusually for Orchidaceae –a

family that is often cited as an example of the evolution of specific pollinator relationships between one orchid species and one pollinator species– many species of *Spiranthes* have overlapping pollinator relationships with multiple numbers of bees. For example, *Bombus fervidus* pollinates *S. cernua, S. diluvialis, S. magnicamporum, S. odorata,* and *S. romanzoffiana*, whereas *B. impatiens* pollinates *S. cernua*, *S. ochroleuca, S. odorata,* and *S. vernalis*. These overlapping pollinator relationships, coupled with the sympatric distributions of many species, have led to the hypothesis that hybridization is a pervasive force within the genus (Sheviak 1982, 1991, 2012, 2013). Prior to the work we present in Chapters 3 and 4, only *S. dilvualis* (southern and mid-Rocky Mountains) and *S. hongkongensis* (endemic to Hong Kong) had supportive genetic data verifying the role of hybridization in their evolution. The phylogenetic (Chapters 3, 4, 5) and biogeographic (Chapter 6) perspectives we provide are a first step toward understanding the role of hybridization within *Spiranthes*.

Catling, P.M. 1982. Breeding systems of northeastern North American *Spiranthes* (Orchidaceae). Canadian Journal of Botany – Revue Canadienne de Botanique, 60(12): 3017–3029.

Catling, P.M. 1983. Pollination of northeastern North American *Spiranthes* (Orchidaceae). Canadian Journal of Botany – Revue Canadienne de Botanique, 61(4): 1080–1093.

Hill, M.O., C.D., Preston, and D.B. Roy. 2004. *PLANTATT. Attributes of British and Irish Plants: Status, Size, Life History, Geography and Habits*. Centre for Ecology and Hydrology, Huntingdon, UK.

Jacquemyn, H., R. Brys, M. Hermy, and J.H. Willems. 2007. Long-term dynamics and population viability in one of the last populations of the endangered *Spiranthes spiralis* (Orchidaceae) in the Netherlands. Biological Conservation, 134: 14–21

Jacquemyn, H. and M.J. Hutchings. 2010. Biological Flora of the British Isles: *Spiranthes spiralis* (L.) Chevall. Journal of Ecology, 98(258): 1253–1267.

Reddoch, J.M., and A.H. Reddoch. 2009. Phenology, Population Dynamics, and Flowering Dynamics of Case's Ladies'-tresses, *Spiranthes casei* var. *casei* (Orchidaceae), in Ottawa, Ontario. The Canadian Field-Naturalist, 123: 19–31.

Salazar, G.A., M.W. Chase, M.A. Soto, and M. Ingrouille. 2003. Phylogenetics of Cranichideae with emphasis on Spiranthinae (Orchidaceae, Orchidoideae): evidence from plastid and nuclear DNA sequences. American Journal of Botany, 90: 777–795.

Salazar, G.A., L.I. Cabrera, S. Madriñán, and M.W. Chase. 2009. Phylogenetic relationships of Cranichidinae and Prescottiinae (Orchidaceae, Cranichideae) inferred from plastid and nuclear DNA sequences. Annals of Botany, 104: 403–416.

Sheviak, C.J. 1982. Biosystematic study of the *Spiranthes cernua* complex. Bulletin of the New York State Museum Science Service, Bulletin Number 448.

Sheviak, C.J. 1991. Morphological variation in the compliospecies *Spiranthes cernua* (L.) Rich.: ecologically-limited effects of gene flow. Lindleyana, 6(4): 228–234.

Sheviak, C.J. 2012. A new and rare *Spiranthes* from the Sierra Nevada. The Native Orchid Conference Journal, 9(4): 1–6.

Sheviak, C.J. 2013. A ctyogeographic survey of *Spiranthes romanzoffiana* Cham. and its taxonomic implications. The Native Orchid Conference Journal, 10(2): 1–31.

Sinker, C.A., J.R. Packham, I.C. Trueman, P.H. Oswald, F.H. Perring, and W.V. Prestwood. 1985. Ecological Flora of the Shropshire Region. Shropshire Trust for Nature Conservation, Shrewsbury, UK.

Wells, T.C.E. 1967. Changes in a Population of *Spiranthes spiralis* (L.) Chevall. at Knocking Hoe National Nature Reserve, Bedfordshire, 1962-65. Journal of Ecology: 55(1): 83–99.

Table of Contents

Chapter 1: Reinstatement, redescription, and emending of *Spiranthes triloba* (Orchidaceae): Solving a 117 year old cryptic puzzle 1–53

Dichotomous Key 22–23

Appendix 24–30

Figures 34–53

Chapter 2: *Spiranthes igniorchis*, a new and rare cryptic endemic of the Central Florida Ridges and Osceola Plain fire-maintained subtropical grasslands 54–75

Figures 69–75

Chapter 3: Cutting Gordian's Knot—Molecular phylogenetic, morphological, and biogeographic evidence supports dividing Spiranthes cernua (Orchidaceae) into two morphologically variable species and two geographically specific natural hybrids 76–139

Figures 110–139

Chapter 4: Intrinsic nature or sporadic individuals? Assessment of the frequency and evolutionary significance of hybridization in the Western North American Cordillera *Spiranthes* clade (Orchidaceae) 140–200

Figures 175–200

Chapter 5: Phylogenetics, species boundaries, and character evolution in *Spiranthes* (Orchidaceae) 201–280

Figures 239–260

Supplementary figures 261–280

Chapter 6: The biogeographic evolutionary history of *Spiranthes* (Orchidaceae) reveals repeated speciation within and dispersal from the North American Geological Coastal Plain, an underappreciated biodiversity hotspot 281–339

Figures 311–339

CHAPTER 1

Reinstatement, Redescription, and Emending of *Spiranthes triloba* (Orchidaceae): Solving a 117 year old cryptic puzzle.

Matthew C. Pace and Kenneth M. Cameron

Abstract*—Spiranthes* (Orchidaceae) is a genus of primarily North American orchids that have traditionally presented numerous taxonomic challenges. Many species display extreme intra-species morphological variation and/or inter-species morphological similarities, such that cryptic speciation may be common; *Spiranthes odorata* is a species that encompasses these challenges. Molecular phylogenetic, morphological, field, and herbarium research concerning the delimitation of *Spiranthes odorata* has led to the reinstatement and redescription of *Spiranthes triloba*, a name alternately synonymized with *Spiranthes cernua* and *Spiranthes odorata* for more than a century. While lost under synonymy, plants matching the morphology of *Spiranthes triloba* were ambiguously described as *Spiranthes* ×*itchetuckneensis* and *Spiranthes* ×*folsomii*. Although the species has a descriptive specific epithet, the labellum of the holotype is decidedly not tri-lobed; *Spiranthes triloba* is redescribed and emended here so as to reflect its actual morphology. *Spiranthes triloba* is restricted to frequently burned calcareous prairies and flatwoods of Florida and likely warrants protection at the state level. A key is provided to distinguish this species from other *Spiranthes* found in Florida.

Keywords —Florida, herbaria, John Kunkel Small, species delimitation, *Spiranthes odorata*, taxonomic confusion.

Spiranthes (Orchidaceae), the ladies' tresses orchids, is the second largest orchid genus in North America north of Mexico with 23 currently recognized species (Sheviak and Brown 2002). In the context of orchid species-richness (e.g., *Bulbophyllum*: ca. 2,000 spp., *Epidendrum*: ca. 1,500 spp.), *Spiranthes* is relatively small in size with just ca. 32 species worldwide. Yet many *Spiranthes* taxa have complicated nomenclatural and taxonomic histories (e.g., *Spiranthes sinensis* (Pers.) Ames has 3 homotypic synonyms and 47 heterotypic synonyms); more than a few orchidologists have been mired in confusion over this genus (e.g., Ames 1947). Difficulties in species delimitation are a result of, and response to, the presence of small flowers (3–14 mm long) with few discrete and/or unique characters, intra-species morphological plasticity, inter-species morphological similarities, cryptic speciation, widely disjunct populations, cycles of taxonomic lumping and splitting, confusion over types, the acceptance of ubiquitous hybridization between species, and a general lack of systematic studies across the full geographic distribution of any given *Spiranthes* taxon.

A level of clarity has been accomplished in understanding species diversity within *Spiranthes* through the work of Sheviak and Catling in more accurate and thorough species description (Catling and Catling 1988; Catling and Sheviak 1993; Sheviak 1973, 1984, 1989, 1990), and defining specific species complexes (Catling 1980; Sheviak 1982, 1991). However many taxa remain poorly understood, and some morphological characters may have been overlooked or underutilized. Furthermore, some authors have arguably added to the confusion within the genus through an overreliance on cytology to infer species-status and a presumption of pervasive hybridization (e.g., *Spiranthes romanzoffiana* Cham. [Sheviak 2013]), and the prolific publication of names that likely represent aberrant individuals or populations (e.g., *Spiranthes eatonii* Ames ex P.M. Br. (Brown 1999a)) and/or are taxonomically illegitimate (e.g., *Spiranthes*

×*australis* P.M. Br. nom. illeg. (Brown 1999b)). Molecular phylogenetic evidence for the species-status of most *Spiranthes* has just begun to be presented (e.g., Dueck and Cameron 2008), but this is likely to change as more studies are published. Since many putative species of *Spiranthes* exhibit marked morphological variation, a clearly stated species concept is critical to future taxonomic work in *Spiranthes*: we maintain that an integrated history-bound phylogenetic species concept (Baum and Donoghue 1995; Dayrat 2005) in which monophyly is emphasized – with supporting morphological and ecological data– should be implemented when addressing questions regarding species-status in this genus.

One species with a long history of confusion due to many of the issues presented above is *Spiranthes odorata* (Nutt.) Lindl. (Fig. 1, 2). Originally described as "the largest and finest *Spiranthes* in the United States" under the basionym *Neottia odorata* Nutt. (Nuttall 1834), it was diagnosed based on its leafy character, long roots, and wavy subfimbriate labellum margin. As indicated by the specific epithet "*odorata*," the flowers of this species are intensely fragrant, presenting a complex and heady mixture of vanilla, jasmine, and coumarin that can be discerned from several meters away from the plant. When described, the habitat of this species was given as "*Hab. in Carolina; ripis lutosis inundatis fluvii Neuze, prope Newbern*." Although his formal Latin description and English discussion of *N. odorata* could be considered quite thorough for the time, Nuttall did not designate a type nor did he include a drawing of his new species.

Lindley (1840) transferred *Neottia odorata* to *Spiranthes*, adding details concerning the morphology of its callosities (basal labellum nectar glands) and designated Drummond 333 as the type (this specimen has yet to be found). As with many other taxa in *Spiranthes*, the distinctiveness and morphological boundaries of *S. odorata* came into question within a few decades of its description. Small (1913) synonymized *S. odorata* under *Spiranthes cernua* (L.)

Rich; doubts as to the status of *S. odorata* were shared by Ames (1921), who was "convinced" *S. odorata* should be synonymized with *S. cernua*. Ames (1921) also noted that specimens of *Spiranthes ochroleuca* (Rydb.) Rydb., a denizen of the Appalachian Mountains and Great Lakes Basin, were often misidentified as *S. odorata* due to morphological similarities between those two species. Correll (1940) subsumed *S. odorata* into *S. cernua* as *S. cernua* var. *odorata* (Nutt.) Correll, a position continued by Luer (1972). Gleason and Cronquist (1991) implausibly maintained both the varietal status of var. *odorata* and the species status of *S. odorata* simultaneously. Although most current authors and floristicians treat *S. odorata* as a distinct species, the combination *S. cernua* var. *odorata* survives in the horticulture trade as the name for the widely available ornamental cultivar 'Chadds Ford', originally grown and distributed by the Mt. Cuba Center. However, the exact identity of this cultivar is itself in question: it is uncertain if it is a cultivar of *S. cernua* or *S. odorata*. Despite confusion and differences of opinion as to the identity of *S. odorata*, Fernald (1950), Luer (1975), Sheviak (1982), and Sheviak and Brown (2002) maintained it as a distinct species worthy of recognition, though its exact boundaries in relation to *S. cernua* –morphological delimitation, geographic range, putative hybridization– remained unclear. The circumscription of *S. odorata* has been further hindered by a lack of clarity over the species-boundaries of *S. cernua* and the taxa of the so-called *S. cernua* complex. The designation by Catling in 1980 of an unattributed PH specimen from the type locality, with an original label likely in Nuttall's handwriting, as the lectotype for *Neottia odorata* added some clarity to the situation (PH barcode 00019246).

Although the lectotype of *S. odorata* is a large fluvial plant with the lowest wholly visible flower (the second lowest/oldest flower of the inflorescence) 13.4 mm in length (based on lateral sepal length), large leafy cauline bracts, an acuminate labellum apex, and conical, incurved

callosities 1.3 mm in length, the limits of *S. odorata* have come to include plants that do not easily fit within this ideal. Specifically, small-statured plants from Florida, commonly with green labella and often found growing in flatwood prairies, have been included as part of the morphological variability expressed by *S. odorata*. Among citizen scientist, local orchid enthusiasts, and conservation agencies in Florida, these plants are colloquially known as "green-lipped *S. odorata*" or "dwarf *S. odorata*" (Menk 2007; Stewart and Richardson 2008; here referred to as "green-lipped *S. odorata*"). Dueck and Cameron (2007) and Dueck et al. (2014) found evidence for the non-monophyly of *S. odorata*; however the morphological characters or limits and taxonomic status of these disparate *S. odorata* clades were not explored.

In the course of a systematic revision of the genus incorporating expanded phylogenetic and morphological sampling and analyses by M.C. Pace, it became apparent that a formal taxonomic and morphological review of both "green-lipped *S. odorata*" and *S. odorata* sensu stricto was warranted. This would provide long-sought clarity in regard to species delimitation for citizen scientists and local naturalists, orchidologists, systematists, conservation organizations, federal and states agencies, and floristicians.

MATERIALS AND METHODS

Taxonomic Sampling—Specimens of all *S. odorata* and *S. cernua*, were carefully reviewed from AMES, BH, CLEM, F, FLAS, FSU, LSU, MO, NY, NYS, SEL, US, and USF (acronyms follow Thiers [continuously updated]). Type specimens of all *Spiranthes* recorded from Florida (the primary area of interest) were reviewed, either through the Global Plants web portal (http://plants.jstor.org/) or in person. Particular attention was given to the types and descriptions of *S. odorata*, *S. cernua*, and names currently synonymized under them.

Fieldwork based on georeferenced herbarium specimens was conducted throughout Florida from 2012–2014, focusing on "green-lipped *S. odorata*." Samples were collected for herbarium and spirit vouchers, morphological measurements, and phylogenetic reconstructions. Live plants were collected and grown at the University of Wisconsin – Madison botany greenhouses for additional research.

Morphometrics—Twenty-nine herbarium specimens identified by the first author as *S. triloba* and 90 specimens of *S. odorata* from BH, F, FLAS, FSU, LSU, MO, NY, NYS, SEL, US, and USF were measured for the following characters: lateral sepal length, widest-point lateral sepal width, flower length in profile, labellum length, plant height, basal leaf length, and widest-point basal leaf width (fig. 3). Additionally, categorical variables were assigned for taxon, state of collection, and labellum surface texture. These characters were chosen because they are easily measured on most pressed herbarium specimens of *Spiranthes* and are also of use to botanists in the field. Specimens were included from throughout the ranges of the included taxa, and included the holotype or lectotype specimens of *Spiranthes triloba*, *Spiranthes odorata*, *S. ×itchetuckneensis*, and *S. ×folsomii*. To analyze and understand the morphological variability of these species, principal components analyses (PCA) were performed and visualized in RStudio (2012) using rehydrated labella from herbarium samples.

Phylogenetic Methods—Phylogenetic analyses incorporated and expanded upon the sampling of Dueck et al. (2014). Only species of *Spiranthes* found in the southeastern United States were included in our current analyses. *Deiregyne diaphana* (Lindl.) Garay, *Dichromanthus cinnabarinus* (Lex.) Garay, *Sacoila lanceolata* (Aubl.) Garay, and *Schiedeella llaveana* (Lindl.) Schltr. were included as outgroups based on the tribal and sub-tribal work of Salazar et al. (2003). For new accessions, 3–4 unopened floral buds or ca. 1 cm sq^2 of leaf tissue

were collected and silica gel-dried for later extraction of total gDNA. IBI Plant Isolate Kits (Peosta, IA) were used for all newly collected samples. Samples of *S. triloba* were collected throughout Florida by the first author based on herbarium research; additional samples of other species, particularly *S. cernua*, *S. odorata*, and *S. magnicamporum* were also included. In total 8 samples of *S. cernua* (including *S. parksii*), 2 samples of *S. ochroleuca*, 3 samples of *S. ovalis*, 13 samples of *S. triloba*, 10 samples of *S. odorata*, 10 samples of *S. magnicamporum*, and 2 samples of *S. longilabris* (representing the *S. cernua* species complex, in addition to 21 other in-group samples) were used (fig. 4). All accessions were amplified for the plastid gene regions *mat*K, *ndh*J, *trn*L-F intron, *trn*S-fM, and *ycf*1, nuclear ribosomal ITS, and the low-copy nuclear region *ACO*. Amplification of loci and cycle sequencing followed standard protocols based on annealing temperatures of the associated primers. PCR products were purified using ExoSap-It (Cleveland, OH). Cycle sequencing products were cleaned using Agencourt CleanSeq (Beverly, MA) magnetic beads. Direct sequencing of cleaned cycle sequencing products was performed at the University of Wisconsin – Madison Biotechnology Center. Resulting sequences were edited and aligned using software modules available in Geneious 7.1 including MUSCLE. Ambiguities were coded with standard IUPAC-IUB Symbols for Nucleotide Nomenclature (Cornish-Bowden 1985). If samples failed to amplify after repeated attempts for a given locus, they were coded as missing data.

The data were analyzed as 1) combined plastid data, 2) combined nuclear data, and 3) total evidence combined nuclear + plastid data. Analyses were performed under Bayesian Inference (MrBayes on XSEDE (3.1.2)) implemented through CIPRES Portal V. 3.3 (Miller et al. 2010). Based on Dueck et al. (2014), the GTR+G (general-time-reversible with a gamma distribution) model was implemented for all datasets and partitions. Analyses were run for

10,000,000 generations, with a sample frequency of 100,000, nruns = 2, nchains = 6, temp = 0.2, and a burnin of 500,000. Phylogenetic inference of the 50% majority-rule consensus tree was constructed using the "sumt" option based on the remaining trees. The topology of this tree was visualized as trees in FigTree (Rambaut 2014).

.

RESULTS

Taxonomy—Review of herbarium and field specimens indicates that there are several morphological characters that quickly and unequivocally distinguish "green-lipped *S. odorata*" from *S. odorata* s.s. Furthermore, individuals of "green-lipped *S. odorata*" clearly do not match the lectotypes of *Neottia* (*Spiranthes*) *odorata* or *Ophrys* (*Sprianthes*) *cernua* L. (fig. 5). Instead, plants of "green-lipped *S. odorata*" match the holotype of *Gyrostachys triloba* Small (*Spiranthes triloba* (Small) K. Schum.) (fig. 6), a name that has alternately been synonymized under both *S. cernua* and *S. odorata* for 117 years. Plants previously called "green-lipped *S. odorata*" should instead be identified as *Spiranthes triloba*; for the remainder of this article "green-lipped *S. odorata*" will be referred to as *S. triloba*. Curiously, these plants also match the holotype of *Spiranthes* ×*itchetuckneensis* P.M. Br., but not all of its paratypes. The plants also correspond to a portion of the *Spiranthes* ×*folsomii* P.M. Br. holotype (see discussion).

Morphometrics—The resulting PCA of 119 measured *S. odorata* and *S. triloba* herbarium specimens produced two well-defined groups that corresponded to their *a priori* taxon identification (*S. odorata*, *S. triloba*) (Fig. 7). Although a few individuals of each species approach the other, and both are fairly variable in all characters measured, the two species are on the whole discrete. The presence of a patch of papillae on the upper labellum surface of *S. triloba*

–vs. a glabrous labellum for *S. odorata*– unambiguously distinguishes individuals that might be otherwise misidentified (fig. 14).

Phylogenetic Analysis—The combined nuclear+plastid Bayesian majority-rule tree (Fig. 8) supports and strengthens the conclusion of Dueck and Cameron (2007) and Dueck et al. (2014): that *S. odorata* as currently defined is strongly non-monophyletic. A strongly supported monophyletic clade of *S. odorata* s.s. is sister to most of the other species found in southeastern North America (minus *S. lucida*, *S. praecox*/*S. sylvatica* and *S. tuberosa*); a distantly related and strongly supported *S. triloba* clade is sister to *Spiranthes magnicamporum* Sheviak. The included individual sample of *S. cernua* from Florida clustered within the clade of other *S. cernua* (including *Spiranthes parksii* Correll) and is clearly distinct from both *S. triloba* or *S. odorata*. Additionally, the sample of *S. cernua* 'Chadds Ford' is embedded within the *S. cernua* clade. Nuclear *ACO* and nrITS did not yield sufficient phylogenetic resolution among otherwise strongly supported clades of species for the taxa of interest (data not shown); however *S. triloba* was never recovered within *S. cernua*, *S. longilabris*, *S. odorata*, or *S. ovalis*. Though additional nuclear loci may provide evidence of hybridization, the data we present here does not support that hypothesis, and we feel confident in stating *S. triloba* is not of hybrid origin. Samples from the general type localities of *S.* ×*itchetuckneensis* and *S.* ×*folsomii* were recovered within the *S. triloba* clade.

DISCUSSION

Taxonomy—Spiranthes odorata as currently defined is clearly non-monophyletic. Plants previously referred to as "green-lipped *S. odorata*" should instead be named *S. triloba*, first described more than 100 years ago. *Spiranthes triloba* is here formally reinstated as a valid

species distinct from *S. odorata* and *S. cernua*, or any other currently accepted species of *Spiranthes*. In order to more satisfactorily reflect its actual morphology based on multiple specimens studied in and ex situ, *S. triloba* is here formally redescribed and emended below. Chief among the morphological characters used to indisputably separate *S. triloba* from *S. odorata* and *S. cernua* is a labellum that is lime-green in color at anthesis but matures to a sunny yellow and has a dense patch of papillae on its adaxial surface. Based on observations in the field and greenhouse this color change is unrelated to removal or depositing of pollinaria, or the age of the individual flower or plant. In some plants individual flowers open as lime-green and change to yellow within 2–5 days, whereas in other plants the labellum remains green until just before senescence.

Spiranthes triloba was described by Small (1898) under *Gyrostachys* based on a single collection of multiple stems from Fort Meyers, Florida (Simpson s.n.; NY barcode 00008874, fig. 6). In the description of this new species Small stated "the crucial character that separates it from all of its relatives is the three-lobed lip" (Small 1898). On the holotype sheet there is a pencil drawing depicting a labellum with small notches or lobes. However this supposedly definitive character is completely lacking from any of the many flowers present on the physical specimen or accompanying slide, adding to the mystery surrounding this species. Furthermore, no other herbarium specimens or live plants were found to display a three-lobed labellum. The labella of many *Spiranthes* are small, delicate, and easily torn during dissection. Perhaps Small based his description on an aberrant flower not included in the herbarium sheet, discarded after dissection, or damaged in some way. Alternatively, the reflextion and curling of the labellum is such that it can sometimes appear to be lobed. However upon closer examination it is evident that the lip is in no way lobed. Despite the misleading specific epithet, the name *Gyrostachys*

triloba must be honored as the basionymn for the "green-lipped *S. odorata*" and given priority as *S. triloba*.

Gyrostachys triloba was transferred to *Spiranthes* by Schumann (1898) without additional observations or comments, whereas Small (1913) transferred it to *Ibidium* Salisb. (=*Spiranthes*), and it was transferred by House (1920) to *Triorchis* Millán (=*Spiranthes*). *Spiranthes triloba* was first synonymized under *S. odorata* by Ames (1905), a position followed by Luer (1972, 1975). In contrast, *S. triloba* was synonymized under *S. cernua* by Small himself (1933), a stance followed by Correll (1950). Sheviak via a 1976 annotation label on the *S. triloba* holotype sheet, identifies that specimen as *S. odorata*, yet later synonymized the name under *S. cernua* (Sheviak 1982). Catling, writing on a large sheet attached to the holotype of *S. triloba* dated 1980, takes a middle-of-the-road approach: "[this name] is definitely synonymous with either *S. cernua* or *S. cernua* var. *odorata* [= *S. odorata*] but its definite placement with either will have to wait until the two varieties have been studied in detail in the southwest and their most constant distinguishing features have been elucidated." Our research presented here answers this call from Catling, supporting *S. triloba* as an unequivocally molecularly and morphologically distinct species from both *S. cernua* and *S. odorata*.

Phylogenetics—Our molecular data show that *S. odorata* s.s. is sister to most of the other *Spiranthes* species found in the southeastern United States, and is not closely related to any member of the *S. cernua* species complex (s.s. or s.l.) (Fig. 8), with which it has been previously placed (Sheviak 1982, 1991; Sheviak and Brown 2002). *Spiranthes odorata* s.s. includes populations across the North American Atlantic and Gulf Coastal Plain, from mid-Virginia south to Florida and west to Texas, in addition to small disjunct populations in Tennessee and Kentucky. This is the first time these widely disjunct populations of *S. odorata* in TN and KY

(where they are state endangered) have been included in any molecular analyses. As originally noted by Nuttall, *S. odorata* is morphologically the largest member of the genus.

In contrast, *S. triloba* is distantly related to *S. odorata*; rather it is within the *S. cernua* species complex s.l. and sister to *S. magnicamporum* (Fig. 8). Overall, *S. triloba* is a smaller and more gracile plant compared to *S. odorata* (Fig 7 & 8). Although phylogenetic analyses place *S. triloba* within the greater *S. cernua* species complex, and *S. triloba* has been under synonymy of *S. cernua*, it is does not share a most recent common ancestor with any member of the *S. cernua* complex s.s. (Fig. 8). *Spiranthes cernua* does occur in Florida, but is restricted to one known population in the Apalachicola National Forest, Liberty Co. (it also occurs in southern Alabama and Georgia); *S. triloba* also occurs in Liberty Co., however the two species are not sympatric. The papillate, white margined labellum which matures from lime-green to yellow vs. a glabrous, wholly white labellum easily distinguish *S. triloba* from *S. cernua* (Fig.9 & 10).

The sister relationship of *S. triloba* and *S. magnicamporum* –a species primarily of the North America Great Plains with widely scattered disjunct populations in the eastern Great Lakes Basin, New Mexico, and the cedar glades of the southern Appalachian Mountains and northwestern Gulf Coastal Plain– was initially surprising to us, however a biogeographic relationship between the Great Plains and Florida/the Coastal Plain is exhibited in many plants: e.g., *Agalinis* Raf. (Orobanchaceae), *Baptisia* Vent. (Fabaceae), *Coreopsis* L. (Asteraceae), *Silphium* L. (Asteraceae). We are unaware of another group in which molecular data has supported the sister relationship between a Great Plains species and a Florida species. Furthermore, there are several possible morphological synapomorphies support this pairing. Both species have papillate labella whereas all other species in the *S. cernua* complex s.l. are glabrous. The labellum of *S. magnicamporum* was described as glabrous (Sheviak 1973) –with this

characteristic frequently repeated– yet plants from across its distribution (e.g., Mississippi, Nebraska, New Mexico, Ontario, Virginia, Wisconsin) are papillate. Some individuals of *S. magnicamporum* are glabrous (and the presence of papillae is rarely variable within an inflorescence), however a papillate labellum appears to be the more commonly encountered condition. The lateral sepals of both species typically sweep upward and slightly outward, often meeting or surpassing the dorsal sepal and petals (Fig. 9 & 10); the similarities in gross apparent floral morphology are sometimes so striking that *S. triloba* can occasionally be likened to as a 'dwarf' *S. magnicamporum*. *Spiranthes triloba* and *S. magnicamporum* are both fragrant with vanilla being a major component of their perceivable scent. Although *S. ochroleuca* has been reported to have a slightly musky scent (Fernald 1950), and rare populations of *S. cernua* may be very lightly fragrant, the *S. cernua* complex s.l. generally lacks fragrant flowers.

Status of putative hybrids—As indicated by morphological and phylogenetic analysis, *S.* ×*itchetuckneensis* (*S. odorata* × *Spiranthes ovalis* Lindl.) and *S.* ×*folsomii* (*S. odorata* × *Spiranthes longilabris* Lindl.) are clearly synonyms of *S. triloba*. However, a review of their descriptions (Brown 1999b, 2000) and holotype and paratype specimens yield rather ambiguous and poorly constructed concepts of these plants. Both names are based on the most limited of morphological and field study, with scanty descriptions wholly lacking the essential morphological details necessary to properly identify *Spiranthes*. Moreover, the types are mixed collections or based on sheets of different species. In the case of *S.* ×*itchetuckneensis*, the holotype (A. Will s.n., FLAS barcode 78786) and one paratype (Garber s.n. Gulf Hammock 1877, FLAS barcode 69862) represent *S. triloba*, whereas two other paratypes (Beckner 1602, FLAS barcode 96958; Watson and Mirrill s.n., FLAS barcode 25931) are *S. ovalis*. In an added layer of confusion, the original description of *S.* ×*itchetuckneensis* (Brown 1999b) does not

properly identify the pictures provided as exemplars of the new alleged nothospecies and its supposed parents: the plant labeled "*Spiranthes odorata*" is in fact *S. triloba*, and the image labeled "*S.* ×*itchetuckneensis*" is *S. ovalis*. The holotype of *S.* ×*folsomii* (Garber s.n. Pine barren ponds 1877, FLAS barcode 69863) is a mixed collection: the two whole plants on the sheet are *S. triloba*, but pieces of inflorescences within the packet represent both *S. triloba* and *S. longilabris*. In an attempt to restore clarity, the flowers of *S. longilabris* have been moved to a new labeled packet, and a lectotype for *S.* ×*folsomii* designated (see Taxonomic Treatment). The photograph of *S.* ×*folsomii* in Brown (2000) is an individual of *S. triloba*.

TAXONOMIC TREATMENT

Spiranthes triloba (Small) K. Schum. emend. M.C. Pace. — HOLOTYPE: UNITED STATES OF AMERICA. Florida: Lee County, Fort Meyer, collected 11 December 1891, *J. H. Simpson 368* (NY!). Isotype & drawing of holotype: AMES. Basionym: *Gyrostachys triloba* Small.

Homotypic synonyms: *Ibidium trilobum* Small; *Triorchis triloba* (Small) House

Heterotypic synonyms:

Spiranthes ×*itchetuckneensis* P.M. Br. — HOLOTYPE: UNITED STATES OF AMERICA. Florida: Columbia County, 15 ft. n. of Blue Hole, near Ichetucknee Springs, ca. 4 mi. n.w. of Fort White, S 7, T 6 S, R 15 E, collected 5 Nov 1960, *A. Will s.n.* (FLAS!)

Spiranthes ×*folsomii* P.M. Br. in part — LECTOTYPE [here designated: left most individual plant on the sheet]: UNITED STATES OF AMERICA. Florida: Levy County, Pine barren ponds, collected Oct 1877, *A.P. Garber s.n.* (FLAS!)

Spiranthes triloba (Small) K. Schum. emend. M.C. Pace est simile in aliquibus aspectibus ut *Spiranthes odorata*, *Spiranthes cernua*, et *Spiranthes magnicamporum*. Possit clare et fidenter separari ab omni tres species fundatur in characteres unica comitiua: radices tenuis stoloniferous, foliis lineari-lanceolatis oblanceolate parvis, flores parvis aromatibus 5-8 mm longis, labellum inferiorum dense papillato quod aperit ut tiliagineus-virides et mutationes ad flavi calidum apricis, sepalis lateralibus rectanguli supra flore, et conicis, obtusis, basi papillosis callum 0.5–0.8 mm longis. Videtur inopinatus quamvis epitheton speciem describit, labellum est enim potestas non tri-lobatis.

Spiranthes triloba (Small) K. Schum. emend. M.C. Pace is similar in some aspects to *S. odorata*, *S. cernua*, and *S. magnicamporum*. It can clearly and confidently be separated from all three species based on the following unique suite of characters: slender stoloniferous roots, small linear-lanceolate to oblanceolate leaves, small fragrant flowers 5–8mm long, a densely papillate lower labellum that opens as lime-green and matures to warm sunny yellow, lateral sepals held angled slightly above the flower, and conical, rounded, basally papillate callosities 0.5–0.8 mm long. Paradoxically, despite the descriptive specific epithet, the labellum is emphatically not tri-lobed (Fig. 15).

Terrestrial, acaulescent, deciduous herb, ca. 20–60 cm tall including the inflorescence. Roots fasciculate, fleshy, slender, stoloniferous for up-to ca. 0.5 m from the mother plant with plantlets arising from small offshoots; creamy tannish-white (white in cross-section). Leaves 1–5, forming a basal rosette, held upright; remaining until the appearance of the inflorescence (occasionally fugacious at the time of flowering), withering either during flowering or shortly thereafter; linear-lanceolate to slightly oblanceolate, 55–236 mm long, 7–21 mm wide; bluntly acuminate; leaf base tapered and decurrent, shiny medium-green. Inflorescence strongly erect

and perpendicular to the ground; peduncle to 20–60 cm, medium-green; glabrous; 1–2 small lefty cauline bracts occasionally present (frequently absent), quickly reducing to adpressed, clasping, lanceolate, acute bracts; raceme a single row of flowers in an open to tightly coiled spiral (appearing as 1–4 ‘ranks’, very rarely succund or nearly so), ca. 3–13 cm long; moderately to densely pubescent with blunt-tipped septate trichomes; trichomes to 0.5 mm long; flowers opening from the base to the apex of the inflorescence, basal flowers beginning to senesce as the uppermost flowers are opening. Floral bracts glabrous but pubescent at the base and with ciliate margin; broadly lanceolate, acuminate; concave around the ovary; 7–14 mm long; medium-green. Flowers resupinate; campanulate; slightly to strongly nodding and becoming more open with age; white; with a pleasing moderately-strong sweet fragrance of vanilla, talcum powder, and faint cinnamon. Sepals white; free; moderately to densely pubescent with blunt-tipped capitate septate trichomes. Dorsal sepal slightly convex, slightly to strongly reflexed near the tip; lanceolate, bluntly acuminate; 5–7 mm long, 2.3 mm wide when flattened. Lateral sepals lanceolate, acute; straight to just barely falcate, angled slightly outward and upward, the tips often surpassing the dorsal sepal and petals; 5.2–8.8 mm long, 1.3–2 mm wide. Dorsal petals white; slightly concave; lanceolate, bluntly acute; slightly to strongly reflexed at tips, with the dorsal sepal appearing stellate; 5–7 mm long, 2.5 mm wide when flattened. Labellum shortly clawed, free but clasping the column; keeled/concave for its length; reflexed strongly downward at about 1/3 the distance from the claw to labellum apex; centrally densely papillate at the region of reflextion and below to the apex, area of papillae 1.5–3.5 mm long; margin entire to very slightly undulating from the base until the area of reflextion, below point of reflextion margin becoming shallowly laciniate and crisped; 2 basal callosities/nectar glands, white to yellow, conical, rounded, upright, 0.5–1.3 mm tall, with long, dense papillae at the base, papillae 0.5–1

mm long; margin white, central area of labellum opening as a bright lime-green, maturing to warm sunny yellow (very occasionally remaining lime-green until just before the flower senesces), area of coloration 5 mm long, 2.5 mm wide, labellum 5–8mm long, 4–8 mm wide below the callosities, 3–5 mm wide at the area of reflextion when flattened; apex subacute, often curling back-in on itself, pointing back toward the raceme. Column protandrous, slightly rhombic, medium-green, narrow, 2–3.1 mm long, 2 mm wide; with a fringe of minute glands or papillate in a thin crescent just below the stigmatic surface; with a pair of upright flaps or wings at each side and clasping the column, the wings green basally, mostly white to translucent; column foot glabrous. Rostellum well-developed, white to ivory (eventually dark drown), tapering to thin acute membranes at the apex, 1 mm long. Stigmatic surface glabrous, shiny, base angled away from the flower opening, roundly diamond-shaped when viewed from below, medium-green to yellowish-green near the rostellum, 1 mm long, 2 mm wide. Anther 1, dorsal, dark coffee-brown, triangular-ovoid, 2 mm long; pollinium very pale buttery yellow, attached to a well-developed viscidium, pollinaria 2.8 mm long; viscidium dark coffee-brown, linear, slightly sticky, immersed the rostellum, leaving behind a narrow V-shaped rostellar remnant after removal, 1 mm long. Ovary moderately to densely pubescent with septate trichomes, medium-green. Fruit a capsule, light brown.

Etymology—The specific epithet "*triloba*", as given by Small (1898) in his original description means "three-lobed," referring to the supposedly three-lobed labellum. Although a drawing on the holotype sheet shows a slightly tri-lobed lip, the labellum of the physical specimen is clearly not tri-lobed. A suggested common name is the "panther ladies' tresses" or "panther *Spiranthes*", an homage to the Florida panther (*Puma concolor* subsp. *coryi* Bangs). Both orchid and panther are restricted to Florida, and both are dependent on the same frequently

burned flatwood and calcareous prairie habitats for their survival. This common name also refers to Florida Panther National Wildlife Refuge, one of the global strongholds for both species.

Distribution, habitat, and ecology— Herbarium specimens, fieldwork, and phylogenetic analyses indicate *S. triloba* is restricted to Florida (Fig. 11), whereas *S. odorata* occurs throughout the Coastal Plain of the southeastern U.S., including Florida. One population of *S. triloba* in Gadsden Co. grows within ca. 8 km of the Georgia border, but despite an expansive search among herbaria, no specimens have been found for Georgia; similarly fieldwork in southern Alabama and Georgia in 2013 did not locate this species. Thus *S. triloba* should be regarded as endemic to Florida. Historically *S. triloba* occurred in Alachua, Citrus, Collier, Columbia, Gadsden, Hernando, Hillsborough, Jefferson, Lee, Levy, Liberty, Miami-Dade, Palm Beach, Pasco, Polk, Taylor, Sumter, and Wakulla counties; populations are still (or most likely) found in all of these counties.

The eastern populations in Miami-Dade and Palm Beach counties, owing to their geographic separation and small size, merit additional comment. One population in Miami-Dade Co. is almost certainly extirpated as it was centered in the historic Lemon City neighborhood of Miami on the Florida Atlantic Coastal Ridge, an area which has been completely urbanized. The other Miami-Dade population is the vicinity of Hammer Key and represents the southern-most and only known station for this species within the Everglades physiographic province and Everglades National Park [ENP] (Small 860, NY; Austin et al. 1987). As frequently burned calcareous prairies continue to occur in this region of the Everglades, it is possible that *S. triloba* still exists in ENP (Mr. Jimi Sadle (ENP), personal communication). The extreme rarity of *S. triloba* within the Everglades is likely due to the hydrology of the region; we hypothesize that although this species can withstand seasonal inundation (as in FPNWR), it cannot compete in

most of the Everglades' permanently flooded, peat-dominated conditions. Similarly, the outline of *S. triloba*'s distribution to the west of the Everglades largely mirrors the northwestern boundaries of the Tamiami Formation, which is composed of highly permeable light colored fossiliferous sands and quartz pockets (Missimer 1992). The Palm Beach population, which sits near the southern portion of the highly permeable Anastasia Formation, only discovered in 2015 by Mr. Dylan Scott (Avon Park Air Force Range), is particularly important as it represents the lone know extant population of *S. triloba* in eastern Florida. Prior to the herbarium review and fieldwork conducted for this study, *S. triloba* was only known from the type location, and "green-lipped *S. odorata*" was only known from FPNWR and two other stations in Collier Co. in southern Florida, and Wakulla Co. in northern Florida.

Within Florida *S. triloba* and *S. odorata* are ecologically separated: *S. triloba* is predominantly found in frequently burned calcareous prairies and open flatwoods, whereas *S. odorata* principally grows in flooded *Taxodium* Rich. and *Nyssa* L. forests and cypress domes, and is frequently submerged at the time of flowering (Fig. 2 & 12). These distinct habitats commonly occur in close proximity or are contiguous with each other in a mosaic over the larger landscape, and both species rarely occur in damp roadside ditches, yet they have never been observed to co-occur in a shared location. Temporally, there is a wide overlap for most of their blooming period, but *S. triloba* starts and finishes later: late-October to early-February for *S. triloba* (occasional individuals may bloom sporadically into May); early-October to mid-December for *S. odorata*.

Like most *Spiranthes*, *S. triloba* appears to require frequent disturbances to persist and bloom; here recurrent fire likely reduces competition and encroachment from taller herbs, shrubs, and trees, in particular *Sabal palmetto* (Walter) Lodd. ex Schult. & Schult. f. and *Serenoa repens*

(W. Bartram) Small. In FPNWR and Avon Park Air Force Range, these frequently burned calcareous prairies and open flatwoods typically burn once every 2–4 years. As such, fire may be a key abiotic factor enabling the persistence of *S. triloba*. This hypothesis is anecdotally supported by return visits to populations in FPNWR: in the second year after burning, *S. triloba* was extremely abundant with hundreds of stems; by the fourth year after burning, only a dozen flowering stems were visible. The relationship between *S. triloba*, fire, blooming, and asexual vs. sexual reproductive success deserves to be investigated more rigorously. Interestingly, *S. magnicamporum*, the sister species to *S. triloba*, also grows in frequently burned calcareous prairies in the Great Plains of the United States and Canada. Typical associates in prairie habitats throughout the range of *S. triloba* include *Aristida beyrichiana* Trin. & Rupr., *Asclepias verticillata* L., *Cladium jamaicense* C.B. Clarke, *Coreopsis leavenworthii* Torr. & A. Gray, *Eriocaulon* L. spp., *Eryngium yuccifolium* Michx., *Heliotropium polyphyllum* Lehm., *Lobelia feayana* A.Gray, *Lobelia glandulosa* Walter, *Muhlenbergia capillaris* (Lam.) Trin., *Pinguicula lutea* Walter, *Pinguicula pumila* Michx., *Rhynchospora* Vahl spp., *Sabal palmetto*, *Serenoa repens*, and *Spiranthes longilabris*. Only two populations –Ichetucknee Springs and a station in Jefferson Co. – occur at the sunny edges of calcareous wooded habitats dominated by *Taxodium distichum* (L.) Rich., *Acer rubrum* L., and *Carpinus caroliniana* Walter. Possible indicator species include *Lobelia glandulosa*, *Pinguicula lutea*, *P. pumila*, and *Rhynchospora* spp.

Statement on rarity—Given its endemism to Florida (a highly developed state at severe risk for climate-change induced oceanic flooding), a preference for a rare and fragile fire-dependent habitat (Orzell and Bridges 2006), strongly clonal nature, history of taxonomic confusion, and probable extirpation of one of the three geographically unique eastern populations, *S. triloba* is suggested to be of high conservation concern. Many populations grow

in areas less than 20 m in elevation, putting them at risk of salt water intrusion or submersion from predicted sea-level rise. Invasive species such as *Sus scrofa* L. (feral boar), *Imperata cylindrical* (L.) P. Beauv. (cogon grass), and *Schinus terebinthifolius* Raddi (Brazilian pepper tree) also pose a threat through the destruction or degradation of habitat.

Cultivated *S. triloba* grown in greenhouses without bees (the pollinators of *Spiranthes* (Darwin 1862; Catling 1982, 1983; Sheviak 1982)) have not produced any fruits, indicating that *S. triloba* is not apomictic or autogamous (a common condition in the *S. cernua* species complex s.l.), and most likely requires insect-mediated pollination for sexual reproduction –the presence of a well-developed rostellum also supports this conclusion. Plants examined in situ frequently had pollinaria removed and fruits were regularly encountered. Potential pollinators were only observed at Avon Park Air Force Range: *Apis mellifera* L. (Hymenoptera: Apidae) and cf. *Augochlora pura* Say (Apoidea: Halictidae) (fig. 13). *Apis mellifera* was observed late afternoon, climbing up the inflorescence in the typical fashion (Darwin 1861, 1862; Catling 1983), and cf. *A. pura* was observed mid-morning, climbing downward from the top of the inflorescence. This top-down progression of *A. pura* is the same movement observed for the Halicitid pollinators of *Spiranthes lucida* (H.H. Eaton) Ames (Catling 1983). It is thought that the non-protandrous nature of *S. lucida* is in some way related to its exclusive pollination by Halictid bees, yet *S. triloba* is protandrous. Most North American *Spiranthes* are pollinated by *Bombus* Lat. (bumble bees) (Catling 1983); in neither of the two instances described here for *S. triloba* were pollinaria observed to be removed and other potential pollinators may yet be observed. If protandrous *S. triloba* is exclusively or mainly pollinated by Halicitid bees, that would be quite unexpected and intriguing, challenging previously held hypotheses for the pollination relationships of this genus. Though populations may consist of dozens to hundreds of flowering stems, the clonal nature of

S. triloba via stoloniferous roots likely indicates low levels of within population genetic diversity and should affect the development of potential conservation strategies; analyses to address the question of genetic diversity are on-going.

DICHOTOMOUS KEY TO THE SPECIES OF *SPIRANTHES* OCCURRING IN FLORIDA

1a. Racemes moderately to densely pubescent 2.

1b. Racemes glabrous or sparsely pubescent 8.

2a. Trichomes sharply pointed, not capitate *S. vernalis*

2b. Trichomes blunt, not pointed, capitate 3.

3a. Labellum glabrous 4.

3b. Labellum with patch of papillate 7.

4a. Leaves large, to 49 cm; flowers intensely fragrant *S. odorata*

4b. Leaves small or absent, to 13 cm; flowers not fragrant 5.

5a. Labellum centrally yellow *S. laciniata*

5b. Labellum centrally white 6.

6a. Leaves oblancelate *S. ovalis*

6b. Leaves linear or linear-lanceolate *S. cernua*

7a. Flowers fragrant; lateral sepals usually sweeping upward *S. triloba*

7b. Flowers not fragrant; lateral sepals held parallel or below the flower *S. brevilabris*

8a. Flowers less than 4 mm long *S. tuberosa*

8b. Flowers larger than 4 mm long 9.

9a. Leaves oblanceolate or obovate 10.

9b. Leaves linear, linear-lanceolate, or absent 11.

10a. Labellum centrally yellow *S. floridana*

10b. Labellum centrally green *S. lacera* (including cf. *S. eatonii*)

11a. Labellum coloration restricted to prominent veins *S. praecox* (including cf. *S. sylvatica*)

11b. Labellum coloration solid 12.

12a. Lateral sepals held slightly oblique, moderately downwardly falcate *S. torta*

12b. Lateral sepals held moderately oblique to widely-spreading, slightly upwardly falcate *S. longilabris*

Appendix: Representative Specimens Examined.

Spiranthes odorata: U.S.A.

Alabama:

Cleveland Co., Moro Bottoms Natural Area, floodplain of Moro Creek, ca. 6 mi S of Kingsland, 9 Oct 1989, *Guldin s.n.* (WILLI).

North Carolina:

Brunswick Co., Along Moore's Creek, north of Southport, 21 Oct 1949, *Godfrey 50163* (FSU).

South Carolina:

Charleston Co., near McClellanville, 4 Nov 1961, *Batson s.n.* (BH)

Colleton Co., Edisto River north of Canadys on US highway 15, 12 Oct 1956, *Bell 5383* (FSU).

Louisiana:

Assumption Par., NW of Paincourtville, 16 Oct 1939, *Brown 8343* (LSU).

Baton Rouge Par., Six miles SE on Jefferson Highway on right side of road, 10 Sep 1972, *Abdullah s.n. (*LSU).

East Baton Rouge Par., Four miles south of Baton Rouge. 6 Nov 1938, *Brown s.n.* (LSU). Near 7th Ward School, 30 Oct 1927, *Brown 1511* (LSU). South of Baton Rouge, 300 m north of Chandler Drive, 3 Nov 1972, *Schroer 84* (LSU).

Jefferson Par., Jean Lafitte National Historic Park, 15 Nov 1997, *Maliakal 168* (LSU). South side of Horse Show Lake, 100 yards north of Hoo Shoo Too Road, 12 Nov 1971, *Ginn 2*2 (LSU). Next to Iberville Terrace, 6 mi South of LSU campus on east side of Highland Road, 10 Nov 1976, *Aycock 221* (LSU).

Lafayette Par., I-10 West. Take LA 347 exit. Turn left onto LA 347. Go about 1 mile to the first light, take a right-heading into Lafayette. Travel about 14 miles and take a left going towards Lake Martin, 11 Nov 1999, *Turner 6* (LSU).

Lafourche Par., North part of Lake Boeuf, ca. 1.4 miles NNW of mouth of Theriot Canal, ca. 6 air miles NNW of Raceland, 29°48'0.00"N 90°38'11.00"W, 14 Oct 2008, *Reid 6860* (LSU).

Livingstone Par., 1.2 miles west of Highway 447 and 1 mile south of Interstate 10, 27 Oct 1973 *Miller 78* (LSU). west side of LA 477 at I-12 junction, 3 road miles south of Walker, 22 Oct 1972, *Rekas 65* (LSU). Junction of I12 and LA631/4 mile west of intersection, 29 Oct 1972, *Rekas s.n.* (LSU). 100 yards north of Highway 63 and Highway 449 junction; 25-100 yards east of Highway 449, 27 Oct 1973, *Lee 141B* (LSU). Highway 22 near Amite River at Clio, 10 Nov 1970, *Brown 21784* (LSU). Near Denham Springs, 20 Oct 1940, *Exner s.n.* (LSU).

Natchitoches Par., Louisiana Highway 478, 1.0 mi SW of junction with LA Highway 120. E side of creek crossing, N side of creek, swamp on N side of road, 15 Oct 1994, *Nesom 7854* (NY).

Rapids Par., Kisatchie National Forest; FS273 to left on FS201, 21 Dec 1993, *Bruser 100* (LSU).

Terrebonne Par., along Bayou Grand Caillou, 3 miles above Dulac, 22 Oct 1936, *Archeneaux 245* (BH).

St. Helena Par., About 4 ¾ miles SE of Montpelier, T4S R6E S35, 20 Nov 1971, *Allen 1784* (LSU).

St. John the Baptist Par., Under I-55 bridge, 1/2 mile north of junction I-10 and I-55, north of LaPlace, 28 Nov 1992, *Montz 5970* (LSU).

St. Landry Par., south of US 190 east of US 167 and east of Opelousas; T6S, R4E, S73, 31 Oct 1982, *Thomas 82702* (FLAS). Highway 190, 2 miles from Krotz Springs, 8 Nov 1971, *Peng 7* (LSU).

St. Martin Par., Unknown, Oct 1981, *Urbatsch s.n.* (LSU).

St. Tammany Par., White Kitchen TNC Preserve, W side of West Pearl River, ca. 0.75 mi upstream from US 90 Bridge, ca. 1 mi N of White Kitchen, 5 Nov 2003, *Reid 4772* (LSU). 1/2 mile west of Madisonville, 23 Oct 1940, *Brown 8750* (LSU).

Washington Par., west bank of Pearl River south of LA 10 bridge east of Bogalusa, 23 Oct 1992, *Thomas 132800* (BH). Unknown, unknown, Oct 1880, *Louglois 330* (F). Washington Par., north and south of LA 10 E of Bogalusa at west bank of Pearl River; T3S, R14E, S40, 4 Nov 1979, *Thomas 69546* (FLAS). North and south of LA 10 E of Bogalusa at west bank of Pearl River, T3S, R14E, S40, 4 Nov 1979, *Thomas 69546 & 483* (LSU).

North Carolina: Currituck Co., 2.4 mi north of Knotts Island (P.O.) then 0.3 mi west, 15 Oct 1958, *Ahles 51328* (FLAS).

Florida:

Unknown: Bank of the Apalachicola, s.d. *Chapman s.n.* (NY). Bank of the Apalachicola, s.d., *Chapman s.n.* (NY).

Alachua Co., Ca. 1 mi. n of High Springs; Spring run of Camp Kullaqua; ca 200 m from junction with Santa Fe River, just upstream from FL 441 bridge, 13 Dec 1998, *Whitten s.n.* (FLAS). Hogtown Creek, Gainsville, 27 Nov 1927, *Watson s.n.* (FLAS). Orange Lake, 15 Nov 1932, *Tissot s.n.* (FLAS). Two mi east of Windsor on FL. RT 20, Nov 1979, *Burns 94* (NY).

Bradford Co., 100m east of bridge crossing Santa Fe River on State Road 231, 21 Oct 1980, Vliet 32 (FLAS).

Brevard Co., Okeechobee region, 18 Nov 1903, *Fredholm 6205* (NY).

Broward Co., Ft. Lauderdale, 19 Nov & 25 Nov 1903, *Small & Carter 1042* (F, NY).

Calhoun Co., Clarksville. Jim and Judy Oenbrink Thistle Dew Farms, 4 Nov 2007, *MacClendon 747* (FSU).

Collier Co., 15 mi E of junction of RT 29 on Everglades Pkwy, Big Cypress Swamp, 1980, *Sheviak 816* (NYS). Along Rt 84 (Everglades Pkwy), 20 mi east of Rt 29, Big Cypress Swamp, 12 Dec 1973, *Sheviak 817* (NYS).

Columbia Co., Six miles NW of Fort White off of CR 238 in Ichetucknee Springs State Park. Ca. 1.6 miles south of North Entrance; east side of river; southwest of Grassy Hole Spring; T6S, R16E, S18, 14 Oct 1992, *Herring 999* (FLAS). Along the Santa Fe River, O'Leno State Park, 21 Nov 1980, *Godfrey 78270* (FSU).

Clay Co., Green Cove Springs, 25 Nov 1925, *Murrill s.n.* (FLAS).

Dade Co., Orange Glade, near Miami, 7 Dec 1903, *Eaton W526* (F). Dade Co., Bay Isles in Everglades, northeast part trail, 13 Dec 1941, *Davis s.n.* (FLAS). Everglades National Park, near Pahay-Okee, 3/4 mi from main road, 1/2 mi from Pahay-Okee Tower, 12 Dec 1982, *Miller 10* (FSU).

Escambia Co., Bluff Springs, slough near west bank of Escambia River via Bryneville Road east of US 29, 29 Oct 1983, *Burkhalter 9143* (FSU).

Franklin Co., Apalachicola River floodplain, Ft. Gadsen State Park, 6 air mi south-southwest of Sumatra, 15 Nov 1984, *Anderson 7787* (FLAS). Franklin Co., At end of FS road 101, at Wright Landing on Owl Creek, just southwest of Wright Lake, ca. 2.2 air mi southwest of Sumatra; Apalachicola NF, SEQ, SWQ, NEQ; T5S, R8W, S35, 3 Nov 1991, *Orzell 18949* (FLAS). Franklin Co., South end of Forbes Island between Apalachicola River and Brothers River Slough, ca. 8.5 air mi north-northwest of town of Apalachicola, 15 Oct 1985, *Anderson 8917* (FSU).

Gulf Co., West bank of Apalachicola River about halfway between mouths of Owl, 17 Nov 1995, *Anderson 16103* (FSU).

Hendry Co., Seminole Indian Reservation, southeast corner of Hendry Co., 17 Nov 1963, *Ward 3804* (FLAS).

Hillsborough Co., Road 60, east of Tampa, 1 Nov 1953, *Garrett s.n.* (FLAS). English Creek, N side of SR 60, T29S, R22E, S25, 23 Nov 1984, *Lassiter 2212* (FSU). Eight miles south of Zephyr Hills. 19 Nov 1964, *Godfrey 65135* (FSU).

Jackson Co., Chipola River at Marianna Cavers State Park, 19 Nov 1960, *Mitchell 912* (FSU).

Lake Co., tributary of Alexander Run, ca. 1/2 mi below Alexander Spring, Ocala National Forest T16S, R37E, 1 Jan 1965, *Ward 4363* (FLAS).

Levy Co., Gulf Hammock, 17 Nov 1940, *Watson s.n.* (FLAS).

Manatee Co., Along Wauchula Rd., N of Myakka City, 10 Dec 1973, *Sheviak 814* (NYS).

Marion Co., Belleview; L City & L. Worth, s.d., *Swinple s.n.* (F). unknown, unknown, s.d., *Hitchcock s.n.* (F). Marion Co., Reddick, 15 Nov 1936, *Foster s.n.* (FLAS). Marion Co., Oklawaha River, ca. 1 mi south of Eureka, T13S, R24E, S16, 11 Nov 1965, *Ward 5452* (FLAS).

Putnam Co., Mud Springs Welaka, 6 Dec 1940, *Laessle s.n.* (FLAS).

St. Lucie Co., 12 mi east of Okeechobee City, 8 Dec 1919, *Small 9284* (NY).

Sumter Co, Wahoo, 14 Nov 1937, *St. John s.n.* (FLAS).

Walton Co., by the Choctawhatchee River, east of Bruce, 6 Nov 1982, *Godfrey 80201* (FSU).

Texas:

Harris Co., Harrisburg, 13 Nov 1875, *Joor s.n.* (NY).

Wood Co., Mineola, 18 Oct 1900, *Reverehon 2174* (NY).

Virginia:

Charles City Co., at head of Kittewan Creek, 25 Sep 1948, *Sargent 13* (FLAS). Weianoke, 4 mi S of Charles City, 29 Sep 1940, *Fosberg 17527* (MO).

Greensville Co., Fontaine Creek se of Taylor's Millpond, 21 Sep 1939, *Fernald 11308* (NY).

James City Co., 2.55 mi along Long Loop Road, Jamestown Island, 7 Oct 1969, *Loetterie 510* (WILLI). Field about 5 miles west of Toano, 24 Sep 1939, *Menzel 346* (WILLI). Next to Colonial Parkay, at Cam Lightfoot, at end of State Route 617, 4.9 miles S of Williamsburg, 27 Oct 1993, *McCahon 32* (WILLI).

Norfolk Co., Bowers Hill, 8 Oct 1944, *Hubricht 82740* (MO).

Princess Anne Co., Along a small tributary of the North Landing River, 0.9 mi WSW of Pungo Ferry Road bridge, 2.1 mi WNW of Creeds, 18 Oct 1995, *Fleming 11537* (WILLI). Along SE side of Milldam Creek 0.25 mi NW of its confluence with the North Landing River, 2.3 mi SW of Creeds, 18 Oct 1995, *Fleming 11540* (WILLI).

Spiranthes trioba: U.S.A.

Florida:

Unknown Co., unknown, 18 Mar 1905, *Eaton 779 ½* (NY).

Citiris Co., South of road to Ozello, 1 mi west of US 19, T19S, R17E, S4, 11 Nov 1967, *Ward 6529* (FLAS).

Columbia Co., 15ft n of Blue Hole, near Ichetucknee Springs, ca. 4 mi nw of Fort White, T6S, R15E, S7, 5 Nov 1960, *Will s.n.* (FLAS).

Collier Co., Kissimmee Billy, north of Alligator Alley, 1 Dec 1976, *Correll 47714* (NY).

Dade Co., Lemon City, 27 Dec 1903, *Eaton 726 ½* (F). Lemon City, 16 Dec 1903, *Eaton 606 ½* (F). Lemon City, 28 Dec 1903, *Eaton s.n.* (F). Lemon City, 16 Dec 1903, *Eaton s.n.* (NYS).

Gadsden Co., S of Dolan Road and north of I-10, ca. 2.5 air miles SSW of Chattahoochee, 8 Nov 2005, *Anderson 21485* (FSU).

Hernando Co., Annutteliga Hammock north of Brooksville, 28 Nov 1921, *Small 10070* (NY). along unimproved E to W road into Chassahowitzka Swamp, ca 4.9 air mi NNW of junction FL 50 and US19 in Weeki Wachee, 1 Nov 1991, *Orzell 18880* (SEL).

Hillsborough Co., 8 miles south of Zephyr Hills, 19 Nov 1964, *Godfrey 65134* (FSU).

Jefferson Co., near Waukeenah, 30 Nov 1923, *Small 11048* (NY). Beside O'Neal/Tram Rd, 4 mi S of Thomas City / Walker Springs Rd, ca. 7 air miles SE of Wacissa, 31 Oct 2005, *Neel 503* (FSU).

Lee Co., Ft. Meyers, 11 Dec 1891, *Simpson 368* (NY). Alva, 1 Dec 1917, *Francis 76* (US).

Levy Co., Gulf Hammock, Oct 1877, *Garber s.n.* (F, NY). Gulf Hammock, Oct 1877, *Garber 2403* (NY). Unknown, s.d., *Garber 2404* (NY).

Polk Co., Auburndale, 5 Dec 1930, *McFarlin 4021* (FLAS).

Taylor Co., Along US Highway 98 ca 12.3 miles W of western limit of Perry, 2 Nov 1970, *Dress 10331* (BH). Bradley Springs at first dirt road crossing downstream from springs, 29 Oct 1977, *Leonard 7046* (FSU).

Wakulla Co., St. Marks National Wildlife Refuge (St. Marks Unit), S side Aucilla Tram Rd, 1.5 mi E of Rt 59, 6 Nov 2009, *Anderson 24790* (NY). Along the Wakulla River at Upper Bridge, 10 Nov 1973, *Godfrey 73132* (FSU). Upper Bridge, Wakulla River, 29 Nov 1954, *Godfrey 52789* (FSU). St. Marks National Wildlife Refuge (St. Marks Unit). S side Aucilla Tram Rd, 1.5 mi E of Rt 59, 6 Nov 2009, *Anderson 24757* (FSU).

Ames, O. 1905. A synopsis of the genus *Spiranthes* north of Mexico. Orchidaceae, fasc. 1: 22-156.

Ames, O. 1921. Notes on New England orchids. I. *Spiranthes*. Rhodora 23: 3-85.

Austin, D.F., A.F. Cholewa, R.B. Lassiter, and B.F. Hansen. 1987. The Florida of John Kunkel Small: his species and types, collecting localities, bibliography and selected reprinted works. Contributions from the New York Botanical Garden, 18.

Baum, D.A., and M.J. Donoghue. 1995. Choosing among alternative "Phylogenetic" species concepts. Systematic Botany, 20(4): 560-573.

Brown, P.M. 1999a. Recent taxonomic and distributional notes from Florida 1. North American Native Orchid Journal, 5(1): 7-15.

Brown, P.M. 1999b. Recent taxonomic and distributional notes from Florida 4. North American Native Orchid Journal, 5(4): 358-362.

Brown, P.M. 2000. Recent taxonomic and distributional notes from Florida 5. North American Native Orchid Journal, 6(1): 64-66.

Catling, P. M. 1980. Systematics of *Spiranthes* L. C. Richard in northeastern North America. Ph.D. thesis, University of Toronto, Toronto.

Catling, P.M. 1982. Breeding systems of northeastern North American *Spiranthes* (Orchidaceae). Canadian Journal of Botany, 60: 3017–3039.

Catling, P.M. 1983. Pollination of northeastern North American *Spiranthes* (Orchidaceae). Canadian Journal of Botany, 61: 1080–1093.

Catling, P.M. and V.R. Catling. 1988. *Spiranthes nebulorum* (Orchidaceae), a new species from southern Mexico and Guatemala. Rhodora, 90(862): 139–146.

Catling, P.M., and C.J. Sheviak. 1993. Taxonomic Notes on some North American Orchids. Lindleyana, 8: 77–81.

Cornish-Bowden, A. 1985. Nomenclature for incompletely specified bases in nucleic acid sequences: recommendations 1984. Nucleic Acids Research, 13(9): 3021–3030.

Correll, D.S. 1940. A contribution to our knowledge of the orchids of the southeastern United States. Botanical Museum Leaflets 8(4): 69–85.

Darwin, C.R. to A. Gray, 17 Sep 1861. *In* F. Burkhardt, J. Browne, D.M. Porter, and M. Richmond (eds). 1994. The Correspondence of Charles Darwin, Vol. 9. Cambridge University Press.

Darwin, C.R. 1862. On the various contrivances by which British and foreign orchids are fertilised by insects, and on the good effects of intercrossing. London: John Murray.

Dayrat, B. 2005. Towards integrative taxonomy. Biological Journal of the Linnean Society, 85(3): 407–415.

Dueck, L.A., and K.M. Cameron. 2007. Sequencing re-defines *Spiranthes* relationships, with implications for rare and endangered taxa. Lankesteriana, 7(1-2): 190–195.

Dueck, L.A., and K.M. Cameron. 2008. Molecular evidence for new species – *Spiranthes stellata*. North American Orchid Journal, 14(1): 12–21.

Dueck, L.A., D. Aygoren, and K.M. Cameron. 2014. A molecular framework for understanding the phylogeny of *Spiranthes* (Orchidaceae), a cosmopolitan genus with a North American center of diversity. American Journal of Botany, 101(9): 1551–1571.

Fernald, M.L. 1950. Gray's Manual of Botany. ed. 8. American Book Co., Boston, MA.

Gleason, H.A., and A. Cronquist. 1991. *Manual of Vascular Plants of Northeastern United States and Adjacent Canada*. Second Edition. The New York Botanical Garden, Bronx, NY.

House, H.D. 1920. A consideration of certain genera proposed by Ehrhart. American Midland Naturalist, 6(9): 200–207.

Lindley, J. 1840. The Genera and Species of Orchidaceous Plants. Ridgways, London, UK.

Menk, A. 2007. An extreme expression of color in *Spiranthes odorata*. North American Native Orchid Journal 13:26.

Miller, M.A., W. Pfeiffer, and T. Schwartz. 2010. "Creating the CIPRES Science Gateway for inference of large phylogenetic trees"; in Proceedings of the Gateway Computing Environments Workshop (GCE), 14 Nov. 2010, New Orleans, LA pp. 1 – 8.

Missimer, T.M. 1992. "Stratigraphic relationships of sediment facies within the Tamiami Formation of southwestern Florida: Proposed intraformational correlations"; in Scott, T.M., and Allmon, W.D., (eds.), The Plio-Pleistocene stratigraphy and paleontology of southern Florida; Florida Geological Survey Special Publication 36, pp. 63 – 92.

Nuttall, T. 1834. A Description of some of the rarer or little known plants indigenous to the United States, from the dried specimens in the herbarium of the Academy of Natural Sciences in Philadelphia. Journal of the Academy of Natural Sciences of Philadelphia, 7(1): 61–115.

RStudio. 2014. RStudio: Integrated development environment for R (Open Source Edition; Version v0.98.932) [Computer software]. Boston, MA. Retrieved September 25, 2014.

Rambaut. A. 2014. FigTree v1.4.2, released 2014-07-09. http://tree.bio.ed.ac.uk/software/figtree/

Salazar, G.A., M.W. Chase, M.A. Soto, and M. Ingrouille. 2003. Phylogenetics of Cranichideae with emphasis on Spiranthinae (Orchidaceae, Orchidoideae): evidence from plastid and nuclear DNA sequences. American Journal of Botany, 90: 777–795.

Schumann, J (H.K.). 1898. Just's botanischer Jahresbericht. 2(1): 339.

Sheviak, C.J. 1973. A New *Spiranthes* from the Grasslands of North America. Botanical Museum Leaflets, 23(7): 287–297.

Sheviak, C.J. 1982. Biosystematic study of the *Spiranthes cernua* complex. New York State Museum Bull. 448: 1–73.

Sheviak, C. J. 1984. *Spiranthes diluvialis* (Orchidaceae): a new species from the western United States. Brittonia 36(1): 8–14.

Sheviak, C.J. 1989. A new *Spiranthes* (Orchidaceae) from Ash Meadows, Nevada. Rhodora, 91: 225–234.

Sheviak, C.J. 1991. Morphological variation in the compilospecies *Spiranthes cernua* (L.) L.C. Rich.: Ecologically-limited Effects of Gene Flow. Lindleyana, 6:228–234.

Sheviak, C.J. 2013. A cytogeographic survey of *Spiranthes romanzoffiana* Cham. and its taxonomic implications. The Native Orchid Conference Journal, 10 (2): 1–22.

Sheviak, C. J., and P. M. Brown. 2002. *Spiranthes*. In *Flora of North America north of Mexico*, Volume 26. Oxford University Press, New York, NY, USA.

Small, J.K. 1898. Studies in the Botany of the Southeastern United States. -XV. Bulletin of the Torrey Botanical Club, 25 (12): 605–621.

Small, J.K. 1913. *Flora of the Southeastern United States*. Ed. 2. J.K. Small, New York, NY, USA.

Small, J.K. 1933. Our largest ladies'-tresses. Journal of the New York Botanical Garden, 34: 106–108.

Stewart, S.L., and L.W. Richardson. 2008. Orchid flora of the Florida Panther National Wildlife Refuge. North American Native Orchid Journal, 14(2): 70–104.

Thiers, B. [continuously updated]. Index Herbariorum: A global directory of public herbaria and associated staff. New York Botanical Garden's Virtual Herbarium. http://sweetgum.nybg.org/ih/

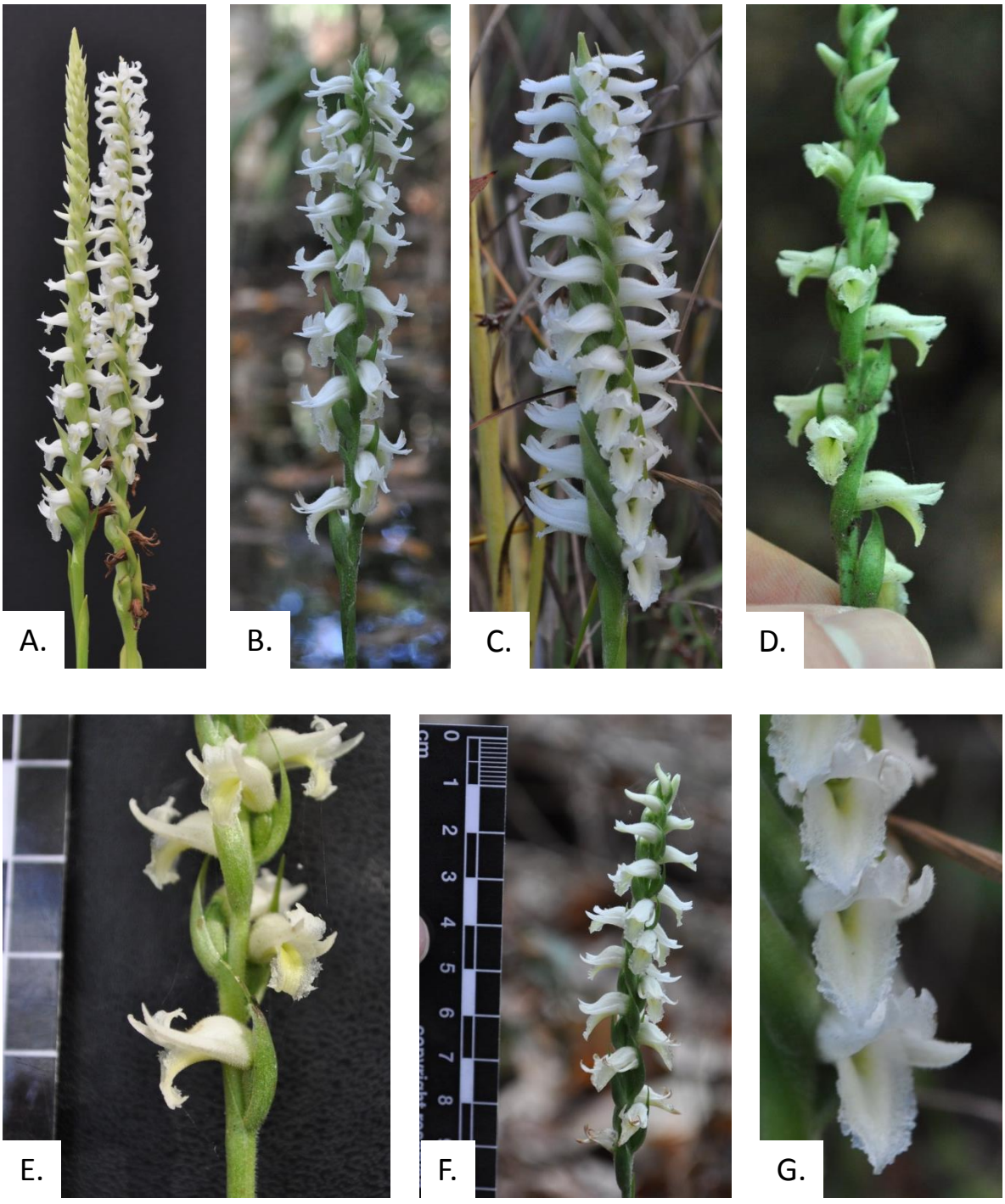


Fig. 1. *Spiranthes odorata* s.s.. **A.** Greenhouse grown plants; originally from Brunswick Co., NC (same population as sample so5h). **B.** Dorchester Co., NC. **C & G.** Taylor Co., FL (same plant; sample so10). **D & E.** Leon Co., FL (different plants, sample population). **F.** Bibb Co., GA.

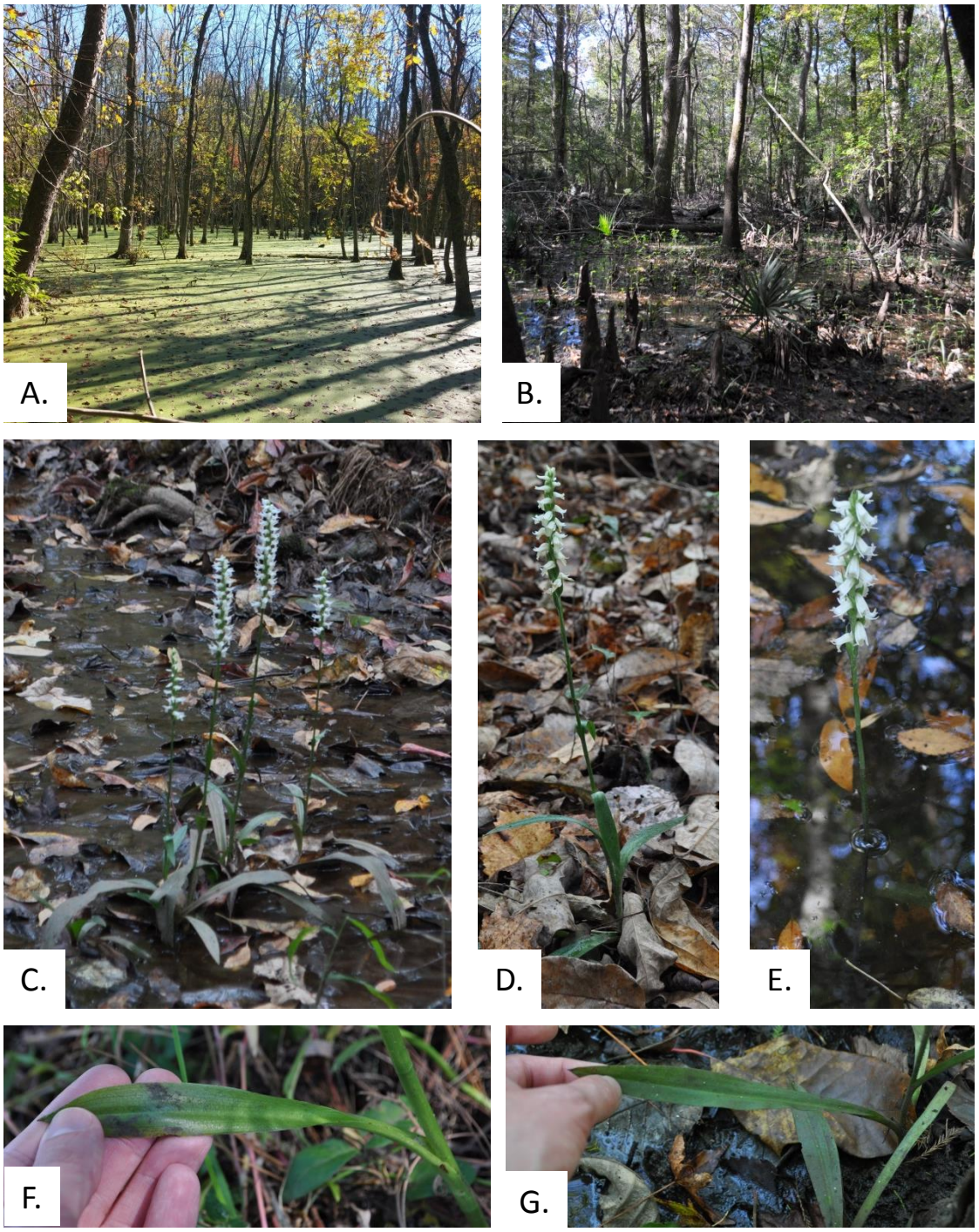


Fig. 2. *Spiranthes odorata* s.s.. **A**–**B.** Typical habitat. **A.** Flooded *Acer*–*Celtis*–*Fraxinus*–*Ulmus* forest, Woodford Co., KY (photo T. Littlefield). **B.** Flooded *Nyssa*–*Taxodium*–*Acer*–*Liquidambar* forest, Leon Co., FL. **C**–**E.** Habit. **C.** Richland Co., SC. **D.** Bibb Co., GA. **E.** Dorchester Co., NC. **F**–**G.** Leaves. **F.** Currituck Co., NC (sample plant so1). **G.** Taylor Co., FL (sample site of so10).

Fig. 3. *[Legend for following three pages]. Morphological data matrix used in the Principal Components Analysis.*



Collector, collection number	species	state	labellum surface	plant height (cm)	leaf length (mm)	leaf width (mm)	inflorescence length (cm)	doral floral length (mm)	lateral sepal length (mm)	lateral sepal width (MM)	labellum length (mm)	floral bract length (m)	floral bract width (mm)
Wills.n.	xitchetucknensis	FL	pap	34	110	14	7.8	7	5.8	1.2	5.3	9	3
Simpson368	triloba	FL	pap	27.5	90	13.5	5.5	7	8	1.3	7	11.5	2
W.J. Dress10331	triloba	FL	pap	49	135	16	12.4	8.8	8.8	1.5	7.5	14	3
A.P. Garbers.n.	triloba	FL	pap	52.5	60	8.1	7.7	5.1	5.2	1	5.5	8.2	3
A.A. Eaton726_1/2	triloba	FL	pap	49.5	80	9	6.6	7	7.5	1.1	7.5	13	3
A.A. Eaton606_1/2	triloba	FL	pap	58	110	8	9	7	6	1	6	11	3
A.A. Eatons.n.	triloba	FL	pap	33	88	6	5.5	7				14	5
J.B. McFarlin4021	triloba	FL	pap	25.5	75	8.5	3.9	7.1	7	1.5	7.5	10	3
D.B. Ward6529	triloba	FL	pap	37.7	105	12	72	6	6	1.6	6	13	3.2
J.H. Simpson368	triloba	FL	pap	27.5	90	13.5	2.2	7	8	1.3	7	11.5	2.4
A.P. Garber2403	triloba	FL	pap	38	135	19	8.2	8	7.5	2	6.2	11.5	2
A.P. Garber2404	triloba	FL	pap	43	77	11	7	6.3	6	1	9	10	2
D.S. Correll47714	triloba	FL	pap	35	85	10	7.3	7	7	1.9	7	12	3.4
J.K. Small10070	triloba	FL	pap	21			13	5	7	1	7.2	10	3
A.P. Garbers.n.2	triloba	FL	pap	49.1	170	21	3.9	7.5	8.2	2	7	12	2.5
J.K. Small11048	triloba	FL	pap	32.4	105	7	6.4	5.2	6	1.6	4.8	12	3
A.A. Eaton779_1/2	triloba	FL	pap	28.5	55	4	6.3	6	6	1.6	7.6	12	2.9
L.C. Anderson24790	triloba	FL	pap	48.5			8.4	8	8	2	7.8	13.5	3.5
C.J. Sheviak6255a/6342	triloba	FL	pap	35.5	98	7.2	10.5	7.5	8	2	7	15	3
A.A. Eatons.n.2	triloba	FL	pap	34.5	76	11	7.1	6	6	1.7	7.5	7	4.1
S.W. Leonard7046	triloba	FL	pap	56	228	18.8	11.6	7.9	7	2	7.1	11	4.3
R.K. Godfrey73132	triloba	FL	pap	27.9	80	13	7.4	6.7	7.1	1.7	6.6	11.9	3.1
R.K. Godfrey52789	triloba	FL	pap	28.8	120	16	6.6	6.2	8	1.4	7.7	12.5	3.5
R.K. Godfrey65134	triloba	FL	pap	41.5	118	8.9	7.7	7.6	7.6	1.5	7.2	10	3
L.C. Anderson21485	triloba	FL	pap	32	58	8.8	8.4	8	7.5	1.8	7.4	14.5	3.1
L.C. Anderson24790	triloba	FL	pap	48.5			8.4	8	8	2	7.8	13.5	3.5
H. Neel503	triloba	FL	pap	31			10.2	8	8	2	7	13	3.2
L.C. Anderson24757	triloba	FL	pap	24.7	72	12.6	6.5	6.5	6	2	7	12	3.4
R.K. Godfrey73132	triloba	FL	pap	36.5	95	13	8.8	6.7	7.1	1.7	6.6	11.9	3.1
S.L. Orzell18880	triloba	FL	pap	52.75	236	12	8.1	8	8.1	2	7.5	10	3.1
M.E. Francis76	triloba	FL	pap	37	75	7.3	6.6	6.4	7.1	1.5	7.3	12	3.4
R.K. Godfrey73132	triloba	FL	pap	36.5	95	13	8.8	6.7	7.1	1.7	6.6	11.9	3.1
W.Batsons.n.	odorata	SC	glab	51	350	25.8	11.6	11	10.5	2.1	9	18	3.5
R.D.Thomas132800	odorata	LA	glab	49	195	11	12.2	9	9	2	8	13	3
G.Archeneaux245	odorata	LA	glab	52.8	300	15	20.9	14	14	2.1	10.5	18	4
A.S.Hitchcocks.n.	odorata	FL	glab	31			6.7	11	8.5	2.1	9.5	15	3.5
M.S.Swinples.n.	odorata	FL	glab	38	75	11	7	9	9.2	2	8.3	12.5	4
J.K.Small_J.J.Carters.n.	odorata	FL	glab	54.7	240	8	7	9.9	9	2.5	10	14.8	3.2
A.A.EatonW526	odorata	FL	glab	57.3	237	12.5	8.6	8.5	7.7	2.1	10	16	3
A.B.Louglois330	odorata	LA	glab	76.3	375	18	11.8	12	12	2.2	13	23	5
H.Fosters.n.	odorata	FL	glab	35	153	6	9.5	10	10	3	12	6	3
D.B._S.S.Ward4363	odorata	FL	glab	44.3	82	8	9.2	11.5	10	3.8	12	16	3.5

Tissot_Wests.n.	odorata	FL	glab	62.5	215	22.5	11.3	10	10	2.8	9.2	15	4
J.R.Watsons.n.	odorata	FL	glab	41.1	80	10.5	7.4	13	12.8	2	14	17	4.8
W.M.Whittens.n.	odorata	FL	glab	58.1	195	23.9	13.5	11.1	12	2.1	9.4	14	4.1
K.A.Vliet32	odorata	FL	glab	42.5	200	23	13	11	11	3	12.6	18	5.1
W.A.Murrills.n.	odorata	FL	glab	55.7	200	14	11.5	8.5	9	1.9	9.5	11	3.2
B.J.Herring999	odorata	FL	glab	52.1	185	25.5	11.6	11.1	11.1	2	11.4	17	4.1
L.C.Anderson7787	odorata	FL	glab	49.8	239	32.5	14.2	9	9	2.5	8.5	17	4.5
S.L.Orzell18949	odorata	FL	glab	46.3	192	24	14.5	11	11	3.3	11.1	20	5
D.B.Ward3804	odorata	FL	glab	57.5	70	10	8.5	12	11	2.5	12	15	4.5
R.Garretts.n.	odorata	FL	glab	58	160	8	5	8	8	2	8.4	15	4.2
E.P.St.Johns.n.	odorata	FL	glab	48.5	135	14	10.4	11.5	11.5	2.4	13	16.5	5
R.D.Thomas82702	odorata	LA	glab	67.5	173	16	15	11	11	2	10	18	4
R.D.Thomas69546 _483	odorata	LA	glab	48	205	15	11	9	9	2	9.6	12	3.2
Watson_Murrills.n.	odorata	FL	glab	41	153	10	9.2	9.1	10	1.5	10.8	13.5	3.5
H.E.Ahles51328	odorata	NC	glab	69	368	20.5	10.5	11	11	2.5	12	16	5
F.H.Sargent13	odorata	VA	glab	61	490	16	11.7	12.2	12.2	2.5	11.5	18.5	4
A.M.Laessles.n.	odorata	FL	glab	49.1	130	8	8.9	11	11	1	10.8	12	4
D.W.Ward5452	odorata	FL	glab	84.7	390	38	12	12	12	2.4	11	16	3.8
J.H.Daviss.n.	odorata	FL	glab	47.5	210	9	6.7	11.5	11.5	2.1	11.6	16.5	2
R.K.Godfrey78270	odorata	FL	glab	32.8	150	12	10.3	9	10	2.2	10.5	12	4
T.MacClendon747	odorata	FL	glab	36	105	6.8	10.5	9	9	2.9	11.6	16	5.1
C.R.Bell5383	odorata	SC	glab	31.8	174	18	8.7	10	10	2	11	13	5
L.C.Anderson8917	odorata	FL	glab	48	300	37.5	13.5	10.3	11	2	11.5	16	4
L.C.Anderson7787	odorata	FL	glab	39	239	32.5	14.2	12	13	3	11.7	17	4.5
L.C.Anderson18547	odorata	FL	glab	43.1	216	25	10.7	10	10	2.2	11.2	17	4
R.K.Godfrey80201	odorata	FL	glab	38	150	18.4	11.2	9	9	1.8	9	14	4
J.R.Burkhalter9143	odorata	FL	glab	41.5	340	23	10.4	9.5	13	2	10.5	13	3.5
R.K.Godfrey50163	odorata	NC	glab	55	126	20	12	9.5	9	1.4	10.6	13.5	3.2
R.Lassiter2212	odorata	FL	glab	63	290	812	16	12	11	1.9	12	17	3.5
R.S.Mitchell912	odorata	FL	glab	45.8	210	24.8	8.7	9.2	9	1.7	11	13	4.2
L.C.Anderson16103	odorata	FL	glab	45.2	120	16	16.4	10	10	1.5	10.5	18	4.5
T.Miller10	odorata	FL	glab	61.4	180	13.6	4.2	8	9	2	12.2	9	4
R.K.Godfrey73132	odorata	FL	glab	70.4			11	9.2	9	2.6	11	14.5	3.5
R.K.Godfrey65134	odorata	FL	glab	42.9	74	6.8	9.3	11	11	1.8	9.1	14.3	3
K.M.Neubig92	odorata	LA	glab				10	8	9	2	9.7	14	3.5
H.Abdullahs.n.	odorata	LA	glab	34	84	19.5	7.4	9	9.5	1.2	9.5	12.8	3.1
K.K.Turner6	odorata	LA	glab	45.8			8.7	9	9	1.5	10.5	15	3.5
S.Maliakal168	odorata	LA	glab	51	133	14	8.3	14	14	3	13.5	16	4
T.C.Miller78	odorata	LA	glab	34.4	273	14.8	6.8	8.9	14	3	8.6	22	3
A.M.B.Rekas65	odorata	LA	glab	43.7	140	11	11	9	9	1.2	9.5	25	2
A.M.B.Rekass.n.	odorata	LA	glab	40.5	240	13	9.7	9	9	2	10	16.5	3.5
F.N.Lee141B	odorata	LA	glab	45.5	297	16	5.9	10	12.5	2	14	13	3.2
C.A.Brown21784	odorata	LA	glab	64	240	15	13.1	12	11	2	11	15	5

H.P.Naquin236	odorata	LA	glab	44.9			14.5	11	11	1.4	10	12	4.2
J.Bruser100	odorata	LA	glab	49	225	19	7.8	9.4	9.5	2	12.1	14	3.3
C.Reid4772	odorata	LA	glab	81.4	375.6	28	18	10	12.8	2	11	16	5
C.A.Browns.n.	odorata	LA	glab	50.5	280	23	12.4	12.1	12	1.9	10	16.5	4.5
R.L.Ginn22	odorata	LA	glab	38.3	140	13.8	4.7	10	10	1.6	9.5	14.5	2
R.Aycock221	odorata	LA	glab	44	345	15	13	11	11.5	1.6	10	22	4.5
U.A.Brown8750	odorata	LA	glab	52.3	235	9	7.1	11	11.5	1.4	11	17	4
R.D.Thomas82702	odorata	LA	glab	67.5	173	16	15	11	11	2	12	18	4
S.Scarce349	odorata	LA	glab				9.8	12	12	2.5	13.5	19	4.2
L.Urbatschs.n.	odorata	LA	glab	39.5	160	16.5	10	10	11.1	3	12	12	4
G.L.Montz5970	odorata	LA	glab	76	235	18	12.5	11.6	11.5	1.9	12	19	5
C.M.Allen1784	odorata	LA	glab	27.5	175	5	11.9	11	11	1.7	11	14	3.5
C.Reid6860	odorata	LA	glab	55.2	74	6	6.5	10	11.1	2	11	14.5	4
B.B.Exners.n.	odorata	LA	glab	67.2	478	24.1	12.6	13	13	1.3	11	17	4.3
C.A.Brown1511	odorata	LA	glab	59.8	369	19	14.5	10	10	2.1	11.3	16	5
J.H.Peng7	odorata	LA	glab	39	130	14	7.5	9.5	10	3	10.2	13	4
J.D.Schroer84	odorata	LA	glab	68.3	220	20	14	12	12	2	10.8	16	6
C.A.Brown8343	odorata	LA	glab	36.1	135	17	7	11	11	2.1	12	11	3.3
R.D.Thomas69546_483	odorata	LA	glab	23.5	205	15	11	10	10	2	10.6	12	3.2
R.K.Godfrey73132	odorata	FL	glab	70.4			11	9.2	9	2.6	11	14.5	3.5
F.R.Fosberg17527	odorata	VA	glab	50.5	420	17.6	6.7	9.9	11	2.5	10.4	12	3.9
L.HubrichtB2740	odorata	VA	glab	51			14.5	11	12	1.5	10.9	20	5
R.K.Godfrey73132	odorata	FL	glab	70.4			10.6	9	9	2.6	11	14.5	3.5
G.P.Fleming11537	odorata	VA	glab	56	230	16	9.2	10	10	2.1	13.5	16	5
G.P.Fleming11540	odorata	VA	glab	64.5			8.6	10	10	2.5	11.6	16	4
L.E.Loetterle510	odorata	VA	glab	60.1	205	13.7	8.6	9.9	9.9	2.5	9.1	17	3.8
R.W.Menzel346	odorata	VA	glab	52.3	288	10	9	10.1	10.1	2	10.6	13	4
J.Guldins.n.	odorata	AL	glab	46.4	323	10.5	8.4	8	9	2.1	12	12	3.5
T.McMahon32	odorata	VA	glab	59.3	90	7	8.4	10.2	10.3	2	12	18	5
C.Burns94	odorata	FL	glab	73.8			13	9	9	2.9	11.3	14	5.1
J.K.Small_J.J.Carter1042	odorata	FL	glab	74.1	209	10	8.2	11	10	2.1	12	12	3
J.F.Joors.n.	odorata	TX	glab	30.4			9.7	9	9	1.4	8	10.1	3.1
J.Reverehon2174	odorata	TX	glab	48.5	155	12	12.4	10.1	10	2.1	10	12	3
A.W.Chapmans.n.	odorata	FL	glab	47.5	285	29	10.5	10.2	11	2	11	16.5	4.5
A.W.Chapmans.n.	odorata	FL	glab	37.5	210	24	8.4	10.2	10	2	11.4	15	4
T.Miller10	odorata	FL	glab	82.9	228	18	8.9	9	9	2	12.2	9	4
J.K.Small9284	odorata	FL	glab	62.6	234	20	12.2	10	10	1.8	11	18	3.9
A.Fredholm6205	odorata	FL	glab	67.9	185	12	10	10.1	11	2	12	19	3
M.L.Fernald11308	odorata	VA	glab	36.7	318	26	8.7	11	10	2.9	11.9	15	3.5
G.Nesom7854	odorata	LA	glab	42.8	220	17	11	10.5	10.5	2.1	12	17	3.3
C.J.Sheviak816	odorata	FL	glab	52.7	80	10	7.5	10	10	2	11	12	3
C.J.Sheviak817	odorata	FL	glab	45.7	113	19	9.5	9	10	2.8	12	14.9	5
C.J.Sheviak814	odorata	FL	glab	46.5	200	12.5	7	9	9	2	11	13	4.2

Fig. 4. [Legend for following two pages]. Molecular data matrix used in the molecular phylogenetic analyses (an 'X' indicates successful amplification).



ID #	Species/Variety	Collection Site	Collector	Coll.Date	ACO	ITS	matK	ndhj	trnLF	trnSM	ycf1 3'	Herbarium specimen	Photo Voucher
	S. brevilabris												
1e	S. brevilabris	Walker Co., TX	Eric Keith	4/6/07		x	x	x	x	x	x	82213	yes
1g	S. brevilabris	Levy Co. (Goethe St.For.), FL	Scott Stewart	4/1/06	x	x	x	x	x	x	x		
	ochroleuca/casei												
16a	S. ochroleuca	Oconee Co., SC	Jim Fowler	10/25/02		x	x	x	x	x	x	80494	yes
16b	S. ochroleuca	Craig Co., VA	Stan Bentley	9/13/03	x	x	x	x	x	x	x		
	cernua/parksii /Coastal Plain/Piedmont												
4m	S. cernua (deep south race)	Liberty Co., FL (via UFL)	Scott Stewart	7/3/06	x	x	x	x	x	x	x		yes
4cc	S. cernua (JF-B1)	Marlboro Co., SC. (Bennettsvl.)	Jim Fowler	10/21/06	x	x	x	x	x	x	x		yes
4dd	S. cernua (JF-B3)	Brunswick Co., NC (BoilSprLk)	Jim Fowler	10/29/06	x	x	x	x	x	x	x		
4ee	S. cernua - Chadd's Ford (17k)	cultivar - White Flwr. Farm, CN	Lucy Dueck/KC	8/19/06	x	x	x	x	x	x	x		
sc9a	S. cernua	Little Creek Reservoir, James City, VA	M.C. Pace	10/25/13	x	x		x	x	x	x	Pace s.n.	yes
sc15b	S. cernua	Wayne Co, GA	M.C. Pace	11/6/13	x	x	x	x	x	x	x	Pace s.n.	yes
20a	S. parksii - a (sp5)	Brazos Co., TX	Cathy Walters (DNA)	10/8/03		x	x	x	x	x	x	TAMU	yes
20b	S. parksii - b (sp137)	Brazos Co., TX	Cathy Walters (DNA)	10/22/03		x	x	x	x	x	x	TAMU	
	S. eatonii												
7a	S. eatonii	Clay Co., FL	PMBrown	5/8/03		x	x	x	x	x	x		
	S. floridana												
8b	S. floridana	Bradford Co., FL	PMBrown	4/2003	x	x	x		x	x			
8f	S. floridana	Duval Co. (Jennings St.For.), FL	Scott Stewart	4/2/06	x	x	x	x	x	x	x		
	S. lacera												
10d	S. lacera var. lacera	Oconto Co., WI	Kip Knudson	7/28/06	x	x	x		x	x			yes
	S. lacera var. gracilis												
11a	S. lacera var. gracilis	Pickens Co., SC	Jim Fowler	7/12/03	x	x	x	x	x	x	x	80497	
11c	S. lacera var. gracilis	Forsyth Co., NC	David McAdoo	8/8/2004	x	x	x	x	x	x	x	80504	
	S. igniorchis												
12b	S. laciniata	Charlton Co., GA	Jim Fowler	6/18/05	x	x	x	x	x	x	x	80503	
slt5a	S. laciniata	LA			x		x	x	x	x	x		
	S. longilabris												
13a	S. longilabris	Brunswick Co., NC	Frank Galloway	10/31/03	x	x	x	x	x	x	x		
13c	S. longilabris	Levy Co., FL	Scott Stewart	11/20/04	x	x	x	x	x	x	x		
	S. lucida												
SL13d	S. lucida	Ozark National Forest, AR	M.C. Pace	19-May-13	x	x	x	x	x	x	x	M.C. Pace 576	yes
sl23a	S. lucida	TN	M.C. Pace		x	x	x	x		x	x	Pace s.n.	yes
	S. magnicamporum												
15c	S. magnicamporum	Ozark Co., MO	Bill Summers	10/18/04		c	x	x	x	x		80501	
15e	S. magnicamporum	Grimes Co., TX	Joe Liggio	10/31/04	x	x	x	x	x	x		80489	
15f	S. magnicamporum - (sm)	Walworth Co., WI	Jeff Hapeman	9/16/06	x	x	x	x	x	x	x	82207	
sm7h	S.magnicamporum	NM	M.C.Pace	9/21/13	x	x	x	x	x	x	x		
sm11a	S.magnicamporum	Russell Co., VA		10/17/13	x	x	x	x	x	x	x		
sm12a	S.magnicamporum	GA	Jim Fowler	11/3/13	x	x	x	x	x	x	x		yes
sm15a	S.magnicamporum	Marthaville, Lambton Co, Ontario, Canada	Oldham	9/7/11	x	x	x	x	x	x	x	Oldham 39307 (NYS)	
sm16a	S.magnicamporum	Black Belt Prairie, Starkville, MS	McCabe	10/29/91		x	x		x	x		McCabe s.n. (NYS)	
sm17a	S.magnicamporum	Cowan, Franklin co., TN	Sheviak	11/8/06	x	x		x	x	x	x	Sheviak 7064 (NYS)	
sm19a	S.magnicamporum	Chandlerville, Cass Co., IL	Sheviak	9/25/81	x	x	x		x	x	x	Sheviak 2142 (NYS)	

	S. odorata												
17g	S. odorata	Levy Co., FL	Scott Stewart	11/20/04		x	x	x	x	x	x		
17i	S. odorata	San Jacinto Co., TX	Joe Liggio	10/24/04		x	x	x	x	x	x	80488	
17opq	S. odorata	Effingham Co., GA	Darlene (L) Vincent	12/2/06	x	x	x	x	x	x	x	82211	yes
17z	S. odorata	Levy Co. (Goethe St.For.), FL	Scott Stewart	11/20/04		x	x	x	x	x	x		
So1d	S. odorata	Piney Island, NC	M.C. Pace	10/26/2013	x	x	x	x	x	x	x	Pace s.n.	yes
So5h	S. odorata	Bridge, Brunswick Co., NC	M.C. Pace	11/4/2013	x	x	x		x	x	x	Pace s.n.	yes
So10i	S. odorata	Roadside, Taylor Co., FL	M.C. Pace	11/9/2013	x	x	x		x	x	x	Pace s.n.	yes
so11	S. odorata	Kentucky	Sheviak			x	x		x	x	x	Sheviak 2408	
so12	S. odorata	Tennessee	Durr	10/1/2001	x	x	x	x	x	x	x	Durr s.n.	
so13	S. odorata	Back Bay NWR, Princess Anne Co., VA	Statler	11/4/2006	x	x			x	x	x	Statler s.n. (NYS)	
	S. ovalis var. ovalis												
18b	S. ovalis var. ovalis	Columbia Co., FL	PMBrown	11/11/06		x	x		x	x	x		
	S. ovalis var. erostellata												
19c	S. ovalis var. erostellata - c	Rutherford Co., NC	Jim Fowler	10/11/03		x	x	x	x	x	x		
19d	S. ovalis var. erostellata/hybrid?	McCormick Co., SC (F659D)	Lucy Dueck	10/11/06		x	x	x	x	x	x	82210	yes
	S. praecox												
22c	S. praecox (spr3)	Brazos Co., TX	Cathy Walters (DNA)	4/30/03		x	x		x	x	x	TAMU	yes
22d	S. praecox	Bradford Co., FL	Jim Fowler	4/2003		x	x	x	x	x			
22i	S. praecox	Alachua Co., FL	Scott Stewart	5/20/05		x	x	x	x	x	x		
	S. triloba												
FL36	S. triloba	Florida Panther NWF, FL	M.C. Pace	11-Jan-13	x	x	x		x		x	Pace s.n.	yes
FL130	S. triloba	Fakahatchee Strand, FL	M.C. Pace	16-Jan-13	x	x	x	x	x	x		Pace s.n.	yes
FL135	S. triloba	Corkscrew Swamp Sanctuary, FL	M.C. Pace	17-Jan-13	x	x	x	x	x	x	x	Pace s.n.	yes
FL146	S. triloba	Wakulla Co., FL	M.C. Pace	9-Nov-13		x	x			x	x	Pace s.n.	yes
Flpolk	S. triloba	Polk Co., FL	Steve Orzell			x	x	x		x	x	Orzell 26717	yes
FL164	S. triloba	Citis Co., FL	M.C. Pace				x			x	x	Pace s.n.	yes
FL165	S. triloba	Jesserson Co., FL	M.C. Pace				x			x	x	Pace s.n.	yes
17h	S. odorata	Collier Co. (Naples), FL	Scott Stewart	5/25/05			x		x	x	x		
17m	S. odorata	Columbia Co.,FL (Ich.Spr.S.P.)	PMBrown	11/11/06			x	x	x	x	x		
17n	S. odorata (dwarf)	Levy Co., FL	PMBrown	11/11/06	x	x	x	x	x	x	x		
Ich2	S. x ichetuckneensis	Columbia Co., FL	PMBrown	11/11/06		x	x	x	x	x			
si2	S. triloba	Ichetucknee Springs, Columbia Co., FL	M.C. Pace	11/13/13	x	x	x	x		x	x	Pace s.n.	yes
	S. sylvatica												
25d	S. sylvatica	Alachua Co., FL	Scott Stewart	5/20/05	x	x	x	x	x	x	x		
	S. torta												
28b	S. torta (sm flwr & stem)	Miami-Dade Co., FL	PMB/RH	6/20/06	x	x	x		x	x	x	82221	yes
28c	S. torta (flwr only)	Miami-Dade Co., FL	PMB/RH	6/20/06	x	x	x		x	x	x	82221	yes
	S. tuberosa												
26b	S. tuberosa (late blm.)	S. Aiken Co., SC	Lucy Dueck	8/19/03	x	x	x	x	x	x	x	82218	yes
26c	S. tuberosa	Monroe Co., WV	Stan Bentley	9/6/03	x	x	x	x	x	x	x		
	S. vernalis												
27k	S. vervalis	Alachua Co., FL	Scott Stewart	5/20/05		x	x		x	x	x		
27m	S. vernalis	Bradford Co., FL	Scott Stewart	5/25/05	x	x	x		x	x	x		
	Outgroup taxa												
Sala	Sacoila lanceolata var. lanc.	Levy Co., FL	Scott Stewart	6/1/04	x	x	x		x	x	x		
Dici	Dichromanthus cinnabara		G. Salazar		x	x	x	x	x	x	x		
Mepo	Mesadenella polyantha		G. Salazar		x	x	x		x	x	x		
Dedi	Deiregyne diaphana		G. Salazar			x	x		x	x	x		
Scll	Schiedeella llaveana		G. Salazar			x	x		x		x		



Fig. 5 Comparison of holotype and lectotype specimens. Red box indicates sheets/names which are included under *Spiranthes triloba*.



Fig. 6. Holotype specimen of *Gyrostachys* (*Sprianthes*) *triloba*. Florida: Lee County, Fort Meyer, collected 11 December 1891, J. H. Simpson 368 (NY).

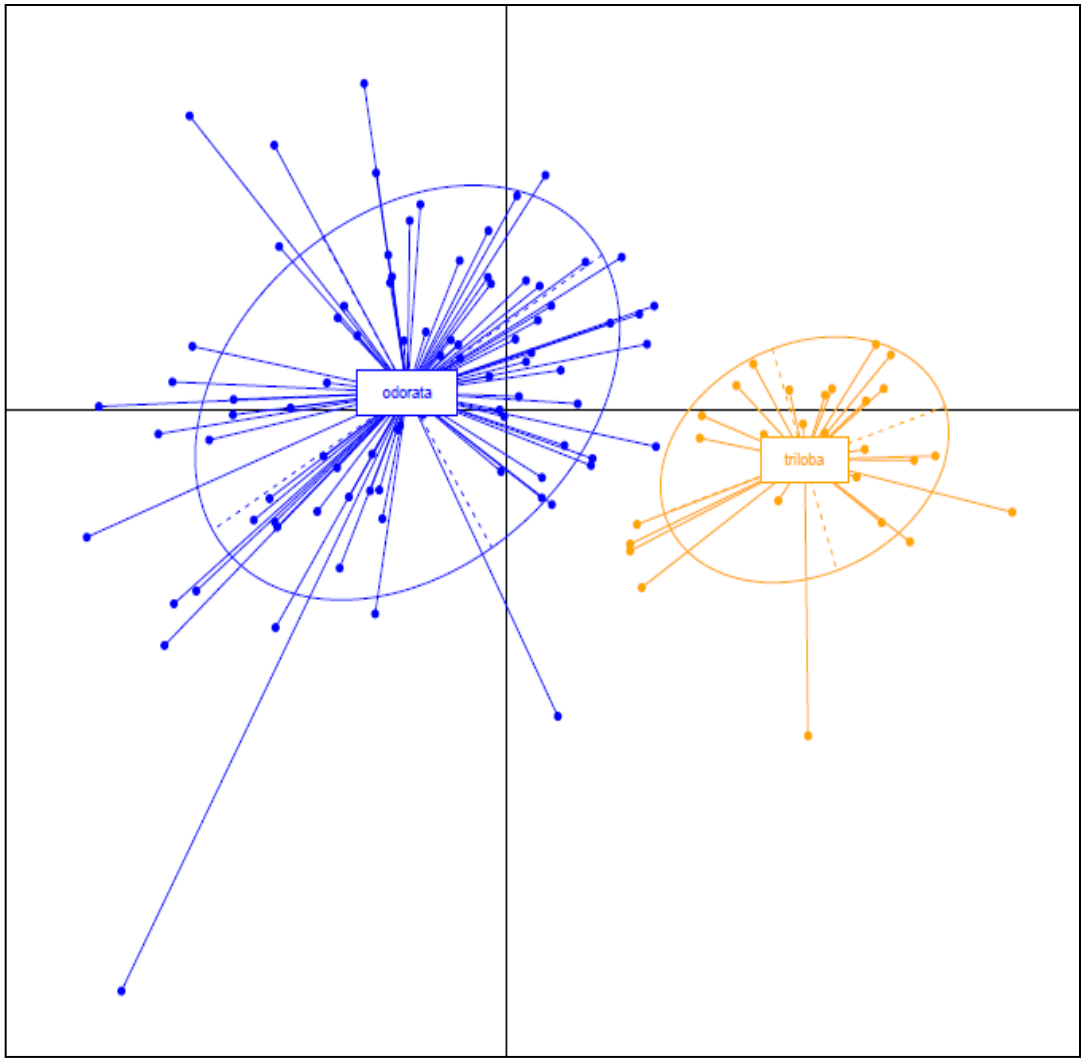


Fig. 7. Morphometric analysis of pressed herbarium specimens. Features measured include plant height, raceme length, leaf length and width, lateral sepal length and width, labellum length and width. Blue indicates *S*. *odorata*, orange indicates *S*. *triloba*. This color scheme also corresponds to glabrous vs. papillate individuals.

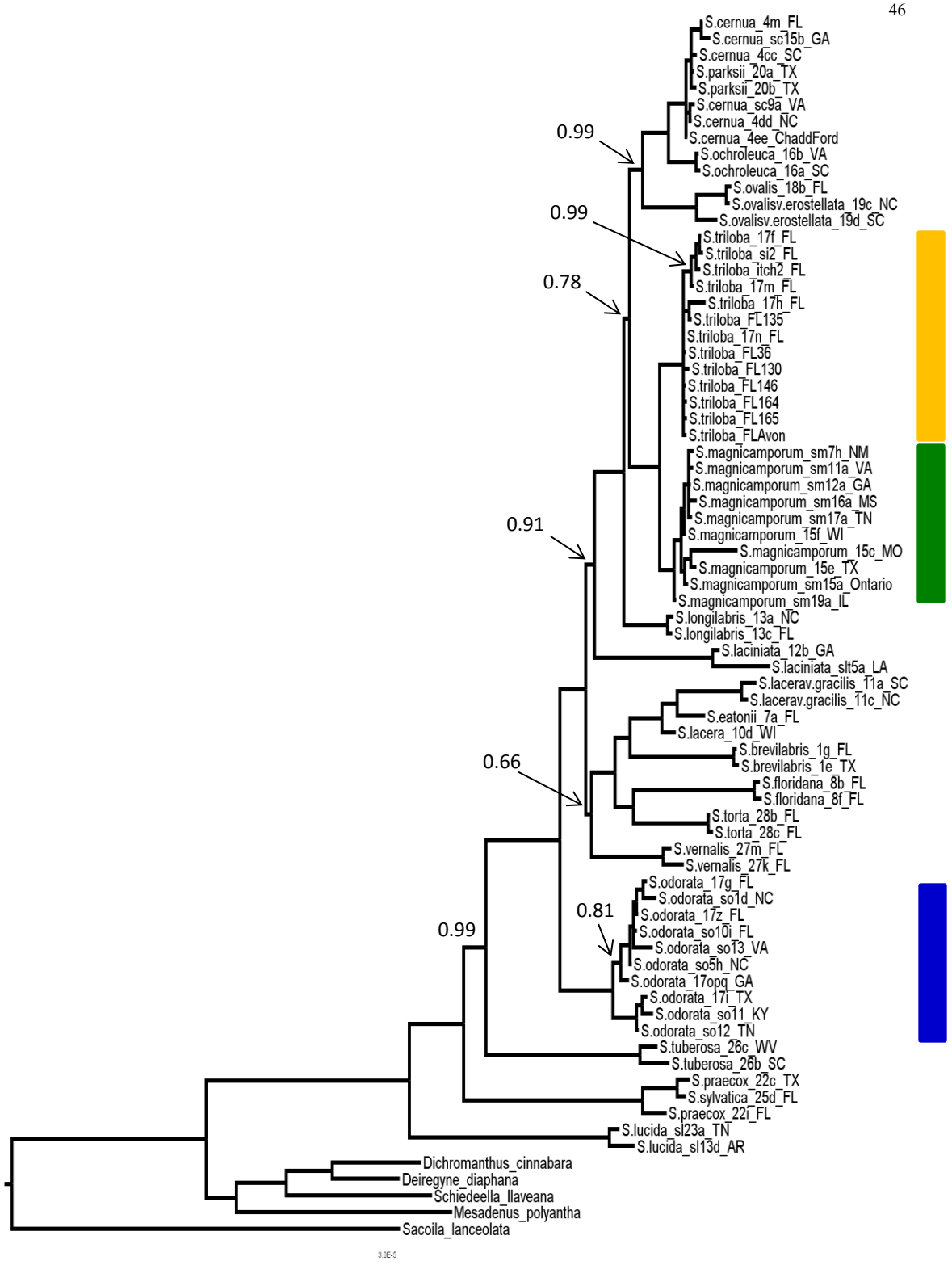


Fig. 8. Bayesian Majority Rule tree, combined nuclear + plastid data. Posterior probabilities = 1, unless otherwise noted. Position of *S. triloba* indicated by the blue bar, *S. odorata* s.s. indicated by the orange bar, *S. magnicamporum* indicated by the green bar.

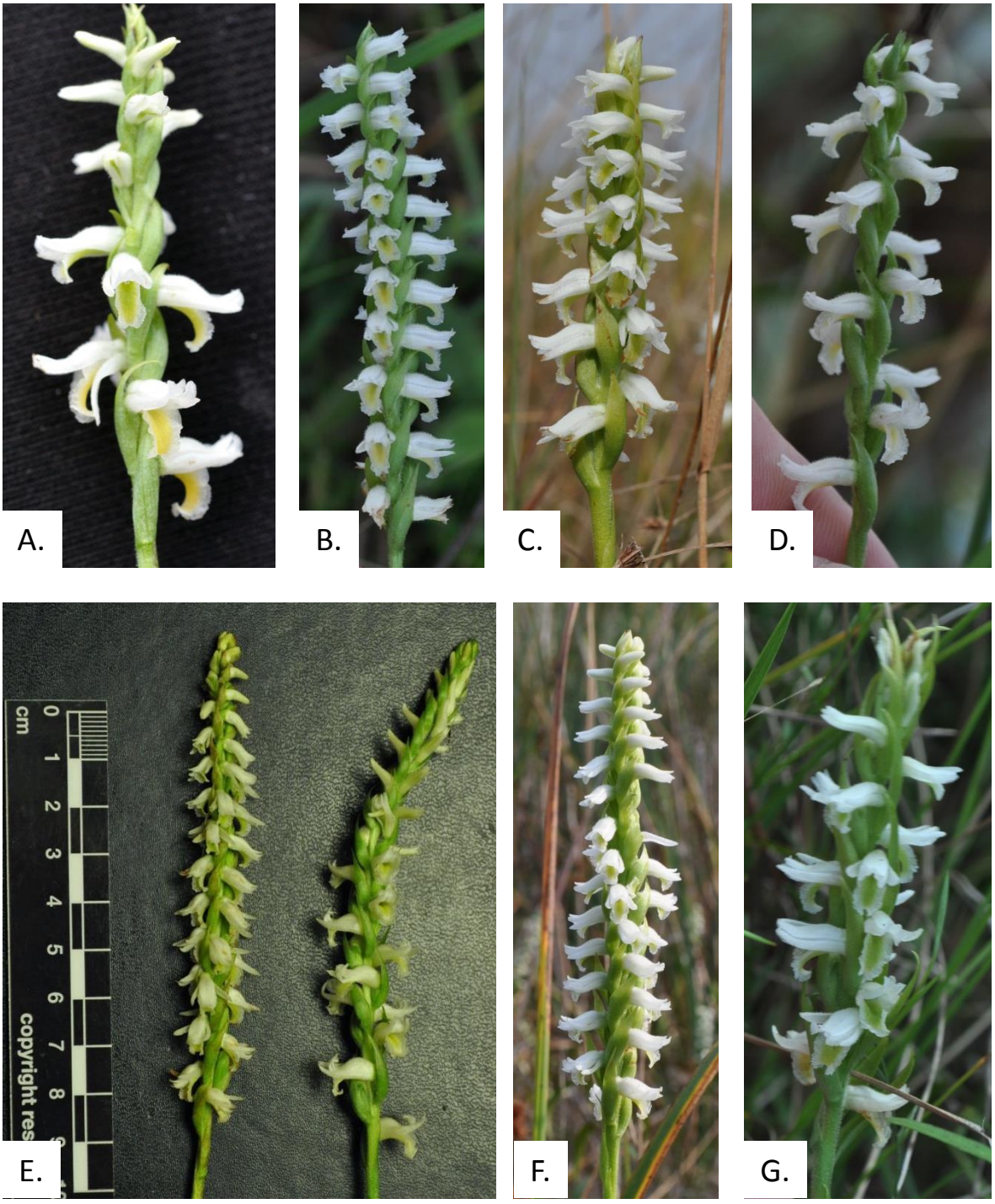


Fig. 9. *Spiranthes triloba*. **A.** Collier Co., FL, sample FL36 . **B.** Citrus Co., FL, sample FL164. **C.** Polk Co., FL, sample FLAvon. **D.** Columbia Co., FL, sample si2, holotype location of *S*. ×*itchetucknensis*. **E.** *S*. *triloba* Wakulla Co., FL, sample FL146 (L) & *S*. *odorata*, Leon Co., FL, sample so5h (R). **F.** Wakulla Co., FL. **G.** Collier Co., FL, sample FL135.

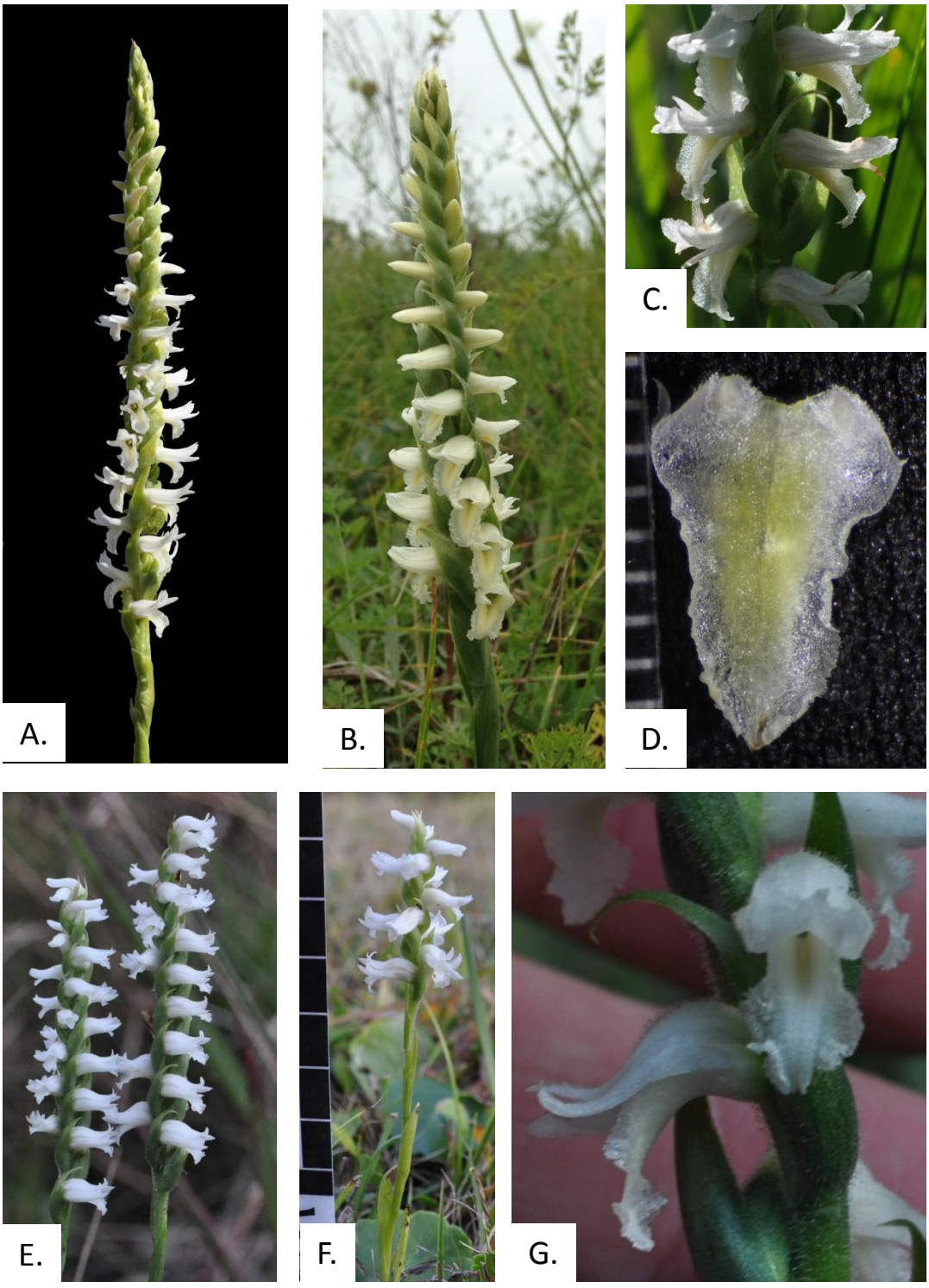


Fig. 10. A-D. *Spiranthes magnicamporum*. **A.** Guadalupe Co., NM, sample sm7h. **B & D.** Waukesha Co., WI. **C.** Dane Co., WI. **D.** Labellum, flattened. **E-G.** *Spiranthes cernua*. **E.** Wayne Co., GA (sample sc15b). **F.** Brunswick Co., NC, sample location of 4dd. **G.** Sussex Co., DE.

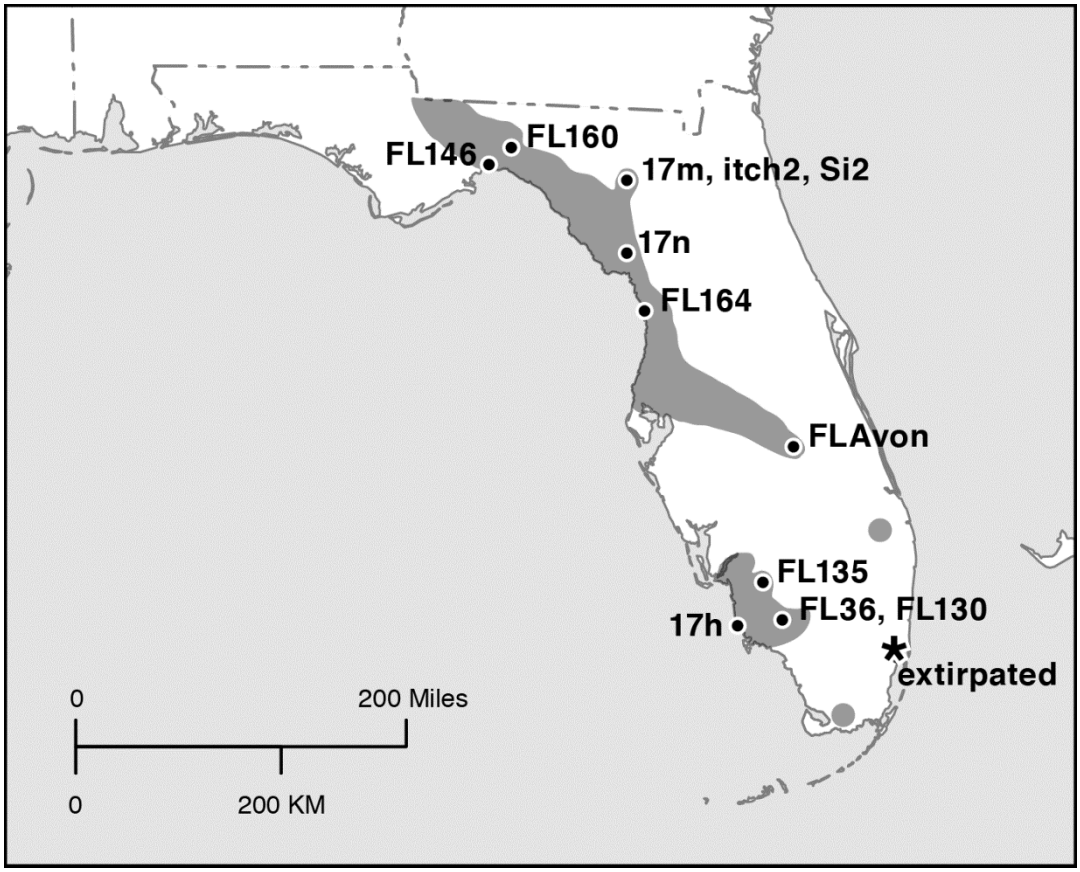


Fig. 11 Distribution map of *Spiranthes triloba*. Gray area indicates the known range based on annotated herbarium specimens and field work (including two disjunct populations in southern and southeastern Florida. Labeled black circles denote the locations of molecular phylogenetic *S*. *triloba* samples. The asterisk indicates the now extirpated population centered on the Lemon City neighborhood of Miami.



A.



B.



C.

Fig. 12. Habitat of *Spiranthes triloba*. **A.** Frequently burned calcareous prairie (foreground) with pine flatwood (background), Collier Co., FL. **B.** Wet roadside, Citrus Co., FL. **C.** *Taxodium-Acer* wet forest, holotype location of *S.* ×*itchetucknensis*, Columbia Co., FL.



Fig. 13. Observed pollinators & potential pollinators. **A & B.** *Bombus impatiens* removing pollinaria from *S. odorata*. Brunswick Co., NC. 2 November 2013. **C.** cf. *Augochlora pura* visiting *S. triloba*. Polk Co., FL. 16 December 2014. **D.** *Apis mellifera* visiting *S. triloba*. Polk Co., FL. 16 December 2014.

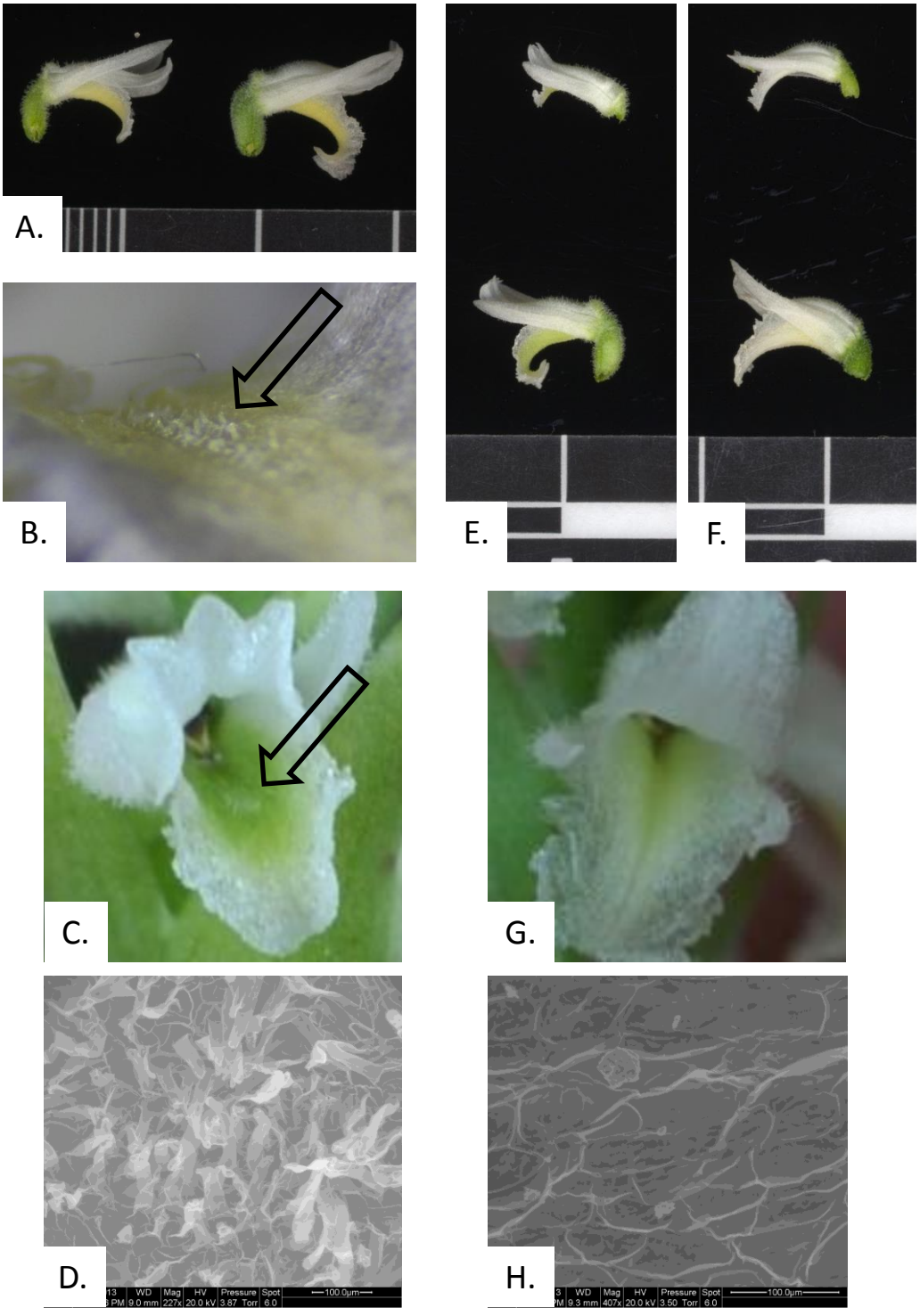


Fig. 14. Floral morphology. **A, C, D, E.** *S. triloba*. **B.** *S. magnicamporum*, arrow indicates labellar papillae. **F, G, H.** *S. odorata*. (A, E, F depicted at the same scale.) **A.** Lowest-most flowers of plants growing within 2 km of each other. **B & E.** younger flowers above, older flowers below. **C.** Arrow indicates labellar papillae **D.** SEM image of *S. triloba* papillate labellum. **H.** SEM image of *S. odorata* glabrous labellum.

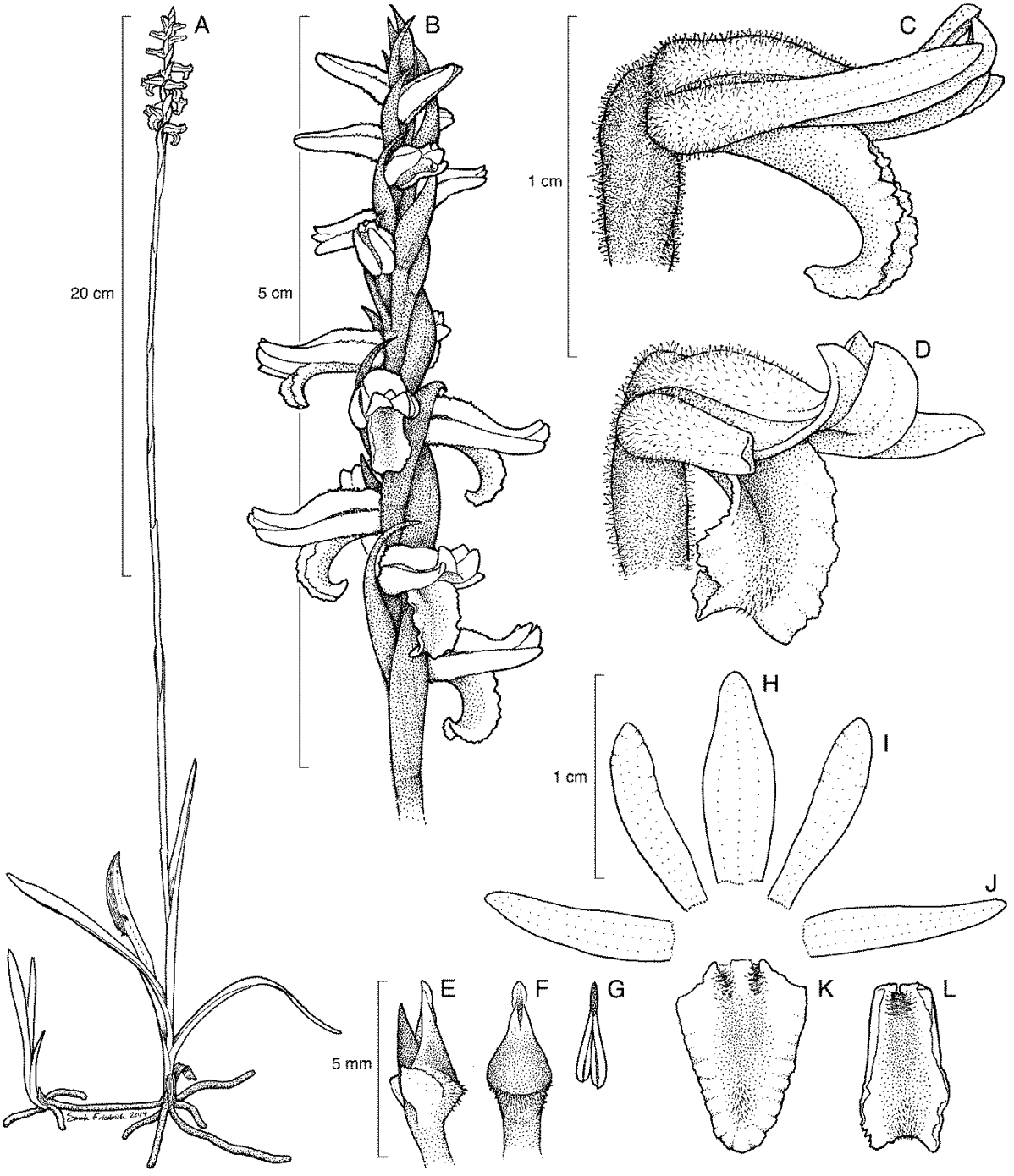


Fig. 15. Line drawing of *Spiranthes triloba* (from live plants). **A.** Whole plant. **B.** Inflorescence. **C.** Flower in profile. **D.** Flower in ¾ view. **E-G.** Column. **E.** Column, side-view. **F.** Column, ventral-view. **G.** Pollinaria with viscidium. **H-L.** Dissected-view flower. **H.** Dorsal sepal. **I.** Dorsal petal. **J.** Lateral sepal. **K.** Labellum, flattened. **L.** Labellum, natural state.

CHAPTER 2

Spiranthes igniorchis, a new and rare cryptic endemic of the Central Florida Ridges and Osceola Plain fire-maintained subtropical grasslands.

Pace, M.C., S.L. Orzell, E.L. Bridges, & K.M. Cameron

Abstract—Species complexes and the possible cryptic species within them are morphologically problematic to identify, very closely related, and often recently evolved, thereby posing inherent challenges to taxonomists, systematists, and conservationists. *Spiranthes* is a systematically challenging orchid genus in which species complexes and cryptic species are a common phenomenon. Following an integrated approach combining molecular phylogenetic, morphometric, and phenological data sources, we propose the description of *Spiranthes igniorchis*, a new and rare species endemic to frequently-burned subtropical grasslands of Avon Park Air Force Range in Central Florida. *Spiranthes igniorchis* is very closely related and morphologically similar to *Spiranthes longilabris*. A discussion of its distinguishing characteristics, and challenges associated with species identification within *Spiranthes*, generally, are presented.

Key words—Avon Park Air Force Range, cryptic species, fire, integrative taxonomy, North American Coastal Plain, phenology, *Spiranthes cernua* species complex, subtropical grasslands

As habitat continues to be lost at alarming rates, a thorough understanding of biodiversity is critical to elucidating ecosystem services, complex patterns of interspecific interactions, and implementing conservation strategies in a time of continually limited resources and global climate change. This is particularly important in underappreciated but heavily developed biodiversity hotspots such as the North American Coastal Plain (Jenkins et al. 2015, Noss et al. 2015). Species complexes –groups of taxa which are morphologically difficult to distinguish, very closely related, and typically recently evolved– pose intrinsic challenges to taxonomists and conservationists alike. Integrative approaches to species delimitation and taxonomic status, in which advances in molecular phylogenetics, morphometrics, and other data sources are used complimentarily, offer an appealing path forward in a time of unprecedented species loss. This approach is especially valuable when delimiting species in genera where putative species frequently share pollinators, often grow sympatrically, and present significant inter-specific morphological variation in addition to intra-specific morphological similarities, such that cryptic speciation and/or species complexes may be an underappreciated phenomenon. *Spiranthes* Rich. (Orchidaceae) –the ladies' tresses orchids– is an excellent example of a plant genus which presents all of these issues.

The genus *Spiranthes* contains at least three broad species complexes –the mostly eastern North American *Spiranthes cernua* (L.) Rich. species complex, the East Asian-Australasian *Spiranthes sinensis* (Pers.) Ames species complex, and the Western North American Cordillera species complex centered around *S. romanzoffiana* and its allies– collectively involving 21 out of 35 worldwide currently accepted species in the genus (Govaerts et al. 2015). Within North American *Spiranthes*, the *S. cernua* species complex has traditionally posed the most serious challenge for systematists, field botanists, lay-naturalists, and conservations (Sheviak 1982);

recent molecular evidence suggests it can be discussed in terms of the *S. cernua* complex s.s. (= *S. casei* Catling & Cruise, *S. cernua*, *S. ochroleuca* (Rydb.) Rydb., *S. parksii* Correll), and the *S. cernua* complex s.l. (= the complex s.s., plus *S. longilabris* Lindl., *S. magnicamporum* Sheviak, *S. ovalis* Lindl., and *S. triloba* (Small) Schum. emend. M.C. Pace) (Dueck et al. 2014; Chapter 1).

Within the *S. cernua* complex s.l., *S. longilabris* has traditionally been considered one of the few 'easy to identify', 'unambiguous', and most 'stable' species, presenting a typically secund (or nearly so) inflorescence, oblong labellum with a ruffled margin, widely spreading/oblique linear-lanceolate lateral sepals, and a bloom period of early October to late December. This species is endemic to a narrow band of frequently burned and floristically hyperdiverse longleaf pine (*Pinus palustris* Mill.) savannas, flatwoods, and wiregrass prairie ecosystems in the North American Atlantic and Gulf Coastal Plain –from the Croatan National Forest just west of Cape Lookout in southern North Carolina, south to southern Florida, and west to the eastern edge of Texas. Across this distribution, *S. longilabris* is listed as Critically Imperiled (S1; Alabama, Georgia, North Carolina, South Carolina, Texas), Imperiled (S2; Mississippi), and Vulnerable (S3; Florida), however it does not receive national-level protection. Comparisons of historical herbarium records with current field and citizen scientist observations support these conservation listings, suggesting that *S. longilabris* –although never abundant– is undergoing a dramatic population decline throughout its range, possibly related to habitat destruction and development, and/or fire suppression.

As part of an ongoing systematic revision of the genus that incorporates molecular phylogenetic data, populations of plants from Avon Park Air Force Range [APAFR], Polk Co., FL, originally identified by S. Orzell as putative morphs of *Spiranthes laciniata* (Small) Ames –a

species distantly related to the *S. cernua* species complex– were recovered as very closely related to *S. longilabris*. Following an integrative taxonomic approach as implemented when studying other cryptic or complex groups (e.g., Barrett and Freudenstein 2011; Bacon et al. 2012; Duminil et al. 2012; Pessoa et al. 2012; Rose and Freudenstein 2014), further morphological examination and ecological data support the description of a new species.

MATERIALS AND METHODS

Phylogenetic Methods—We utilized the phylogenetic sampling from Chapter 5. Loci used are as follows: nuclear *ACO*, nrITS, and *Xdh*, and plastid *mat*K, *ndh*J, *trn*L-F, *trn*S-M, and *ycf*1 3'. Sample collection, total genomic DNA extraction, loci amplification, and sequence alignment follow Chapter 5. Molecular phylogenetic samples of taxa from APAFR were collected for by S. Orzell. APAFR was visited my M. Pace in mid-December 2014, after the bloom period of the 'morphs', but during the bloom period of *S. longilabris*. If samples failed to amplify after repeated attempts for a given locus, they were coded as missing data.

Phylogenetic analyses were performed under Bayesian Inference (MrBayes on XSEDE (3.1.2)) implemented through CIPRES Portal V. 3.3 (Miller et al. 2010). Following Dueck et al. (2014), the GTR+G (general-time-reversible with a gamma distribution) model was implemented for all datasets and partitions. Analyses were run for 10,000,000 generations, with a sample frequency of 100,000, nruns = 2, nchains = 6, temp = 0.2, and a burnin of 500,000. Phylogenetic inference of the 50% majority-rule consensus tree was constructed using the "sumt" option based on the remaining trees. The topology of this tree was visualized as trees in FigTree (Rambaut 2014).

Morphometrics—Sixty-eight specimens of *S. longilabris* and 81 specimens of *S. laciniata* were carefully reviewed from AMES, BH, CLEM, F, FLAS, FSU, LSU, MO, NY,

NYS, SEL, US, USF, and WIS. Individual flowers from the lowest quarter of 5 'morphs'/*Spiranthes igniorchis*, 10 *S. laciniata*, and 5 *S. longilabris* inflorescences were judiciously selected for rehydration using a diluted ammonia solution, and were dissected for examination. Flowers were only removed if sufficient material was available and were returned to fragment packets after dissection. Alcohol preserved material of *S. longilabris* collected during a field trip to APAFR was also included in the morphometric analysis. Scaled pictures of the dissected flowers were measured with the ImageJ processing program (Rasband 1997–2014). Measurements were taken for the following features: lateral sepal length and mid-point width; dorsal sepal length and mid-point width; dorsal petal length and mid-point width; labellum length, widest-width of upper labellum, mid-point width, and width just above the labellum apex; callosity length and basal width; viscidium length; rostellum remnant length, stigmatic surface width; and entire column length from viscidium tip to base of the column foot. To evaluate and comprehend the morphological variability of the species in question, principal components analyses (PCA) were performed and visualized in RStudio (2012).

RESULTS

Our molecular, morphological, and phenological data do not indicate a close relationship of these 'morphs' with *S. laciniata*, to which they were originally attributed. Rather these data support their position as a new species –*S. igniorchis*, formally described below– which is a very closely related sister species to *S. longilabris* within the *S. cernua* species complex s.l. Our combined molecular phylogenetic reconstruction of the relationships within *Spiranthes* find *S. igniorchis* as a fully supported sister species to *S. longilabris* (fig. 1). Molecular mutations which support the genetic differentiation between these two species are as follows: base-pair position 386 in the *ACO* alignment shows a differentiation between *S. igniorchis* (thymine) and *S.*

longilabris (guanine), position 356 from the *mat*K alignment presents *S. igniorchis* as having guanine and *S. longilabris* with adenine, whereas *ycf*1 3' presents two changes: base-pair position 533 (*S. longilabris* –guanine; *S. igniorchis* –thymine), and base-pair position 757 (*S. longilabris* –guanine; *S. igniorchis* –adenine).

Our morphometric analyses of *S. igniorchis*, *S. laciniata*, and *S. longilabris* recover three distinct groupings that correspond to the a priori species identifications (fig. 2). Florally, *S. igniorchis* is generally slightly smaller than *S. longilabris*.

DISCUSSION

Our molecular phylogenetic analyses show the putative 'morphs' from APAFR –here described as a new species: *Spiranthes igniorchis*– are not closely related to *S. laciniata* (the species to which they were initially assigned). Rather, they are recovered within the *S. cernua* species complex s.l., sharing a most recent common ancestor with *S. longilabris* (fig. 1). This result was initially surprising given the wide divergence in phenology between *S. igniorchis* (mid-August) and *S. longilabris* (late-October to late-December). However, careful examination finds that *S. igniorchis* is morphologically similar to *S. longilabris*, and our morphometric analyses show slight but significant differences (fig. 2, fig. 3, fig. 5); based on the available samples, *S. longilabris* and *S. igniorchis* do not overlap in terms of the characters we measured, and are distinct taxa. We acknowledge the potential limits of our morphometric sampling of *S. igniorchis*, *S. laciniata*, and *S. longilabris* given that study was hampered by a scarcity of suitable herbarium and living collections. Herbarium specimens of these included species are often entirely glued to the herbarium sheet, are extremely fragile, and/or lack suitable numbers of flowers on the sheet to warrant ethical destructive sampling. However, *S. laciniata* was sampled from populations located throughout its range, *S. igniorchis* was represented by multiple

individuals per sample site, and *S. longilabris* was thoroughly sampled from the area of primary interest: Florida. Thus we think that although the available data is limited, we can confidently designate *S. igniorchis* as a new species.

Sources of general systematic confusion within Spiranthes—Many taxa of *Spiranthes* are quite similar to each other, with only one or two minute (frequently qualitative) characters that can be used to distinguish sympatric or morphologically similar species, and these characters may be overemphasized in the literature and dichotomous keys. Additionally, potentially useful or diagnostic characters have been overlooked (e.g., papillate labellum in *S. triloba* and *S. magnicamporum*; Chapter 1). Confusion over the identity of cryptic *S. igniorchis* was primarily caused by 1) an under appreciation of the frequency of capitate trichomes throughout the genus, and 2) a widely divergent and unexpected phenology compared to its sister species *S. longilabris* and the wider *S. cernua* species complex.

Although our molecular data indicate no close affinities between *S. igniorchis* and *S. laciniata*, the latter requires some discussion given the original assumption that they might be related. Historically, *S. laciniata* has been confused with *S. vernalis* Engelm. & A. Gray: both species bloom from early-April through late-August, have long linear-lanceolate leaves, centrally yellow labella, cupped lateral sepals typically held below the profile of the flower, and grow in open wet to damp meadows, marshes, and roadside ditches. Additionally, molecular phylogenetic evidence indicates *S. laciniata* and *S. vernalis* are somewhat closely related to one another (Dueck et al. 2014). Furthermore, the labellum margin of *S. vernalis* can approach the lacerate/laciniate labellum margin of *S. laciniata*. One feature that is routinely stressed as a defining feature between these species is trichome type: accuminately pointed in *S. vernalis* vs. roundly capitate in *S. laciniata*. This is a valid morphological distinction that unambiguously

distinguishes these two species; however the fact that most North American *Spiranthes* – including *S. longilabris* and *S. igniorchis*– have rounded capitate trichomes is often overlooked or underappreciated. Our research communicates the need for more refined morphological understanding of this genus so that accurate species identification of morphologically similar or cryptic species can be conducted by both professional systematists and non-professional wildflower enthusiasts.

In Florida, *S. laciniata* blooms from early-May to early-July, whereas *S. longilabris* blooms from early-November to late-December, indicating reproductive isolation. In APAFR *S. laciniata* blooms in mid-June, *S. longilabris* blooms a full six months later in mid- to late-December, whereas *S. igniorchis* blooms between the two –but not overlapping them– in late-August. A few, mostly south Florida species of *Spiranthes* (e.g., *S. triloba*), occasionally bloom outside of their typical bloom period, but these rare sporadic events are not consistent year to year, and are often extensions of the normal bloom period. Slight differences in phenology have been used as evidence by others to support the species status of *S. eatonii* P.M. Brown as distinct from *S. lacera* (Raf.) Raf. However the taxonomic status of *S. eatonii* is suspect: its slightly early phenology is continuous with the range of *S. lacera*, morphological differences intergrade between the two, and molecular phylogenetic data recovers *S. eatonii* embedded within *S. lacera* (Dueck et al. 2014). In contrast, our integrative approach provides statistically significant molecular and morphological data, as well as supporting evidence in the form of widely discontinuous phenology, and thus reproductive isolation, to separate *S. igniorchis* from *S. longilabris*.

The description that follows will allow for focused conservation strategies to be developed for this rare Florida endemic, while simultaneously recognizing the clear and close evolutionary relationship between *S. igniorchis* and *S. longilabris*.

TAXONOMIC TREATMENT

Spiranthes igniorchis M.C. Pace. — HOLOTYPE: UNITED STATES OF AMERICA. Florida: Polk County, Avon Park Air Force Range, collected 25 August 2014, *S.L. Orzell & E. Bridges 26735* (NY!). Isotypes: BH, FLAS, WIS, US.

Spiranthes igniorchis is most similar to *Spiranthes longilabris*, from which it can be differentiated by its August (vs. December) flowering period in Central Florida, more strongly coiled inflorescence, smaller proportioned flowers, less-oblique lateral sepals, and slightly larger callosities. It can be distinguished from *Spiranthes laciniata* by the presence of a ruffled to undulate labellum margin (vs. a laciniate or lacerate margin) and position of the lateral sepals as being held in-line with the flower, not below.

Terrestrial, acaulescent, deciduous herb, ca. 22–43 cm tall including the inflorescence. Roots fasciculate, fleshy, slender. Leaves, few, basal, held upright; fugacious or occasionally remaining until the appearance of the inflorescence, withering either during anthesis or shortly thereafter; linear-lanceolate, to 20 3mm long, 5 mm wide; acuminate; leaf base tapered and decurrent, shiny medium-green. Inflorescence strongly erect and perpendicular to the ground; peduncle glabrous, with adpressed, clasping, lanceolate, acute bracts; raceme a single row of flowers in a slightly to moderately coiled spiral; moderately pubescent with blunt-tipped capitate septate trichomes; trichomes to 0.5 mm long; flowers opening from the base to the apex of the inflorescence, basal flowers beginning to senesce as the uppermost flowers are opening. Floral bracts moderately pubescent; broadly lanceolate, acuminate; concave around the ovary; 6–10

mm long; medium-green. Flowers resupinate; campanulate; held roughly perpendicular to the raceme; white; lacking a perceivable fragrance. Sepals white; free; moderately pubescent with blunt-tipped capitate septate trichomes. Dorsal sepal slightly convex, slightly to moderately reflexed near the tip; lanceolate, bluntly acuminate; 6.5–7.6 mm long, 1.2–2.5 mm wide when flattened. Lateral sepals lanceolate, acute; straight to just barely upwardly falcate, angled slightly outward and upward from the base, becoming more so at the mid-point; 6.9–8.8 mm long, 1.3–1.6 mm wide. Dorsal petals white; slightly concave; lanceolate, bluntly acute; slightly to moderately reflexed at tips; 6.5–7.5mm long, 1.4–2.2 mm wide when flattened. Labellum shortly clawed, free but clasping the column; widely concave for its length; reflexed slightly downward at about 2/3 the distance from the claw to labellum apex; glabrous; margin entire to very slightly undulating; 2 basal callosities/nectar glands, white to yellow, conical, rounded, upright, 0.7–1.1 mm tall, glabrous but with minute papillae at the upper-most area of the labellum in-between the callosities and the labellum margin; margin white, central area of labellum light greenish-yellow to pale yellow, labellum 6.3–8.8 mm long, 3.3–4.3 mm wide below the callosities, 2–2.9 mm wide at mid-length when flattened; apex blunt. Column protandrous, slightly rhombic, narrow, 2.5–3.5 mm long; with a pair of upright flaps or wings at each side and clasping the column; column foot glabrous. Rostellum well-developed, white to ivory, tapering to thin acute membranes at the apex, 0.9–1.3 mm long. Stigmatic surface glabrous, shiny, roundly diamond-shaped when viewed from below, 0.6–1.6 mm long. Anther 1, dorsal, triangular-ovoid; pollinium attached to a well-developed viscidium; viscidium linear, immersed the rostellum, leaving behind a narrow V-shaped rostellar remnant after removal, viscidium with a thin membranous frill after removal from the rostellum, 1–1.7 mm long. Ovary moderately to densely pubescent

with septate trichomes, medium-green. Fruit not seen (hypothesized to be a capsule, light brown).

Etymology—The specific epithet "*igniorchis*" is a combination of "*ignis*", Latin for "fire", and "*orchis*", Latin for "orchid". Together, the epithet refers to the endemic fire swept prairie habitat of this orchid. The maintenance of these high-diversity frequently burned subtropical grasslands is critical for the continued survival of this rare species. A suggested common name for *S. igniorchis* is the Florida fire ladies' tresses.

Distribution and statement on rarity—Despite an expansive herbarium search, *S. igniorchis* is only known from –and presumed to be endemic to– a number of Central Florida prairies contained within APAFR, part of the wider Florida Ridges and Osceola Plains physiographic regions (fig. 5). Presumably, suitable habitat also occurs to the immediate east of APAFR in Kissimmee Prairie Preserve State Park in Okeechobee Co., however we did not find any herbarium specimens to support this wider distribution; thus *S. igniorchis* should be considered endemic to Polk Co., Florida.

Southcentral Florida is one of the most floristically diverse yet heavily developed regions in the United States, suffering from ongoing large-scale suburbanization, ground water depletion, and agricultural utilization (primarily cultivation of *Citrus* × *sinensis* (L.) Osbeck), such that very few remnants remain of the diverse fire-swept subtropical grasslands that *Spiranthes igniorchis* inhabits (U.S. Fish and Wildlife 1999; Orzell and Bridges 2006a, b, c). The current burning practices conducted by the U.S. Military on Avon Park Air Force Range, which have been ongoing for decades, have preserved one of the best remaining exemplars of this habitat type while simultaneously being supportive of the Strategic Mission of both the Air Force Range and the United States Military. At the same time, the secure nature of the military base ensures this

species' protection from poachers. Given the scarcity of extant suitable habitat, coupled with the extreme rarity of this species, *S. igniorchis* meets the U.S. Endangered Species Act's evaluating criterion of being a species in which a large percentage of its vital habitat has been degraded or destroyed. We suggest *S. igniorchis* be protected under the Endangered Species Act as a federally endangered or at least threatened species.

Habitat and ecology—Curiously, *Spiranthes igniorchis* occurs in both acidic and calcareous subtropical grasslands. Within *Spiranthes*, some species are thought to display calciphytic affinities (e.g., *S. cernua*, *S. magnicamporum*), however the ecological reality and pH affinities of these plants may be less obligate, as exemplified by *S. igniorchis*. Nothing is known of the mychorizal associates of most *Spiranthes*, including *S. igniorchis* (but see Stewart and Kane 2007; JaeYoung et al. 2012; Tondello et al. 2012), and it may be that these species switch symbiotic partners, or establish relationships with a wide-ranging and ecologically generalist suite of fungi, allowing them to inhabit both calcareous and acidic soils.

Spiranthes longilabris and *S. triloba* share the same frequently burned prairie habitats as *S. igniorchis* within APAFR, yet there is a slight but distinct separation among these three species such that *S. longilabris* and *S. triloba* never comingle with *S. igniorchis*. Specifically, *S. igniorchis* appears to prefer slightly wetter microhabitats vs. slightly drier sites for *S. longilabris* (*S. triloba* is restricted to just one known population within APAFR), and *S. triloba* and *S. longilabris* bloom 2.5–4 months after *S. igniorchis*. Fire is clearly a crucial element favoring the continued existence of *S. igniorchis* and the wider ecological community to which it has adapted; historically, these annual or biannual fires were lightning initiated, although human set fires are now critical in maintaining fire on the current fragmented landscape (Noss et al. 2015). Such frequent burning cycles maintain graminoid and short-statured forb dominance while

concurrently suppressing taller and woody vegetation, particularly *Sabal palmetto* (Walter) Lodd. ex Schult. & Schult. f. and *Serenoa repens* (W. Bartram) Small. *Calopogon multiflorus* Lindl., *Eulophia ecristata* (Fernald) Ames, *Spiranthes longilabris*, and *S. triloba* are additional orchid species found in the prairies and subtropical grasslands of central Florida which require frequent fires to flower and persist. Conservation efforts and regulations should be enacted to continue to maintain such frequent fires at APAFR and across Florida and the North American Coastal Plain.

No pollinators have been observed for *S. igniorchis* or its sister species *S. longilabris*. The presence of a well-developed rostellum and viscidium and the lack of evidence for apomixis suggests that both sister species require insect mediated pollination for the production of viable seeds. As with the majority of North American *Spiranthes*, including members of the *S. cernua* species complex s.l., the pollinators of both *S. igniorchis* and *S. longilabris* are hypothesized to be species of *Bombus* Latreille (Catling 1982, 1983). We suggest observational monitoring studies be implemented to determine pollinator identity for these rare orchids.

Bacon, C.D., M.J. McKenna, M.P. Simmons, and W.L. Wagner. 2012. Evaluating multiple criteria for species delimitation: an empirical example using Hawaiian palms (Arecaceae: *Pritchardia*). *BMC Evolutionary Biology* 12: 23–39.

Barrett, C.F. and J.V. Freudenstein. 2011. An integrative approach to delimiting species in a rare but widespread mycoheterotrophic orchid. *Molecular Ecology* 20: 2771–2786.

Catling, P.M. 1982. Breeding systems of northeastern North American *Spiranthes* (Orchidaceae). Canadian Journal of Botany, 60: 3017–3039.

Catling, P.M. 1983. Pollination of northeastern North American *Spiranthes* (Orchidaceae). Canadian Journal of Botany, 61: 1080–1093.

Dueck, L.A., D. Aygoren, and K.M. Cameron. 2014. A molecular framework for understanding the phylogeny of Spiranthes (Orchidaceae), a cosmopolitan genus with a North American center of diversity. *American Journal of Botany* 101(9): 1551–1571.

Duminil, J., D. Kenfack, V. Viscose, L. Grumiau, and O.J. Hardy. 2012. Testing species delimitation in sympatric species complexes: The case of an African tropical tree, *Carapa* spp. (Meliaceae). *Molecular Phylogenetics and Evolution* 62(1): 275–285.

Govaerts, R., P.Bernet, K. Kratochvil, G. Gerlach, G. Carr, P. Alrich, A.M.Pridgeon, et al. 2015. World Checklist of Orchidaceae. Facilitated by the Royal Botanic Gardens, Kew. Published on the Internet; http://apps.kew.org/wcsp/ Retrieved 2015-05-14.

JaeYoung, Y., H. HanKyeol, C. JaeMin, C. YongChan, L. ByungChun, and E. AhmHeum. 2012. Identification of orchid mycorrhizal fungi isolated from five species of terrestrial orchids in Korea. *Korean Journal of Mycology*, 40(3): 132–135.

Jenkins, C.N., K.S. Van Houtan, S.L. Pimm, and J.O. Sexton. 2015. US protected lands mismatch biodiversity priorities. *Proceedings of the National Academy of Sciences*, 112(16): 5081–5086.

Noss, R.J., W.J. Platt, B.A. Sorrie, A.S. Weakley, D.B. Means, J. Costanza, and R.K. Peet. 2015. How global biodiversity hotspots may go unrecognized: lessons from the North American Coastal Plain. *Diversity and Distributions* 21: 236–244.

Orzell, S. L. and E. L. Bridges. 2006a. Floristic composition of the south-central Florida dry prairie landscape. Pages 64-99 *in* R. F. Noss, editor. Land of fire and water. Proceedings of the Florida Dry Prairie Conference. Painter, DeLeon Springs, FL.

Orzell, S. L. and E. L. Bridges. 2006b. Species composition and environmental characteristics of Florida dry prairies from the Kissimmee River region of south-central Florida. Pages 100-135 in R. F. Noss, editor. Land of fire and water. Proceedings of the Florida Dry Prairie Conference. Painter, DeLeon Springs, FL.

Orzell, S.L., and E.L. Bridges. 2006c. Floristic composition and species richness of subtropical seasonally wet *Muhlenbergia sericea* prairies in portions of central and south Florida. Pages 136–175 *in* Reed F. Noss ed. Land of Fire and Water: The Florida Dry Prairie Ecosystem. Proceedings of the Florida Dry Prairie Conference. DeLeon Springs, FL.

Rose, F.P., and J.V. Freudenstein. 2014. Cryptic and overlooked: species delimitation in the mycoheterotrophic *Monotropsis* (Ericaceae: Monotropoideae). *Systematic Botany* 39(2): 578–593.

Pessoa, E. M., M. Alves, A. Alves-Araujo, C. Palma-Silva, and F. Pinheiro. 2012. Integrating different tools to disentangle species complexes: a case study of *Epidendrum* (Orchidaceae). *Taxon* 61: 721–734.

Rambaut. A. 2014. FigTree v1.4.2, released 2014-07-09. http://tree.bio.ed.ac.uk/software/figtree/

Rasband, W.S. 1997-2014. ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/

RStudio. 2014. RStudio: Integrated development environment for R (Open Source Edition; Version v0.98.932) [Computer software]. Boston, MA. Retrieved September 25, 2014.

Sheviak, C.J. 1982. Biosystematic study of the *Spiranthes cernua* complex. *New York State Museum Bulletin* 448: 1–73.

Stewart, S.L., and M.E. Kane. 2007. Symbiotic seed germination and evidence for in vitro mycobiont specificity in *Spiranthes brevilabris* (Orchidaceae) and its implications for species-level conservation. *In Vitro Cellular & Developmental Biology-Plant*, 43(3): 178–186.

Thiers, B. [continuously updated]. Index Herbariorum: A global directory of public herbaria and associated staff. New York Botanical Garden's Virtual Herbarium. http://sweetgum.nybg.org/ih/

Tondello, A., E. Vendramin, M. Villani, B. Baldan, and S. Squartini. 2012. Fungi associated with the southern Eurasian orchid *Spiranthes spiralis* (L.) Chevall. *Fungal Biology*, 116(4): 543–549.

U.S. Fish and Wildlife Service. 1999. South Florida Ecosystem: Multi-Species Recovery Plan for South Florida.

Fig. 1. Bayesian Majority Rule reconstruction of *Spiranthes* from the combined data set (ACO, nrITS, matK, ndhJ, trnF-L, trnS-M, ycf1 3', Xdh). Taken from Chapter 5; please see Chapter 5 for the full tree. Posterior probabilities displayed on branches. Individual terminals represent individual samples.

The black bar denotes *S. igniorchi*s, the white bare denotes *S. longilabris*



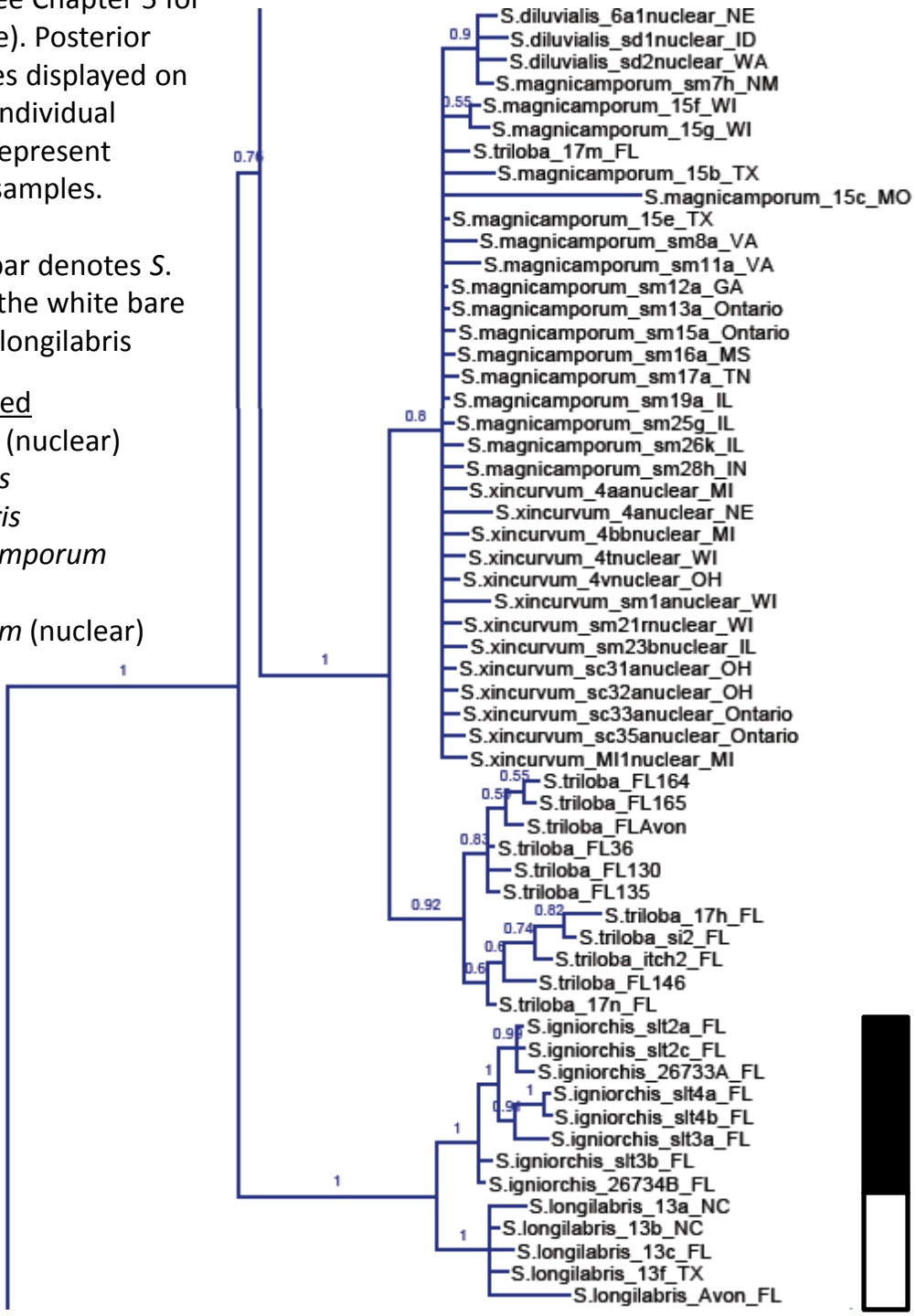
Fig 1

Blue: *Spiranthes cernua* species complex s.l. in part (taken from Chapter 5; please see Chapter 5 for the full tree). Posterior probabilities displayed on branches. Individual terminals represent individual samples.

The black bar denotes *S. igniorchis*; the white bare denotes S. longilabris

<u>Taxa included</u>

S. diluvialis (nuclear)
S. igniorchis
S. longilabris
S. magnicamporum
S. triloba
S. ×incurvum (nuclear)



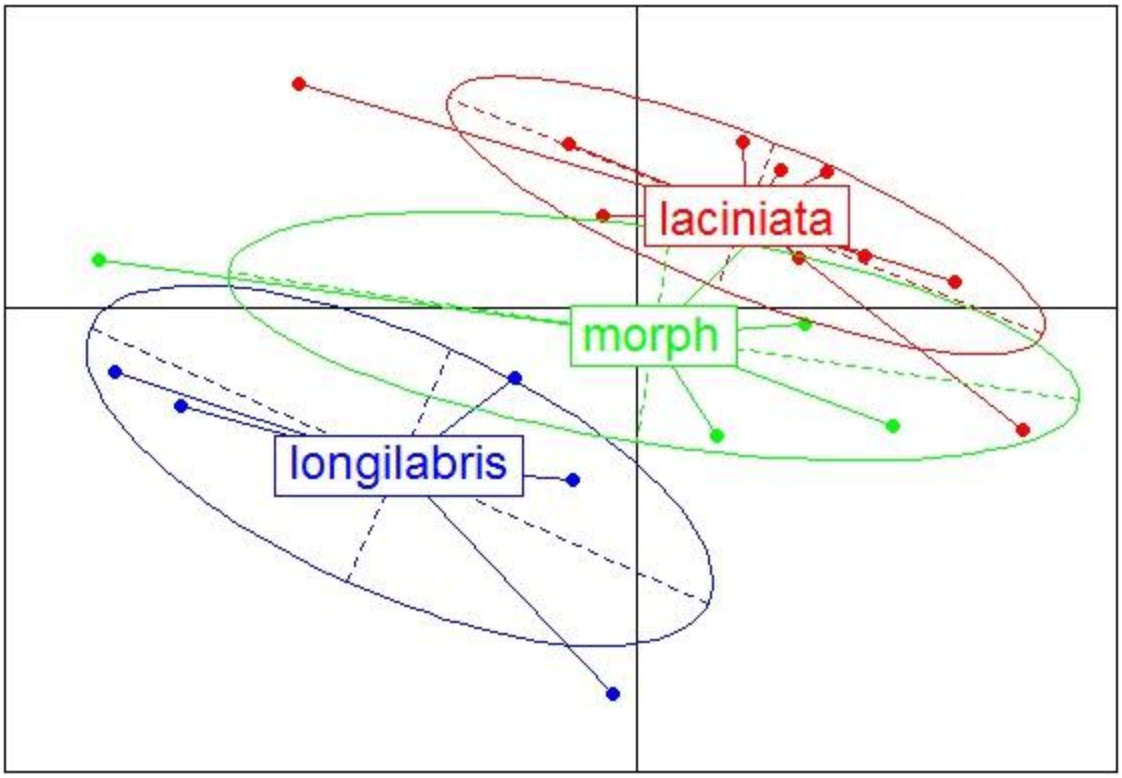


Fig. 2. Visualization of the PCA based on floral measurements of *S*. *longilabris*, *S*. *laciniata*, and *S*. *igniorchis* (called 'morph' in the graph).

Fig. 3. [following page]. A-B. *Spiranthes longilabris*. C-D. *Spiranthes igniorchis*. A. Polk Co., FL. 15 December 2015. B. Brunswick Co., NC. 2 November 2013. C. Polk Co, FL (photo by S. Orzell). D. Polk Co., FL (photo by S. Orzell)





A.



C.



B.



D.

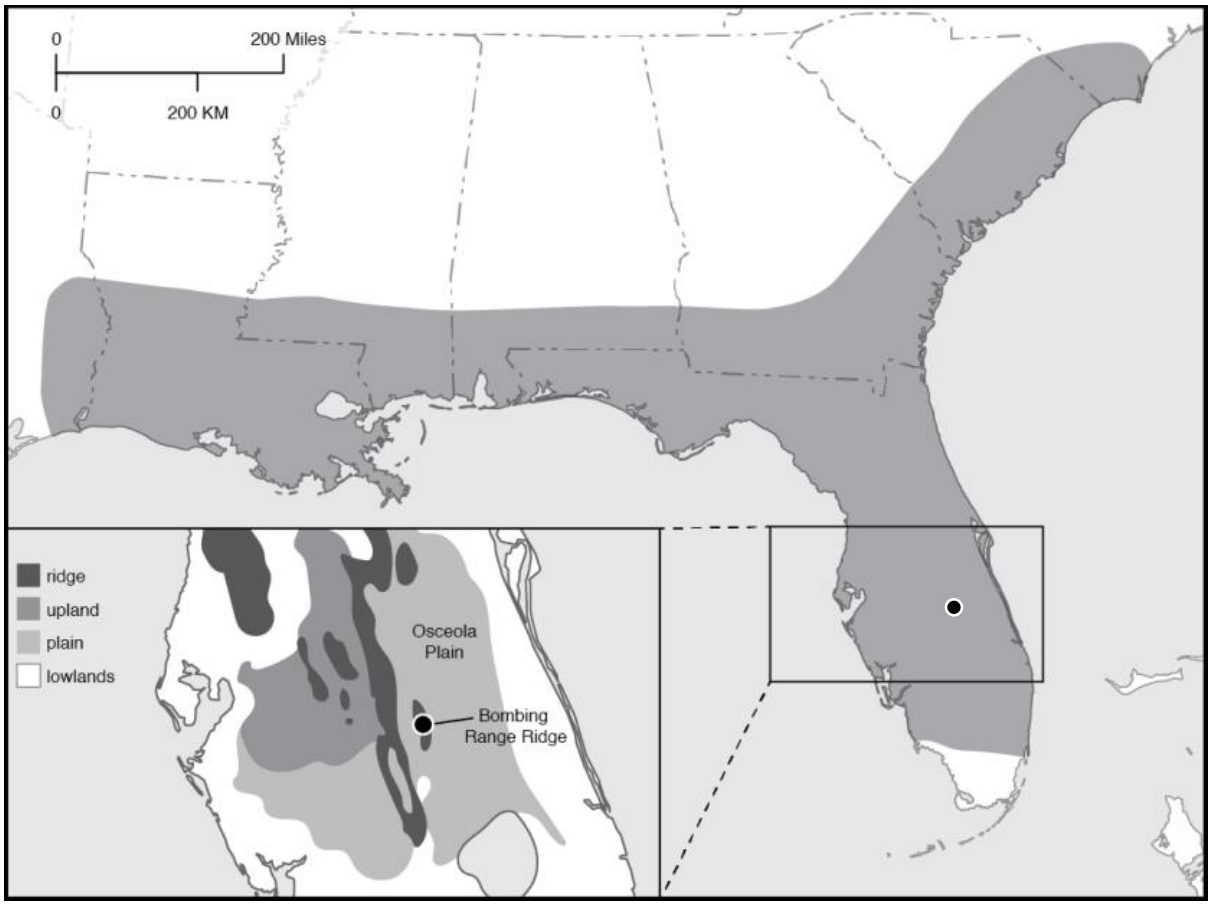


Fig. 4. Distribution map of *Spiranthes longilabris* (gray, large map) and *Spiranthes igniorchis* (black circle). Insert: Close-up map of Central Florida, depicting prominent geographic features. The Bombing Range Ridge and Osceola Plain are highlighted. The black circle in the insert denotes the location of the only known populations of *S*. *igniorchis*. Map created by S. Freidrich.

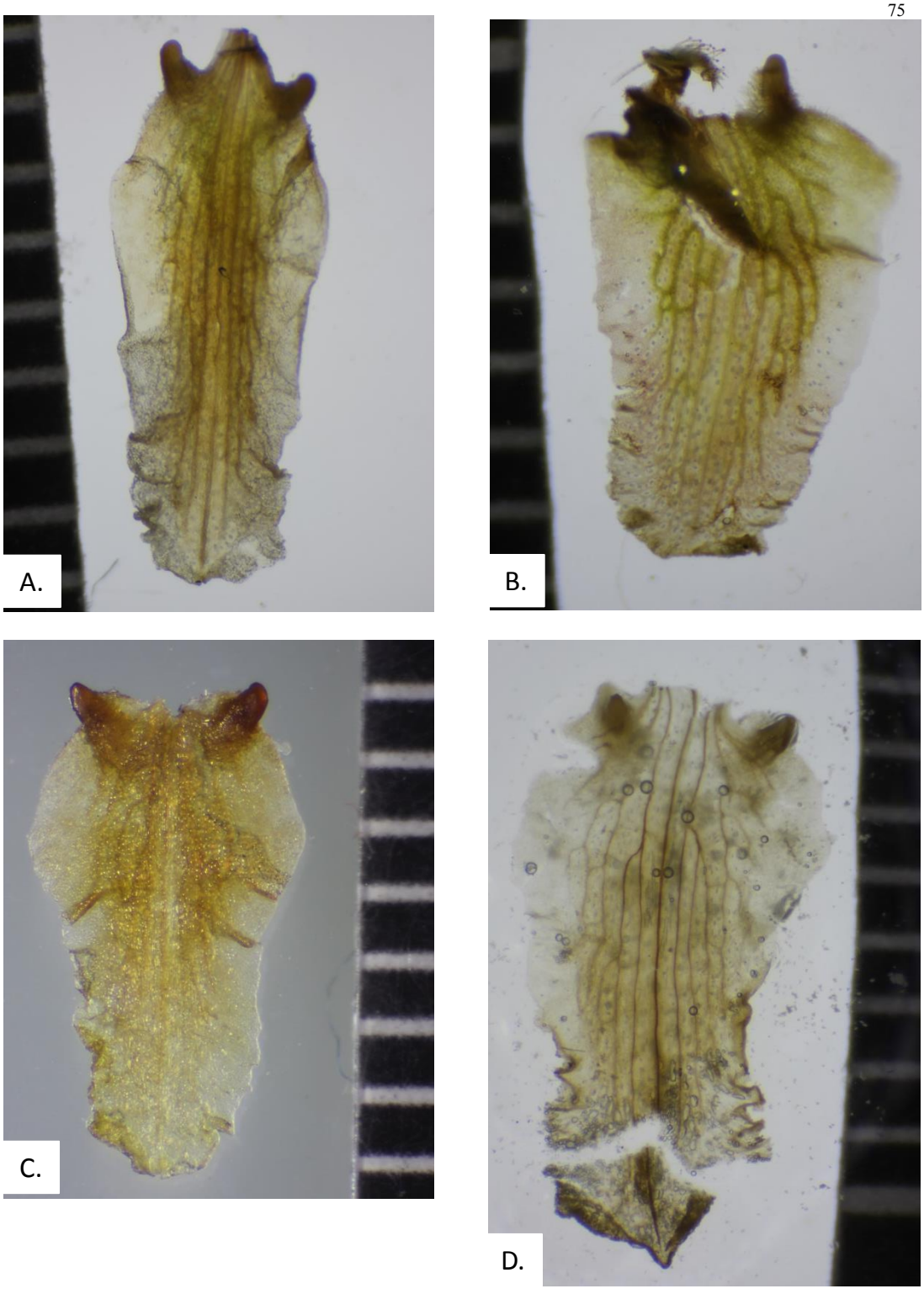


Fig. 5. Representative rehydrated labella of *S*. *igniorchis* (A&B) and *S*. *longilabris* (C&D). All original plants were collected from Avon Park Air Force Range, Polk Co., FL. All images are to scale (tics = 1 mm). A. From holotype, *Orzell 7 Bridges 26735*. B. From isotype, Orzell & Bridges 26735. C. Orzell & Bridges 26720. D. Pace s.n.

CHAPTER 3

Untangling Gordian's Knot—Molecular phylogenetic, morphological, and biogeographic evidence support dividing *Spiranthes cernua* (Orchidaceae) into two morphologically variable species and two geographically specific natural hybrids.

Matthew C. Pace & Kenneth M. Cameron

***Abstract*—**Species identification is both a fundamental goal and challenge of systematic biology. A major challenge in addressing and understanding biodiversity is the evolution of cryptic species that are unequivocally genetically distinct and may not share a most recent common ancestor, but are morphologically overlapping or nearly indistinguishable. The *Spiranthes cernua* species complex is a group of Eastern North American orchids which has long defied easy identification due to morphological variability, overall morphological similarity, and possible hybridization. In an attempt to clarify long standing questions regarding species boundaries within the *S. cernua* species complex, we utilized low copy nuclear, nuclear ribosomal, and plastid molecular phylogenetic datasets, in additional to expansive field and herbarium research. Our results support the description of a new cryptic species –*S. arcisepalum*– as distinct from *S. cernua* s.s., in addition to two biogeographically specific hybrids: S. ×*incurvum* (*S. cernua* s.s. × *S. magnicamporum*), and *S.* ×*kapnosperia* (*S. ochroleuca* × *S. cernua*). This is the first work clarify species boundaries within this challenging group, and to use molecular phylogenetic data to support hybridization as an evolutionary force within the *S. cernua* species complex.

Key words—Appalachian Highlands; cryptic species; Great Lakes Basin; hybridization; morphological variability

The question "what is a species?" is deceptive in its simplicity, yet confounds taxonomists, systematists, and naturalists as much today as it did before the time of Linnaeus. Although challenging, the importance of understanding species boundaries and biodiversity in a time of global climate change, shrinking habitats, and new interspecific hybridization events cannot be underestimated (Wayne and Jenks 1991, Soltis and Gitzendanner 1998, Pettengill and Neel 2011, Rutledge et al. 2012, van Dongen 2012, Becker et al. 2013, Paul et al. 2013, Monzon et al. 2014). How species are defined and delineated impacts the allocation of limited conservation funds, designation of official protection status, the identification of individuals used for ex situ conservation and reintroduction programs, and the general public's perception of systematics and conservation. A major challenge in addressing these issues is the evolution of cryptic species (e.g., Bickford et al. 2007; Adams et al. 2014; Shirley et al. 2014; Chapter 1, Chapter 2) –species that are unequivocally genetically distinct and may not share a most recent common ancestor, but are morphologically overlapping or nearly indistinguishable among individuals. The wider occurrence of species complexes –assemblages of closely related often cryptic species that defy easy delimitation and are typically evolutionarily young– also present a challenging but important window into the process of speciation and diversification, adaptation, and gene flow and hybridization.

The balance between recognition of infraspecific phenotypic variability, identification of cryptic species, taxonomic over-splitting and inflation, and conservation is a thorny and hotly debated topic, especially in charismatic groups such as orchids, primates, and birds (e.g., Pillon and Chase 2007; Dueck and Cameron 2008a; Hopper 2009; Sangster 2009; Markolf et al. 2011; Zachos et al. 2013; Swarts et al. 2014). This complicated set of issues is further obscured by the lack of a universally accepted species concept and the application of varying, ever more

restrictive and minute, sometimes conflicting criteria to elevate organisms to the rank of species (e.g., morphology, reproductive potential and capacity, different molecular tools (traditional phylogenetic loci vs. population genetics), monophyly, floral fragrance, epigenetic differences). The task faced by systematic and conservation biologists is how to best reflect evolutionary pattern and process while simultaneously recognizing diagnosable species, developing useful classifications, and protecting the greatest amount of biodiversity; this challenge is intensified when dealing with putative species that present enormous degrees of phenotypic variation and plasticity.

The genus *Spiranthes* (Orchidaceae) has long posed a significant taxonomic and identification challenge: many of its currently accepted 35 species exhibit marked phenotypic variation at both local and continental scales, flower size and shape varies within a single inflorescence –with younger flowers approaching the morphology of other species– hybridization has traditionally been thought to be nearly pervasive, and there are four species complexes in the genus involving 21 species. Furthermore, cryptic speciation has been documented in the group (Brown et al. 2008; Dueck and Cameron 2008b; Chapter 1, Chapter 2), a few species exhibit polyploidy and apomixis (Catling 1982; Sheviak 1982), the species-level validity of some taxa is questionable (Walters 2005; Dueck and Brown 2008a), incomplete lineage sorting is likely a reality in the group (Dueck et al. 2014), and taxonomic inflation by certain authors is a troublesome issue (e.g., Brown 1999a, b). The group that best encompasses all of these issues is the *Spiranthes cernua* species complex: a true Gordian's Knot comprised of species complexes within species complexes.

Taxonomic History—*Spiranthes cernua* was one of the first species to be named in the genus –under the basionym *Ophrys cernua* L.– in the second edition of *Species Plantarum*

(Linnaeus 1763). Linnaeus's original description is minimalistic (lacking details such as measurements) and the type location is given as a wide ranging "Virginia, Canada," creating an overall picture that is less-than refined. The lectotype (Kalm, s.n., LINN; drawings of lectotype flowers by A. Gray in Sheviak and Catling 1980, pg. 531) displays two somewhat large plants 25.5–36.1 cm tall, with linear to linear-lanceolate leaves, and strongly nodding moderately sized flowers 5.5–7.8 mm long. The labellum is bluntly acute (as denoted in the original description) and slightly dilated near the apex, with two prominent basal callosities (nectar glands). Originally, nearly all autumn flowering North American *Spiranthes* were referred to or placed under synonymy with *S. cernua*. In time several species –*S. casei, S. magnicamporum, S. ochroleuca, S. odorata, S. parksii* (fig. 1–4)– were described to accommodate much of the morphological variation expressed by the autumnal *Spiranthes*. This group came to be known collectively as the *Spiranthes cernua* species complex sensu Sheviak (Sheviak 1982, 1991; Sheviak and Brown 2002).

Even with this added knowledge of species-level diversity among the autumn flowering *Spiranthes, S. cernua* itself still expresses a wide degree of morphological and reproductive variability from within-population to biome level scales (fig. 1–2). Based on the work of Catling and Sheviak (Catling 1982, 1983; Catling and Brown 1983; Sheviak 1982, 1991; Sheviak and Brown 2002), *S. cernua* is traditionally understood to be a facultatively agamospermic polyploid 'compilospecies' (2*n* = 45, 60; sunsu Harlan and deWet 1963) that freely hybridizes with all other species in the *S. cernua* species complex. Sheviak (1991) goes so far as to state "a 'typical' *S. cernua* is difficult to define because of the species' intrinsic hybrid nature." Despite its hypothesized pervasiveness, the morphological identity of potential hybrids has not been well documented, and no primary hybrids involving *S. cernua* have been formally named; the dubious

Spiranthes ×*borealis* P.M. Br. (*S. casei* × *S. ochroleuca*) is the only named hybrid in the complex. Additionally, peloria, cleistogamy, and apomixis are common phenomena within *S. cernua*, with each occurring in different proportions throughout its range. Peloria appears to be especially common in the Midwestern United States and Texas; some peloric individuals of *S. cernua* –particularly in the central Great Plains– are very robust in stature, approaching *S. magnicamporum* in overall appearance. In some regions, only cleistogamous individuals may be found in particular populations. Peloric and non-peloric, cleistogamous and open, and apomictic and sexual individuals may all be found within a single population or populations separated by only a few kilometers.

Repeated attempts have been made to clarify species-relationships and species-status within the *Spiranthes cernua* complex, with different methods favored by various authors including breeding systems (Catling 1981), morphological discriminant analysis (Sheviak and Catling 1980, Catling 1981, Sheviak 1982), and cytology (Sheviak 1982, 1991). Recently, DNA sequences (Dueck and Cameron 2007, Dueck and Cameron 2008a, Dueck et al. 2014, Chapter 1, Chapter 2), AFLPs (Manhart and Pepper 2007), and microsatellites (Walters 2005, Manhart and Pepper 2007) have been utilized to understand the systematics of these orchids. We argue that in taxa that are –even putatively or possibly– morphologically variable, such morphological features are not the most suitable variables to analyze as a primary source of data (and should not be the sole source), and may lead to over splitting based on 1) geographically limited sample size, 2) the presence of locally adapted ecotypes, and/or 3) the fixation of a certain random character due to the predominance of apomixis in specific localized populations. It is possible that the descriptions of the nearly endemic *S. casei* var. *novaescotiae* and *S. parksii* resulted from such an overreliance on morphology and the regional prevalence of apomixis. Instead, we think

viewing morphological variation through the context of molecular phylogenetic relationships offers an attractive path forward.

***Phylogenetic Background*—**Molecular techniques offer an appealing alternative to the traditional morphological approach. However, resolution between the included taxa of the *Spiranthes cernua* species complex has been uniformly low or lacking with the exception of Dueck et al. (2014), Chapter 1, and Chapter 2. This general lack of resolution has been used to argue for opposing positions as exemplified by the debate over the species-status of federally endangered *S. parksii*: Dueck and Cameron (2008a) hypothesized that its peloric morphology, coupled with a lack of molecular resolution between *S. cernua* and *S. parksii* , and especially the fact that recognition of the latter would render the former paraphyletic, indicates the two are the same species, whereas Jacobsen et al. (2009) strongly rejected Dueck and Cameron (2008a), instead attributing the unresolved relationship to the neutral nature of the included markers.

Phylogenetic work dealing with taxonomic confusion in portions of the *S. cernua* species complex by Pace et al. (Chapter 1, Chapter 2) revealed that the *Spiranthes cernua* complex sensu Sheviak does not properly encompass evolutionary history: *Spiranthes odorata* does not belong in this grouping since it does not share a most recent common ancestor with any other member of the complex, instead being sister to most of the other Eastern North American species, and *Spiranthes triloba* –a species lost under synonymy of both *S. cernua* and *S. odorata* for more than a century– is a distinct and valid species, forming a sister relationship with *S. magnicamporum*. Together, Dueck et al. (2014), Chapter 1, and Chapter 2 reveal that the *Spiranthes cernua* complex sensu nov. includes *S. casei*, *S. cernua*, *S. magnicamporum*, *S. parksii*, *S. ochroleuca* (the traditionally included species), plus *S. triloba*, in addition to the unexpected *S. longilabris*, S. *igniorchis*, and *S. ovalis* (Fig 3–4). Furthermore Dueck et al. (2014)

found that *S. cernua* itself is polyphyletic, composed of two genetically and geographically distinct clades/entities. Additionally, *S. casei* was found to be broadly embedded within *S. ochroleuca*. Although support values for individual taxon clades were typically high (pp > 0.90; often 1), the exact relationship between clades was not well resolved, particularly within the *Spiranthes cernua* complex sensu stricto: the two *S. cernua* clades, *S. parksii*, and *S. ochroleuc*a + *S. casei*. Moreover, the morphological limits of these potential clades were unknown. Additionally, the topologies of Chapter 1 and Chapter 2 conflict with Dueck et al. (2014) in the placement of *S. longilabris* and *S. ovalis* in regard to the rest of the complex.

To resolve the long standing problems of species relationships and species status within this complex, we present an expanded sampling of taxa and loci, with integrated morphological and biogeographic work. Together, these data support the division of '*S. cernua*' into two distinct, but morphologically variable species.

METHODS

Taxonomic Sampling—Approximately 500 herbarium specimens species in the *Spiranthes cernua* species complex were carefully reviewed from AMES, BH, CLEM, F, FLAS, FSU, LSU, MO, NY, NYS, SEL, UARK, US, and USF (acronyms follow Thiers [continuously updated]). Individual flowers from the lowermost quarter of the inflorescence were rehydrated for morphological examination from select individual specimens. Fieldwork based on georeferenced herbarium specimens was conducted in DE, MD, GA, FL, IL, IN, NC, NM, NY, OH, SC, VA, VT, and WI from 2012–2014. Samples were collected for herbarium and spirit vouchers, morphological measurements, and phylogenetic reconstructions. For areas we could not visit for fieldwork, 1–10 year old herbarium specimens were judiciously sampled for inclusion in the phylogenetic analysis.

Morphometrics—To analyze and understand the morphological variability of its component species, principal components analyses (PCA) were performed and visualized in RStudio (2012) using rehydrated labella from herbarium samples, scaled drawings in published literature (Sheviak 1982, 1989), and imaged herbarium specimens. Measurements of rehydrated labella included labellum length, width at widest point, width at isthmus/narrowest point, width of dilated tip, length of dilated tip, length from ovary to dilated tip, and callosity height. Measurements of herbarium specimens include height of while plant, leaf length and width at widest point, tightness of spiral (number of twists divided by spike length), flower length in profile, lateral sepal length, lateral sepal width at midpoint, and labellum length.

Phylogenetic Methods—Phylogenetic analyses incorporated and expanded upon the dataset of Dueck et al. (2014), Chapter 1, and Chapter 2. For new accessions, 3–4 unopened virgin buds or ca. 1 cm sq^2 of leaf tissue were collected and silica dried for later extraction of Total gDNA. IBI Plant Isolate Kits (Peosta, IA) and Maxwell® 16 LEV Plant DNA Kits (Madison, WI) were used for all newly collected samples. All accessions were amplified for the plastid gene regions *mat*K, *ndh*J, *trn*L intron, *trn*S-fM, and *ycf*1 3', nuclear ribosomal ITS (internal transcribed spacers 1 and 2 and the 5.8S subunit; nrITS), and the low-copy nuclear regions *ACO* and *Xdh* (fig. 5). PCR amplification protocols are as follows: plastid (except *ycf*1) and nrITS: Dueck et al. (2014), *Xdh*: Górniak et al. (2010), *ACO*: Guo et al. (2012), and *ycf*1: Neubig et al. (2009). PCR products were purified using ExoSap-It (Cleveland, OH), and cycle sequencing products were cleaned using Agencourt CleanSeq (Beverly, MA) magnetic beads. Direct sequencing of cleaned cycle sequencing products was performed at the University of Wisconsin – Madison Biotechnology Center. Resulting chromatograms were edited and aligned using software modules available in Geneious 7.1 including MUSCLE. Ambiguities in plastid

datasets were coded with standard IUPAC-IUB Symbols for Nucleotide Nomenclature (Cornish-Bowden 1985). We interpreted nuclear sequences with ambiguities as possibly being indicative of hybridization. As a proxy for cloning, ambiguities in nuclear ribosomal and low-copy nuclear sequences were dealt with in the following manner: 1) sequences were duplicated as two distinct contigs; 2) based on the ambiguities present across the now doubled sequences, putative parents were identified with complimentary non-ambiguous bases at ambiguous sites; 3) based on the topology of the combined plastid phylogenetic reconstruction, one of these putative parent species was identified as the likely maternal species, the other species with complimentary bases was designated the tentative paternal parental species; 4) ambiguous positions were coded so that one sequence matched the nucleotide of the likely maternal parent, whereas the other matched the nucleotide present in the likely paternal parent; 5) the names of the doubled sequences were changed so that they represented different operation taxonomic units based on their inferred parentage. If ambiguities were present in the apparent hybrid individual which did not correspond to nucleotides found in the putative parental species, non-ambiguous nucleotides were assigned randomly. If nucleotide ambiguities were found in an apparent hybrid, such that only one tentative parent could be identified, the ambiguous sequence was doubled as above, however one copy –designated 'A'– was coded to match the tentative parent, and the other sequence was coded as its compliment, and did not match the nucleotides found in any other taxon –designated 'B'. This coding approach was used in lieu of cloning in an attempt to retrieve both parental copies of potential hybrids, as used in similar research (e.g., Bertrand et al. 2015). If samples failed to amplify after repeated attempts for a given locus they were coded as missing data.

The data were analyzed as 1) distinct individual data partitions, 2) combined plastid data, 3) combined nuclear data, and 4) total evidence combined nuclear + plastid data. In the total evidence combined nuclear + plastid dataset, only the 'A' copy of previously ambiguous nuclear sequences were included. Analyses were performed under Bayesian Inference (MrBayes on XSEDE (3.1.2)) implemented through CIPRES Portal V. 3.3 (Miller et al. 2010). Based on Dueck et al. (2014), the GTR+G (general-time-reversible with a gamma distribution) model was implemented for all datasets and partitions. Analyses were run for 10,000,000 generations, with a sample frequency of 100,000, nruns = 2, nchains = 6, temp = 0.2, and a burnin of 500,000. Phylogenetic inference of the 50% majority-rule consensus tree was constructed using the "sumt" option based on the remaining trees. The topology of this tree was visualized in two ways: 1) as networks in the program SplitsTreeWindow (Huson and Bryant 2006), and 2) as trees in FigTree (Rambaut 2014).

RESULTS

***Phylogenetics*—**Our estimate of phylogenetic relationships fully support the distinction of two clades previously identified together as *S. cernua* s.l. One clade –described below as *Spiranthes arcisepalum* sp. nov.– is strongly supported (pp = 0.94) as sister to a clade of *S. ochroleuca* + *S. casei*. The other clade –including *S. parksii* and here referred to as *Spiranthes cernua* s.s.– is in turn fully supported (pp = 1) as sister to the combined *S. arcisepalum* and *S. ochroleuca* + *S. casei* clade. Only the topology of nrITS failed to support the differentiation of these two clades; nrITS also failed to resolve the relationships between *S. ochroleuca* and the former '*S. cernua* s.l.' (*ycf*1 only recovered weak to moderate support for the formation of *S.*

arcisepalum as distinct from a strongly supported *S. cernua* s.s. clade and a polytomy of *S. ochroleuca* and *S. casei*).

Based on the samples included here, we did recover molecular evidence for geographically-specific hybridization: 1) *S. cernua* s.s × *S. magnicamporum* (*S. ×incurvum* comb. nov. pro. sp., see below) across the northern Interior Lowlands, Great Lakes Basin, and northern Appalachian Highlands; 2) *S. cernua* s.s. × *S. ochroleuca* (*S. ×kapnosperia* hyb. nov., see below) in the southern Great Smoky Mountains of North and South Carolina.

Morphometrics—PCA of the labella of *S. arcisepalum*, *S. cernua*, *S. ×incurvum*, *S. magnicamporum*, and *S. ochroleuca* yield essentially overlapping plots, only *S. casei* is distinct (fig. 6). Based on the morphometric sampling we included, *S. arcisepalum* sp. nov. is the least variable species, however it is entirely contained within the variability of *S. cernua* s.s. (fig. 6). Similarly, the variation of *S. ochroleuca* is also contained within *S. cernua* s.s. *Spiranthes arcisepalum*, *S. cernua*, and *S. ochroleuca* in turn overlap with the morphological variability of *S. ×incurvum*, and all of these species are contained within and surpassed by the morphological variability of *S. magnicamporum*. Thus our morphometric analyses cannot clearly separate a priori groupings based on labellum morphology. A separate morphometric analysis (not shown) of the sepals, lip, leaves, and whole plant fond a slight difference in *S. arcisepalum* vs. *S. cernua* s.s. and *S. ×incurvum*, however all three taxa are widely overlapping, essential similar in all of the features we measured. Qualitative features appear to be more useful in confidently distinguishing *S. arcisepalum* vs. *S. cernua* s.s. vs. *S. ×incurvum*. The lateral sepals of *S. arcisepalum* are typically downwardly falcate, whereas the lateral sepals of *S. ×incurvum* typically sweep upward; similarly in *S. cernua* the lateral sepals usually sweep upwards or are parallel to the ground (i.e., within the plane of the dorsal sepal and petals). The flowers of *S.*

arcisepalum are also slightly smaller than *S. cernua* and *S.* ×*incurvum*. Additionally, the flowers of *S. cernua* –as indicated by the specific epithet–often become strongly nodding as the inflorescence matures, whereas in *S. arcisepalum* and *S.* ×*incurvum* the flowers are more commonly held parallel to the ground, or only slightly nod (the flowers of *S.* ×*incurvum* are occasionally slightly ascending).

TAXONOMIC TREATMENT

Spiranthes cernua (L.) Rich., sensu stricto — Lectotype: UNITED STATES OF AMERICA. "Virginia, Canada" s.d., *Kalm s.n.* (LINN!). Basionym: *Ophrys cernua* L.

Homotypic synonyms: *Neottia cernua* (L.) Sw; *Gyrostachys cernua* (L.) Kuntze; *Ibidium cernuum* (L.) Bouse; *Triorchis cernuus* (L.) Nieuwl.

Heterotypic synonyms: *Limodorum autumnale* Walter; *Spiranthes annua* Lesq. ex Brandeger & Coville; *Gyrostachys constricta* Small; *Spiranthes constricta* (Small) K. Schum.; *Spiranthes parksii* Correll.

Terrestrial, acaulescent, deciduous herb, to 50 cm tall including the inflorescence. Roots fasciculate, fleshy, slender. Leaves 1–5, forming a basal rosette, held upright; remaining until after anthesis (occasionally fugacious at anthesis), withering shortly thereafter; linear-lanceolate to lanceolate, 50–220 mm long, ca. 5-8 mm wide; bluntly acuminate; leaf base tapered and decurrent, shiny medium-green. Inflorescence strongly erect and perpendicular to the ground; medium-green; glabrous; 1–3 small lefty cauline bracts occasionally present (frequently absent), quickly reducing to adpressed, clasping, lanceolate, acute bracts; raceme a single row of flowers in an open to tightly coiled spiral (appearing as 1–4 'ranks'), to 100 cm long; moderately to densely pubescent with blunt-tipped septate trichomes; trichomes to 0.5 mm long; flowers

opening from the base to the apex of the inflorescence, basal flowers beginning to senesce as the uppermost flowers are opening. Floral bracts pubescent; lanceolate, acuminate; concave around the ovary; 7–14 mm long; medium-green. Flowers resupinate; campanulate; slightly to strongly nodding (more so with age); white. Sepals white; free; moderately to densely pubescent with blunt-tipped capitate septate trichomes. Dorsal sepal slightly convex, slightly to strongly reflexed near the tip; lanceolate, bluntly acuminate; 6–12 mm long, 3 mm wide when flattened. Lateral sepals lanceolate, acute; straight to just barely falcate, angled slightly outward and upward, the tips often surpassing the dorsal sepal and petals; 6–12 mm long, 2 mm wide. Dorsal petals white; slightly concave; lanceolate, bluntly acute; slightly to strongly reflexed at tips, with the dorsal sepal appearing stellate; 6–12 mm long, 3 mm wide when flattened. Labellum shortly clawed, free but clasping the column; keeled/concave for its length; reflexed strongly downward at about 1/3 the distance from the claw to labellum apex; centrally glabrous; margin entire to very slightly undulating from the base until the area of reflextion, below point of reflextion margin becoming shallowly laciniate and crisped; 2 basal callosities/nectar glands, white to yellow, conical, rounded, upright, mm tall, with long, dense papillae at the base, papillae mm long; margin white, labellum 7–13 mm long, 3–6 mm wide below the callosities, 2–6 mm wide at the area of reflextion when flattened; apex accuminate. Column protandrous, slightly rhombic, medium-green, narrow, 6 mm long, 2–2.5 mm wide; with a fringe of minute glands or papillate in a thin crescent just below the stigmatic surface; with a pair of upright flaps or wings at each side and clasping the column, the wings green basally; column foot glabrous. Rostellum well-developed, white to ivory, tapering to thin acute membranes at the apex, 1.5 mm long. Stigmatic surface glabrous, shiny, base angled away from the flower opening, roundly diamond-shaped when viewed from below, 2 mm long, 2.5 mm wide. Anther 1, dorsal, triangular-ovoid; pollinium

attached to a well-developed viscidium; viscidium linear, slightly sticky, immersed the rostellum, leaving behind a narrow V-shaped rostellar remnant after removal, mm long. Ovary moderately to densely pubescent with septate trichomes, medium-green. Fruit not seen (hypothesized to be a light brown capsule.

Spiranthes arcisepalum M.C. Pace, sp. nov. — Holotype: United States of America. New York: Hamilton County, just east of Long Lake, town of Long Lake, north of Shaw Pond in wet roadside ditch of Newcomb Road / 28N, collected 4 September 2014, *M.C. Pace 634* (NY!). Isotype: BH.

Spiranthes arcisepalum is most similar to *S. cernua* s.s. and *S. ochroleuca*. It can be distinguished from *S. cernua* s.s. by its more open spiraled inflorescence, smaller flowers, and rounded labellum apex, and it can be distinguished from *S. ochroleuca* by its white colored labellum, and its downward arching lateral sepals.

Terrestrial, acaulescent, deciduous herb, to 30 cm tall including the inflorescence. Roots fasciculate, fleshy, slender. Leaves 1–4, forming a basal rosette, held upright; remaining until the after anthesis, withering shortly thereafter; linear-lanceolate to slightly lanceolate; bluntly acuminate; leaf base tapered and decurrent, shiny medium-green. Inflorescence strongly erect and perpendicular to the ground; peduncle to cm, medium-green; glabrous; 1–2 small lefty cauline bracts occasionally present (frequently absent), quickly reducing to adpressed, clasping, lanceolate, acute bracts; raceme a single row of flowers in an open to slightly tightly coiled spiral (appearing as 1 distinct ‘rank’); moderately to densely pubescent with blunt-tipped septate trichomes; trichomes to 0.5 mm long; flowers opening from the base to the apex of the inflorescence, basal flowers beginning to senesce as the uppermost flowers are opening. Floral

bracts densely pubescent; narrowly lanceolate, acuminate; concave around the ovary; 12.3 mm long; medium-green. Flowers resupinate; campanulate; slightly to moderately nodding and becoming more open with age; white. Sepals white; free; moderately to densely pubescent with blunt-tipped capitate septate trichomes. Dorsal sepal slightly convex, slightly to reflexed to moderately upwardly reclined distally; lanceolate, bluntly acuminate; 8.3 mm long, 2.7 mm wide when flattened. Lateral sepals lanceolate, acute; slightly to strongly downwardly arching and from about 1/3 to 1/2 of their length, the tips often surpassing the labellum margin in profile; 8.5 mm long, 1.4 mm wide. Dorsal petals white; slightly concave; lanceolate, bluntly acute; slightly to strongly reflexed at tips, with the dorsal sepal appearing stellate; 8.3 mm long, 2–2.2 mm wide when flattened. Labellum shortly clawed, free but clasping the column; keeled/concave for its length; reflexed strongly downward at about 1/2 the distance from the claw to labellum apex; constricted near the reflextion and then dilating below; centrally glabrous and thickened; margin entire to very slightly undulating from the base until the area of reflextion, below point of reflextion margin becoming ruffled; 2 basal callosities/nectar glands, white to yellow, conical, rounded, upright, 0.9–1.2 mm tall, with long, dense papillae at the base, papillae mm long; margin white, central area of labellum white to extremely pale yellow back in the throat, labellum 7.2 mm long, mm wide below the callosities, mm wide at the area of reflextion when flattened, 4.8 mm wide at widest point below reflextion. Column protandrous, slightly rhombic, medium-green, narrow; with a fringe of minute glands or papillate in a thin crescent just below the stigmatic surface; with a pair of upright flaps or wings at each side and clasping the column, the wings green basally; column foot glabrous. Rostellum well-developed, white to ivory, tapering to thin acute membranes at the apex. Stigmatic surface glabrous, shiny, base angled away from the flower opening, roundly diamond-shaped when viewed from below. Anther 1,

dorsal, triangular-ovoid; pollinium attached to a well-developed viscidium; viscidium linear, slightly sticky, immersed the rostellum, leaving behind a narrow V-shaped rostellar remnant after removal. Ovary moderately to densely pubescent with septate trichomes, medium-green. Fruit not seen (hypothesized to be a light brown capsule.

Etymology—Latin, '*arcisepalum'* is a combination of '*arcus*'(arching) and '*sepalorum*'(sepals), referring to the downwardly arching lateral sepals –a relatively constant key morphological character– of this species.

Spiranthes* ×*kapnosperia M.C. Pace, hybrid nov. [*Spiranthes ochroleuca* × *Spiranthes cernua*]— Holotype: United States of America. North Carolina: Macon Co. Along Rt. 28, near Highland. 35°01'772"N 83°11'288"W, collected 27 September 2007, *Wen 10003* (US!). Terrestrial, acaulescent, deciduous herb, ca. 30.7–31.1 cm tall including the inflorescence. Roots fasciculate, fleshy, slender. Leaves 1–2, forming a basal rosette, held upright; remaining until the after anthesis, withering shortly thereafter; linear-lanceolate to lanceolate, to 133 mm long, 8.5–10.5 mm wide; narrowly acuminate; leaf base narrowly tapered and decurrent, shiny medium-green. Inflorescence strongly erect and perpendicular to the ground; peduncle to 31.1 cm, medium-green; glabrous; 1–2 small lefty cauline bracts occasionally present, quickly reducing to adpressed, clasping, lanceolate, acute bracts; raceme a single row of flowers in an open to moderately tightly coiled spiral (appearing as single rank), ca. 7 cm long; moderately to densely pubescent with blunt-tipped septate trichomes; trichomes to 0.5 mm long; flowers opening from the base to the apex of the inflorescence, basal flowers beginning to senesce as the uppermost flowers are opening. Floral bracts pubescent; broadly lanceolate, acuminate; concave around the ovary; 11.4–13.7 mm long; medium-green. Flowers resupinate; tubularly campanulate; slightly

to ascending to slightly nodding; white. Sepals white; free; moderately to densely pubescent with blunt-tipped capitate septate trichomes. Dorsal sepal slightly convex, slightly to moderately reflexed near the tip; lanceolate, acuminate; 7.7–9.3 mm long, 1.6 mm wide when flattened. Lateral sepals lanceolate, acute; straight to just barely falcate, angled slightly outward and upward, the tips often surpassing the dorsal sepal and petals; 7.6–9.3 mm long, 1–1.2 mm. Dorsal petals white; slightly concave; lanceolate, bluntly acute; slightly to strongly reflexed at tips, with the dorsal sepal appearing stellate; 7.4 mm long, 1.5 mm wide when flattened. Labellum shortly clawed, free but clasping the column; keeled/concave for its length; reflexed strongly downward at about 2/3 the distance from the claw to labellum apex; centrally glabrous; margin entire to very slightly undulating from the base until the area of reflextion, below point of reflextion margin becoming shallowly ruffled; 2 basal callosities/nectar glands, conical, rounded, upright, mm tall, with long, dense papillae at the base, papillae mm long; margin white, central area of labellum yellowish to ochroleucous, labellum 4 mm wide at the area of reflextion when flattened. Column protandrous, slightly rhombic, medium-green, narrow; with a fringe of minute glands or papillate in a thin crescent just below the stigmatic surface; with a pair of upright flaps or wings at each side and clasping the column, the wings green basally; column foot glabrous. Rostellum well-developed, white to ivory, tapering to thin acute membranes at the apex. Stigmatic surface glabrous, shiny, base angled away from the flower opening, roundly diamond-shaped when viewed from below. Anther 1, dorsal, triangular-ovoid; pollinium attached to a well-developed viscidium; viscidium linear, slightly sticky, immersed the rostellum, leaving behind a narrow V-shaped rostellar remnant after removal. Ovary moderately to densely pubescent with septate trichomes, medium-green. Fruit not seen (hypothesized to be a light brown capsule).

***Etymology*—**From the Greek, '*kapnosperia*' is a combination of '*καπνός*' (smoke) and '*σπείρα*' (spiral), referring to the Smoky Mountains which are the endemic home of this rare hybrid. The choice of Greek (vs. Latin) is also a nod to the Greek-derived specific epithet of *S. ochroleuca*.

Spiranthes* ×*incurvum (Jenn.) M.C. Pace, comb nov., hybrid nov. (pro sp.) [*Spiranthes cernua* × *Spiranthes magnicamporum*]— Lectotype: United States of America. Pennsylvania: Erie Co., Presque Isle. Sandy margins of pond near Fog Whistle, collected 26 August 1905, *O.E. Jennings s.n.* (CM); isolectotypes *O.E. Jennings s.n.*, *18*, 26 August 1905 (CM, NY); isosyntypes: *O.E. Jennings s.n.*, 24 Aug 1905, *O.E. Jennings s.n.*, *18*, *9780*, 25 August 1905 (MICH, MIN, NYS, PH); paratypes: *J.A. Shafer 29, 9781* 9–11 September 1900 (CM, PH), *Gutenberg s.n.* 16 Aug 1880 (CM).

Spiranthes ×*incurvum* is most similar to its parental species: *S. cernua* s.s. and *S. magnicamporum*. It can be distinguished from *S. cernua* s.s.by it more narrowly lanceolate floral parts and more northern distribution, and it can be distinguished from S. *magnicamporum* by its larger callosities and glabrous, white colored labellum.

Terrestrial, acaulescent, deciduous herb, ca. cm tall including the inflorescence. Roots fasciculate, fleshy, slender to slightly tuberous. Leaves 1–3, forming a basal rosette, held upright; rarely remaining until anthesis and withering shortly thereafter but more frequently absent at anthesis; linear-lanceolate to lanceolate; acuminate; leaf base tapered and decurrent, shiny medium-green. Inflorescence strongly erect and perpendicular to the ground; peduncle medium-green; glabrous; 1–2 small lefty cauline bracts occasionally present (frequently absent), quickly reducing to adpressed, clasping, lanceolate, acute bracts; raceme a single row of flowers in a

moderately to tightly coiled spiral (appearing as 1–4 ‘ranks’); moderately to densely pubescent with blunt-tipped septate trichomes; trichomes to 0.5 mm long; flowers opening from the base to the apex of the inflorescence, basal flowers beginning to senesce as the uppermost flowers are opening. Floral bracts moderately to densely pubescent; lanceolate, acuminate; concave around the ovary; 9.5–9.7 mm long; medium-green. Flowers resupinate; slightly tubularly campanulate; slightly ascending to moderately nodding; white. Sepals white; free; moderately to densely pubescent with blunt-tipped capitate septate trichomes. Dorsal sepal slightly convex, slightly to strongly reflexed near the tip; lanceolate, bluntly acuminate; 8.6 mm long, 2 mm wide when flattened. Lateral sepals lanceolate, acute; straight to just barely upwardly falcate, angled slightly upward, the tips often meeting the dorsal sepal and petals; 7.7–9.2 mm long, 1.7 mm wide. Dorsal petals white; slightly concave; lanceolate, bluntly acute; slightly to strongly reflexed at tips, with the dorsal sepal appearing stellate; 8.1 mm long, 2.2 mm wide when flattened. Labellum minutely clawed, free but clasping the column; keeled/concave for its length; reflexed strongly downward at about 1/3 to 1/2 the distance from the claw to labellum apex; centrally thickened and glabrous; margin entire to very slightly undulating from the base until the area of reflextion, below point of reflextion margin becoming shallowly laciniate and crisped; 2 basal callosities/nectar glands, white to yellow, very small, conical to rounded mounds, upright, 0.3–0.8 mm tall, with long, dense papillae at the base, papillae 0.5 mm long; margin white, central area of labellum white to very pale yellow, area of coloration 4.8 mm long, 0.4 mm wide, labellum 7.4–8.1 mm long, 5.4 mm wide below the callosities, 3.3–3.5 mm wide at the area of reflextion when flattened, 3.8–4 mm wide at dialation; apex accuminate. Column protandrous, slightly rhombic, medium-green, narrow, 3.3–3.8 mm long, 1.6–1.7 mm wide; with a fringe of minute glands or papillate in a thin crescent just below the stigmatic surface; with a pair of

upright flaps or wings at each side and clasping the column, the wings green basally, becoming white to translucent; column foot glabrous. Rostellum well-developed, white to ivory, becoming dark drown with age, tapering to thin acute membranes at the apex, 1.6–1.8 mm long. Stigmatic surface glabrous, shiny, base angled away from the flower opening, roundly diamond-shaped when viewed from below, 0.9 mm long, 1.6 mm wide. Anther 1, dorsal, dark coffee-brown, triangular-ovoid, 2.7 mm long; pollinium attached to a well-developed viscidium, yellow, pollinaria 2.5 mm long; viscidium linear, slightly sticky, immersed the rostellum, leaving behind a narrow V-shaped rostellar remnant after removal, 1.2–1.5 mm long. Ovary moderately to densely pubescent with septate trichomes, medium-green. Fruit a light brown capsule.

***Etymology*—**As in Jenning's original description, '*incurvum*', from the Latin, refers to the incurved callosities of this hybrid species. This feature is a key character to distinguish this species from the frequently co-occurring *S. magnicamporum*, one of the parental species, which has highly reduced minute, non-incurved callosities.

DISCUSSION

For the first time, the evolutionary relationships and morphological variation of the *S. cernua* complex s.s. are put into a fully illuminated phylogenetic, biogeographic, and taxonomic context (Fig. 7–20). *Spiranthes arcisepalum* is a newly described and long overlooked cryptic species restricted to the mid- and northern Blue Ridge & Northern Highlands, Ridge & Valley, Great Valley, Appalachian Plateau, and Adirondack systems of the Appalachian Highlands in CT, MA, ME, NH, NJ, NY, OH, PA, VA, VT, WV, and southern Quebec, and the eastern Interior Lowlands of western OH, and it is the sister species to *S. ochroleuca* + *S. casei*. The distribution of *S. arcisepalum* is essentially similar to *S. ochroleuca* apart from the occurrence of

the latter being found further westward into the Great Lakes Basin and southward along the spine of the Appalachian Mountains to KY, NC, and TN (fig. 11). Contrastingly, *S. cernua* s.s. occurs across the Coastal Plain, Piedmont, eastern and southern Appalachian Mountains, and southern Interior Lowlands and Cumberland Plateau, from New England to TX, the western Ouachita Mountains of AR, the southern Cumberland Plateau of KY, and the northern limit of the Mississippi Embayment (fig. 11). Within wide-ranging *S. cernua* s.s., we here formally synonymize *S. parksii* under *S. cernua* as a localized peloric form promulgated through apomixis, supporting the work of Dueck and Cameron (2007; 2008a), Dueck et al. (2014), and Chapters 1 & 2. Additionally, *S. cernua* s.s. and *S. ochroleuca* are documented to form a rare hybrid –*S.* ×*kapnosperia*– in the southern Blue Ridge and Great Smoky Mountains of NC and SC, and *S. cernua* s.s. is entirely replaced by *S.* ×*incurvum* –*S. cernua* s.s. × *S. magnicamporum*– in the middle, northern, and eastern Interior Lowlands, Great Lakes Basin, and Prairie Peninsula, from ca. MO north to MN, and eastward to western NY, southern Ontario and Quebec, and northern Vermont.

Taxonomy—Interpretation of published names –especially older names– in *Spiranthes* is frequently challenging in the absence of accompanying images, well-preserved specimens, and genetic and geographic context. However, particular and/or multiple lines of evidence support the conclusions we reach here. *Spiranthes cernua* can be placed as the correct name for our *S. cernua* s.s clade (fig. 7–10) based primarily on the morphology of its Lectotype. The geographic origin of this specimen cannot be definitively determined, as Kalm traveled throughout the ranges of *S. arcisepalum, S. cernua* s.s., and *S.* ×*incurvum*, from the greater Philadelphia region, north to Montreal, and west to Niagara Falls. Furthermore, the brief formal description provided by Linnaeus could apply to either *S. cernua* s.s. or *S.* ×*incruvum* (though it falls more towards *S.*

cernua). However, the predominantly strongly nodding flowers of the lectotype is a particularly common feature of plants studied and collected for phylogenetic analysis by the first author in northern Delaware and the Delmarva Peninsula (e.g., sample sc1), and the labellum is essentially identical to the broadly oblong, dilated labella of plants from the Coastal Plain –i.e., *S. cernua* s.s.– versus the broadly to narrowly lanceolate labella of *S.* ×*incurvum*. The upward to horizontal position of the lateral sepals and tight coil to the inflorescence do not suggest any affinity to plants from the Appalachian Mountains (i.e. *S. arciseplaum*). Thus, we are confident in assigning the Kalm lectotype specimen from LINN to our phylogenetic clade, as *S. cernua* s.s. As discussed in Sheviak (1982), the type of *Limodorum autumnale* also conforms to plants recovered within our phylogenetic clade *S. cernua* s.s., and should be placed in synonymy with *S. cernua*; the collection location for that specimen of 'Carolina' also supports this conclusion.

In contrast to *S. cernua* s.s., the thorough original description and ample type material of *S.* ×*incurvum* (as *Ibidium*, pro. sp.) creates a rich and detailed concept of this taxon, matching the morphology of phylogenetically sampled individuals we recovered as being hybrid. Combined with its geographic origin from the narrow strip of Central Lowlands along the Lake Erie coast in northwestern Pennsylvania (a region where our phylogenetic data indicate *S. cernua* s.s. does not occur), this name confidently and unambiguously represents plants that we found using molecular data to be hybrid in origin between *S. cernua* s.s. and *S. magnicamporum*. As the oldest confidently placed name for hybrid *S. cernua* s.s. × *S. magnicamporum* individuals, *Ibidium incurvum* is transferred to *Spiranthes* and placed in a new category as a hybrid, serving as the basionym for S. ×*incurvum*.

Ambiguity of the Rafinesque names—The names *S. brevicaulis*, *S. flexuosa*, and *S. petiolaris* –all described by Rafinesque– cannot be placed. All three are lacking type specimens

(likely destroyed), and their descriptions are too brief and ambiguous to ascribe any one name to a particular morphology or phylogenetic clade with any confidence. A similar conclusion was reached by Sheviak (1982) who termed them as "*nomina obscura*". Based on their formal description and geographic origin, *S. brevicaulis* and *S. petiolaris* could represent *S. cernua, S.* ×*incurvum,* or *S. magnicamporum*; all three taxa match aspects of Rafinesque's descriptions, and all three grow in the general vicinity of the Ohio River Valley in Illinois (*S. petiolaris*) and Kentucky (*S. brevicaulis*) (*S. cernua* may occur as far north as the Cumberland Plateau and Ohio and Mississippi River confluence). The description of *S. flexuosa,* collected in the Appalachian Mountains (no state given) is so vague that it could refer to *S. arcisepalum*, *S. cernua* s.s., S. ×*incurvum, S. ochroleuca,* or *S. ovalis*. Although these are validly published names, the destruction of their type specimens and lack of descriptive detail render them unable to be placed, and thus relegated to the side lines of taxonomy in favor or more recently published and confidently placed names.

Phylogenetic and morphological relationships of S. arcisepalum, S. ochroleuca, and status of S. casei—Supporting the earlier work of Dueck et al. (2014), we recovered *S. casei* as broadly embedded within a largely unresolved polytomy with *S. ochroleuca*. Based on this phylogenetic topology and the morphological, ecological, and reproductive context we discuss below, we think the evolutionary reality of '*S. casei*' may be best expressed as an ecologically specific subspecies of *S. ochroleuca*. *Spiranthes casei* can be distinguished from *S. ochroleuca* by its smaller flowers, reduced and slightly peloric labellum, predominantly apomictic nature, and restriction to lichen and bracken barrens of the northern Great Lakes Basin, Adirondack Mountains, Saint Lawrence River Valley, and the Canadian Maritimes. Next generation

sequencing data may afford increased resolution within this *S. ochroleuca*/*S. casei* clade; thus we do not make any formal taxonomic changes here.

Together, *S. arcisepalum*, *S. ochroleuca* and *S. casei* form a sister clade to *S. cernua* s.s. (fig. 7 & 8). Both *S. arcisepalum* and *S. cernua* s.s. retain an ancestral flower color state of white (Chapter 5), whereas *S. ochroleuca* and *S. casei* share a derived flower color of ochroleucous or ivory. The lateral sepals of *S. arciseplaum* and *S. casei* are typically downwardly arching (though occasionally present as parallel to the ground), whereas the lateral sepals of *S. cernua* s.s. and *S. ochroleuca* are angled upward. Similarly, the inflorescences of *S. arcisepalum* and *S. casei* are usually openly spiraled, such that a single helix is obvious. In contrast, *S. cernua* and *S. ochroleuca* are frequently tightly spiraled (though this is variable). The dorsal sepal and petals of *S. casei* do not reflex (or just barely so), whereas in *S. arcisepalum* they are modernly reflexed or inclined, and are strongly reflexed and curling at the apices in *S. ochroleuca*.

Hybridization—Owing to the pioneering work of Catling (1982) and Sheviak (1976, 1982, 1991), *S. cernua* s.l. had traditionally been hypothesized to engaging in frequent unidirectional and geographically-specific hybridization as the "gene recipient" (i.e. maternal parent) with all other members of the traditional *S. cernua* species complex. This notion is discussed for the entire genus more thoroughly in Chapter 4. In all instances, plants of *S. cernua* s.l. that were found within the general range of any of these other species were presumed to be hybrid in origin. Sheviak (1982, 1991) called potential *S. magnicamporum* × *S. cernua* hybrids – here designated as *S.* ×*incurvum*– triploid and tetraploid forms and races of *S. cernua* that arose through "adaptive gene flow" whereby ecologically advantageous genes, characteristics, and morphologies are incorporated into *S. cernua* s.l. through repeated backcrossing. Thereby *S. cernua* s.l., which in eastern North America inhabits moist banks, bogs, fens, meadows, lake

edges, and seeps, could survive in drier and fire prone Midwestern prairies. The overarching premise of this hypothesis –that hybridization might confer some evolutionary benefit in a novel environment on the resulting plants vs. non-hybrid *S. cernua* through incorporation of novel genetic information and neofunctionalization– is plausible and could possibly be supported by the molecular phylogenetic data we present here. Yet *S.* ×*incurvum* does not occur over the entire shared range of *S. cernua* s.s. and *S. magnicamporum* as originally hypothesized by Catling (1982) and Sheviak (1976, 1982, 1991; Sheviak and Brown 2002). Instead, hybrids are restricted to –and entirely replace *S. cernua* s.s.– in the upper Midwest, Great Lakes Basin, Saint Lawrence River Valley, and northern Appalachian Mountains in wet to dry habitats including prairies, dune swales, wet fens, xeric and hot road banks, and alvar escarpments (fig. 11 & 18). Included individuals of *S. cernua* s.s. from Arkansas, Texas (including *S. parksii*), and northern Georgia were not recovered as hybrids despite the occurrence of *S. magnicamporum* throughout those regions (fig. 12). Intriguingly, *S.* ×*incurvum* occurs outside of the range of *S. magnicamporum* (fig. 11 & 12), such as in Wisconsin north of the Tension Zone, the Keweenaw Peninsula of Michigan, and northern Vermont. Furthermore, as indicated by the taxonomically re-interpreted breeding work of Sheviak (1982), *S.* ×*incurvum* appears to be reproductively isolated from *S. magnicamporum*, as crosses between these taxa did not produce seeds. Thus introgression back into *S. magnicamporum* appears to be absent. As all of the samples from this region originally identified as *S. cernua* were recovered as hybrids, it appears likely that all plants previously identified as '*S. cernua*' from the upper Midwest, Great Lakes Basin, Interior Lowlands, and Saint Lawrence River Valley actually represent *S.* ×*incurvum*, and yet we must concede that the exact geographic and/or ecological boundaries among *S.* ×*incurvum*, *S. cernua* s.s., and *S. magnicamporum* are unclear (Fig. 11). Catling and Brown (1983), working on co-occurring

populations of '*S. cernua*' and *S. magnicamporum* in the Eastern Peninsula of Southwestern Ontario, identified only three potential individuals of *S. magnicamporum* × *S. cernua*. Unfortunately, we were unable to incorporate samples of putative pure '*S. cernua*' from this area into our study, although included accessions of '*S. cernua*' from Michigan, Ohio, and Ottawa, Ontario, were recovered as *S.* ×*incurvum*. *Spiranthes magnicamporum* from two stations in southern Ontario were included and recovered as non-hybrid. Additional sampling in Kansas, Kentucky, Missouri, Oklahoma, Ontario, and Quebec are needed to delineate the exact geographic distribution of *S. cernua* s.s, and *S.* ×*incurvum*.

We did not recover evidence supporting the occurrence of hybridization between *S. arcisepalum* and *S. magnicamporum*, even when these plants grow within pollination range in northern Ohio or western Virginia. Furthermore, we did not find evidence of hybridization between *S. arcisepalum* and *S.* ×*incurvum*; these taxa are broadly sympatric in Ohio and comingle at locations in the Adirondack Mountains of NY near Dorset and Indian Lakes. Future population genetic based approaches may reveal evidence supporting low-levels of hybridization or introgression that we were unable to detect given the phylogenetic markers we utilized for the present work.

Discreet quantitative traits to distinguish these cryptic taxa are mostly lacking: the individual parts of the flowers are essentially the same size or encompass other species in their range of morphological variability, and sympatric and co-occurring plants overlap in phenology (fig. 6). However qualitative traits, including leaf shape, labellum shape and apex type, lateral sepal orientation, and geographic distribution are key features that definitively distinguish these four taxa (fig. 1, 2, 11, 13, 14, 15, 16, 17, 20).

Our phylogenetic reconstructions indicate that these orchids are very recently evolved and very closely related, as indicated by the presence of very short branch lengths within the *S. cernua* species complex s.s. It is possible these species and hybrids evolved in response to the glacial cycles of the Quaternary as populations of ancestral species fragmented and/or migrated. The evolution of the *S. cernua* species complex has been dated to have occurred ca. 1.5–2.5 mybp (see Chapter 6).

The role of apomixis in questions of species status & taxonomic rank—The *S. cernua* species complex has a long history of focused study on the occurrence of apomixis, most specifically examining the phenomenon in *S. cernua* s.l. (Leavitt 1900, 1901; Schnarf 1929; Swamy 1948; Catling, 1982; Sheviak 1982; Schmidt and Antifinger 1992). Together, this literature establishes facultative polyembryony as a frequent occurrence across the geographic ranges of *S. cernua* s.s. (including *S. parksii*), *S.* ×*incurvum*, *S. ochroleuca*, and *S. casei*. Populations of some taxa, such as *S.* ×*incurvum* and *S. casei*, are frequently composed entirely of apomicitic individuals (the former *S. parksii* and some populations of other taxa being obligate apomicts), and the frequency of apomixis can vary from year to year, possibly in relation to environmental factors. Apomixis is also frequently tied to instances of cleistogamy (*S.* ×*incurvum*, fig. 15 g) and/or degrees of peloria (typically a reduction of the labellum as exhibited in *S. casei*, some populations of *S.* ×*incurvum*, and the former *S. parksii*).

Apomixis and the accompanying concept of microspecies are important and regionally significant evolutionary forces, particularly when combined with polyploidy and complex patterns of hybridization and reticulate evolution (e.g., Burgess et al. 2014; Dyer et al. 2012). However, we find the use of apomixis to support the species status of mico-endemic, mostly asexually reproduced, hybridizing, genetically homogenous, and morphologically minutely

different individuals and micropopulations (e.g. *Sorbus*, Ludwig et al. 2013) as dubious, especially when hybridization and reticulation occur to a degree –even requiring intra-taxon hybridization– such that it could be argued these microspecies actually represent a nearly panmictic meta-population of a single species. Accordingly, we think apomictic populations must also have additional features distinguishing them from other taxa. In the case of the former *S. parksii*, all of the available molecular phylogenetic and microsatellite data fail to find any reciprocally monophyletic cladogenesis or genetic distinction between this so-called species and *S. cernua* s.s. from surrounding areas in Texas and the wider North American Coastal Plain. Furthermore, the plants are highly restricted in distribution, all apparent floral distinctions appear to be tied to mutant peloria (which is particularly common in the short-grass prairies of the Midwest), and there is no ecological or phenological distinction between it and *S. cernua* s.s. As such, we think the evidence strongly supports the position of '*S. parksii*' as an apomictic regional floral morph and synonym of *S. cernua* s.s. In contrast, predominantly apomictic *S. casei* is distributed over a wide area and is morphologically and ecologically distinct from *S. ochroleuca*; however we did not recover any phylogenetic distinction between these a priori species. It may be possible that *S. casei* may have evolved several times as an ecotype specializing on lichen barrens and disturbed open thickets. Future studies that incorporate next generation DNA sequencing data and methods that address questions at the population genetic level should be conducted in order to continue to evaluate the hypotheses presented here.

CONCLUSIONS

The *Spiranthes cernua* complex, although a group that is still challenging to identify, represents an intriguing natural group through which to explore issues regarding recent

speciation events in the face of hybridization, within-species phenotypical variability and cryptic speciation. Although we have solved some of the long standing problems within the complex with the description and taxonomic refinement of *S. arcisepalum*, *S. cernua*, *S.* ×*incurvum*, and *S.* ×*kapnosperia*, more work is clearly necessary. It is our hope that the *S. cernua* species complex can help biologist understand evolutionary processes within Eastern North America, while also informing conservation practices of these small and challenging, but fascinating, orchids.

Adams, M., T.A. Raadik, C.P., Burridge, A. Georges. 2014. Global Biodiversity Assessment and Hyper-Cryptic Species Complexes: More Than One Species of Elephant in the Room? Systematic Biology, 63(4): 518–533.

Becker, M., N. Gruenheit, M. Steel, C. Voelckel, O. Deusch, P.B. Heenan, P.A. McLenachan, et al. 2013. Hybridization may facilitate in situ survival of endemic species through periods of climate change. Nature Climate Change, 3(12): 1039–1043.

Bertrand, Y.J.K., A. Scheen, T. Macrussen, B.F. Pfeil, F. de Sousa, and B. Oxelman. 2015. Assignment of homoeologs to parental genomes in allopolyploids for species tree inference, with an example from *Fumaria* (Papaveraceae). Systematic Biology, 64(2):

Bickford, D., D.J. Lohman, N.S. Sodhi, P.K.L. Ng, R. Meier, K. Winker, K.K. Ingram, and I. Das. 2007. Cryptic species as a window on diversity and conservation. Trends in Ecology and Evolution, 22(3): 148-155.

Brown, P.M. 1999a. Recent taxonomic and distributional notes from Florida, 1. North American Native Orchid Journal, 5(1): 3–16.

Brown, P.M. 1999b. Recent taxonomic and distributional notes from Florida, 4. North American Native Orchid Journal, 5(4): 358–367.

Brown, P.M., L.A. Dueck, and K.M. Cameron. 2008. *Spiranthes stellata* (Orchidaceae), a new species of ladies'-tresses from the western United States. North American Native Orchid Journal, 14(1): 3–11.

Burgess, M.B., K.R. Cushman, E.T. Doucette, N. Talent, C.T. Frye, and C.S. Campbell. 2014. Efects of apomixis and polyploidy on diversification and geographic distribution in *Amelanchier* (Rosaceae). American Journal of Botany, 101(8): 1375–1387.

Cameron, K.M. 2010. On the Value of Taxonomy, Phylogeny, and Systematics to Orchid Conservation: Implications for China's Yachang Orchid Reserve. Botanical Review, 76 (2): 165–173.

Catling, P.M. 1981. Taxonomy of the autumn flowering Spiranthes species of southern Nova Scotia. Canadian Journal of Botany – Revue Canadienne de Botanique, 59(7): 1253–1270

Catling, P.M. 1982. Breeding systems of northeastern North American *Spiranthes* (Orchidaceae). Canadian Journal of Botany – Revue Canadienne de Botanique, 60(12): 3017–3029.

Catling, P.M. 1983. Pollination of northeastern North American *Spiranthes* (Orchidaceae). Canadian Journal of Botany – Revue Canadienne de Botanique, 61(4): 1080–1093.

Catling, P.M., and J.R. Brown. 1983. Morphometrics and ecological isolation in sympatric *Spiranthes* (Orchidaceae) in southwestern Ontario. Canadian Journal of Botany – Revue Canadienne de Botanique, 61(10): 2747–2759.

Dueck, L.A., and K.M. Cameron. 2007. Sequencing re-defines *Spiranthes* relationships with implications for rare and endangered taxa. Lankesteriana, 7(1-2): 190–195. =

Dueck, L.A., and K.M. Cameron. 2008a. Molecular evidence on the species status and phylogenetic relationships of *Spiranthes parksii*, an endangered orchid from Texas. Conservation Genetics, 9:1617–1631.

Dueck, L.A., and K.M. Cameron. 2008b. Molecular evidence for new species – *Spiranthes stellata*. North American Native Orchid Journal, 14(1): 12–21.

Dyer, R.J., V. Savolainen, and H. Schneider. 2012. Apomixis and reticulate evolution in the *Asplenium monanthes* fern complex. Annals of Botany, 110(8): 1515-1529.

Górniak, M., O. Paun, and M.W. Chase. 2010. Phylogenetic relationships within Orchidaceae based on a low-copy nuclear coding gene, *Xdh*: Congruence with organellar and nuclear ribosomal DNA results. Molecular Phylogenetics and Evolution, 56: 784–795.

Guo, Y-Y, Y-B Luo, Z-J Liu, and X-Q Wang. 2012. Evolution and Biogeography of the Slipper Orchids: Eocene Vicariance of the Conduplicate Genera in the Old and New World Tropics. PLoS ONE 7(6): e38788. doi:10.1371/journal.pone.0038788

Harland, J.R., and J.M. deWet. 1963. The compilospecies concept. Evolution, 17:497–501.

Hopper, S.D. 2009. Taxonomic turmoil down-under: recent developments in Australian orchid systematics. Annals of Botany, 104(3): 447–455.

Huson, D.H., and D. Bryant. 2006. Application of Phylogenetic Networks in Evolutionary Studies. Molecular Biology and Evolution, 23(2): 254–267.

Jacobsen, S., W. Brown, B. McGee, J. McIntyre, and C. Best. 2009. 5-Year Review: Navasota Ladies'-Tresses/*Spiranthes parksii* Correll. U.S. Fish and Wildlife Service Austin Ecological Services Field Office Austin, Texas.

Leavitt, R.G. 1900. Polyembryony in *Spiranthes cernua*. Rhodora, 2: 227–228.

Leavitt, R.G. 1901. Notes on the embryology of some New England orchids. Rhodora 3: 61–63.

Linnaeus, C.V. 1763. *Species plantarum: exhibentes plantas rite cognitas, ad genera relatas, cum differentiis specificis, nominibus trivialibus, synonymis selectis, locis natalibus, secundum systema sexuale digestas*. Editio secunda, aucta.; Holmiae: Impensis Direct, Laurentii Salvii, 1762-63.

Ludwig, S., A. Robertson, T. C.G. Rich, M. Djordjević, R. Cerović, L. Houston, S.A. Harris, and S. J. Hiscock. 2013. Breeding systems, hybridization and continuing evolution in Avon Gorge *Sorbus*. Annals of Botany, 111: 563–575.

Manhart, J.R. and A.E. Pepper. 2007. A genetic study of the rare and endangered orchid *Spiranthes parksii* Correll in a comparative context. Final report. Contract no. 147331.

Markolf, M., M. Brameier, and P.M. Kappeler. 2011. On species delimitation: Yet another lemur species or just genetic variation? BMC Evolutionary Biology, 11: 216.

Martin, C. and L.C. Feinstein. 2014. Novel trophic niches drive variable progress towards ecological speciation within an adaptive radiation of pupfishes. Molecular Ecology, 23: 1846–1862.

Miller, M.A., Pfeiffer, W., Schwartz, T., 2010. Creating the CIPRES science gateway for inference of large phylogenetic trees. Proceedings of the Gateway Computing Environments Workshop (GCE), New Orleans, LA, pp 1–8.

Monzon, J., R. Kays, and D.E. Dykhuizen. 2012. Assessment of coyote-wolf-dog admixture using ancestry-informative diagnostic SNPs. Molecular Ecology, 23(1): 182–197.

Neubig, K.M., W.M. Whitten, B.S. Carlsward, M.A. Blanco, L. Endara, N.H. Williams, and M. Moore. 2009. Phylogenetic utility of *ycf*1 in orchids: a plastid gene more variable than *mat*K. Plant Systematic Evolution, 277: 75–84.

Paul, J., C. Budd, and J.R. Freeland. 2013. Conservation genetics of an endangered orchid in eastern Canada. Conservation Genetics, 14: 195–204.

Pettengill, J.B., and M.C. Neel. 2011. A sequential approach using genetic and morphological analyses to test species status: The case of United States Federally endangered *Agalinis acuta* (Orobanchaceae). American Journal of Botany, 98(5): 859–871.

Pillon, Y., and M.W. Chase. 2007. Taxonomic Exaggeration and Its Effects on Orchid Conservation. Conservation Biology, 21(1): 263–265.

Rambaut. A. 2014. FigTree v1.4.2, released 2014-07-09. http://tree.bio.ed.ac.uk/software/figtree/

Rutledge, L.Y., B.N. White, J.R. Row, and B.R. Patterson. 2012. Intense harvesting of eastern wolves facilitated hybridization with coyotes. Ecology and Evolution, 2(1): 19–33.

Sangster, G. 2009. Increasing numbers of bird species results from taxonomic progress, not taxonomic inflation. Proceedings of the Royal Society: Biological Sciences, 276(1670): 3185–3191.

Schmidt, J.M., and A.E. Antifinger. 1992. The level of agamospermy in a Nebraska population of *Spiranthes cernua* (Orchidaceae). American Journal of Botany, 79(5): 501–507.

Schnarf, K. 1929. *Embryologie der Angiospermen*. Borntrager, Berlin.

Sheviak, C.J. 1973. A new *Spiranthes* from the grasslands of central North America. Botanical Museum Leaflets, 23(7): 285–297.

Sheviak, C.J. 1982. Biosystematic study of the *Spiranthes cernua* complex. Bulletin of the New York State Museum Science Service, Bulletin Number 448.

Sheviak, C.J. 1991. Morphological variation in the compliospecies *Spiranthes cernua* (L.) Rich.: ecologically-limited effects of gene flow. Lindleyana, 6(4): 228–234.

Sheviak, C.J., and P.M. Brown. 2002. *Spiranthes*. In *Flora of North America*, Volume 26. Oxford University Press, USA.

Sheviak, C.J., and P.M. Catling. 1980. The identity and status of *Spiranthes ochroleuca* (Rydberg) Rydberg. Rhodora, 82(832): 525–562.

Shirley, M.H., K.A. Vliet, A.N. Carr, and J.D. Austin. 2014. Rigorous approaches to species delimitation have significant implications for African crocodilian systematics and conservation. Proceedings of the Royal Society: Biological Sciences, 281(1776): 1–10.

Soltis, P.S., and M.A. Gitzendanner. 1998. Molecular Systematics and the conservation of Rare Species. Conservation Biology: 13 (3): 471-483.

Streicher, J.W., T.J. Devitt, C.S. Goldberg, J.H. Malone, H. Blackmon, and M.K. Fujita. 2014. Diversification and asymmetrical gene flow across time and space: lineage sorting and hybridization in polytypic barking frogs. Molecular Ecology, 23: 3273–3291.

Swamy, B.G.L. 1948. Agamospermy in *Spiranthes cernua*. Lloydia, 11: 149–162.

Swarts, N.D., M.A. Clements, C.C. Bower, and J.T. Miller. 2014. Defining conservation units in a complex of morphologically similar, sexually deceptive, highly endangered orchids. Biological Conservation, 174: 55–64.

van Dongen, W.F.D., R.A. Vásquez, H. Winkler. 2012. The use of microsatellite loci for accurate hybrid detection in a recent contact zone between an endangered and a recently-arrived hummingbird. Journal of Ornithology; 153(2): 585–592.

Walters, C. 2005. Genetic relationships among *Spiranthes parksii* and congeneric species. Master's Thesis. Texas A&M University

Wayne, R.K., and S.M. Jenks. 1991. Mitochondrial-DNA analysis implying extensive hybridization of the endangered red wolf *Canis rufus*. Nature, 351(6327): 565–568.

Zachos, F.E., M. Apollonio, E.V. Bärmann, M. Festa-Bianchet, U. Göhlich, J.C. Habel, Elisabeth Haring, et al. 2013. Species inflation and taxonomic artefacts–A critical comment on recent trends in mammalian classification. Mammalian Biology, 78: 1–6.

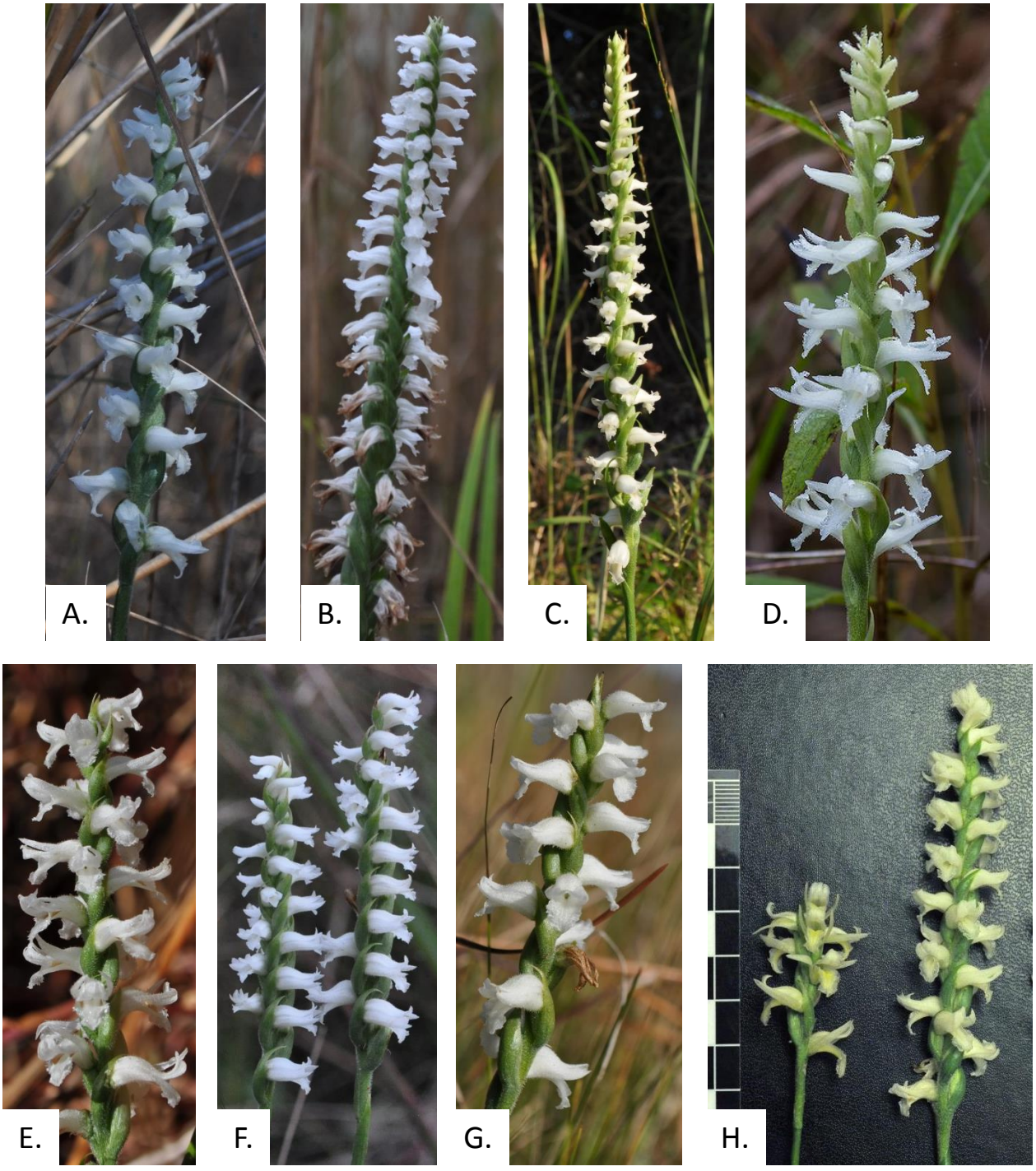
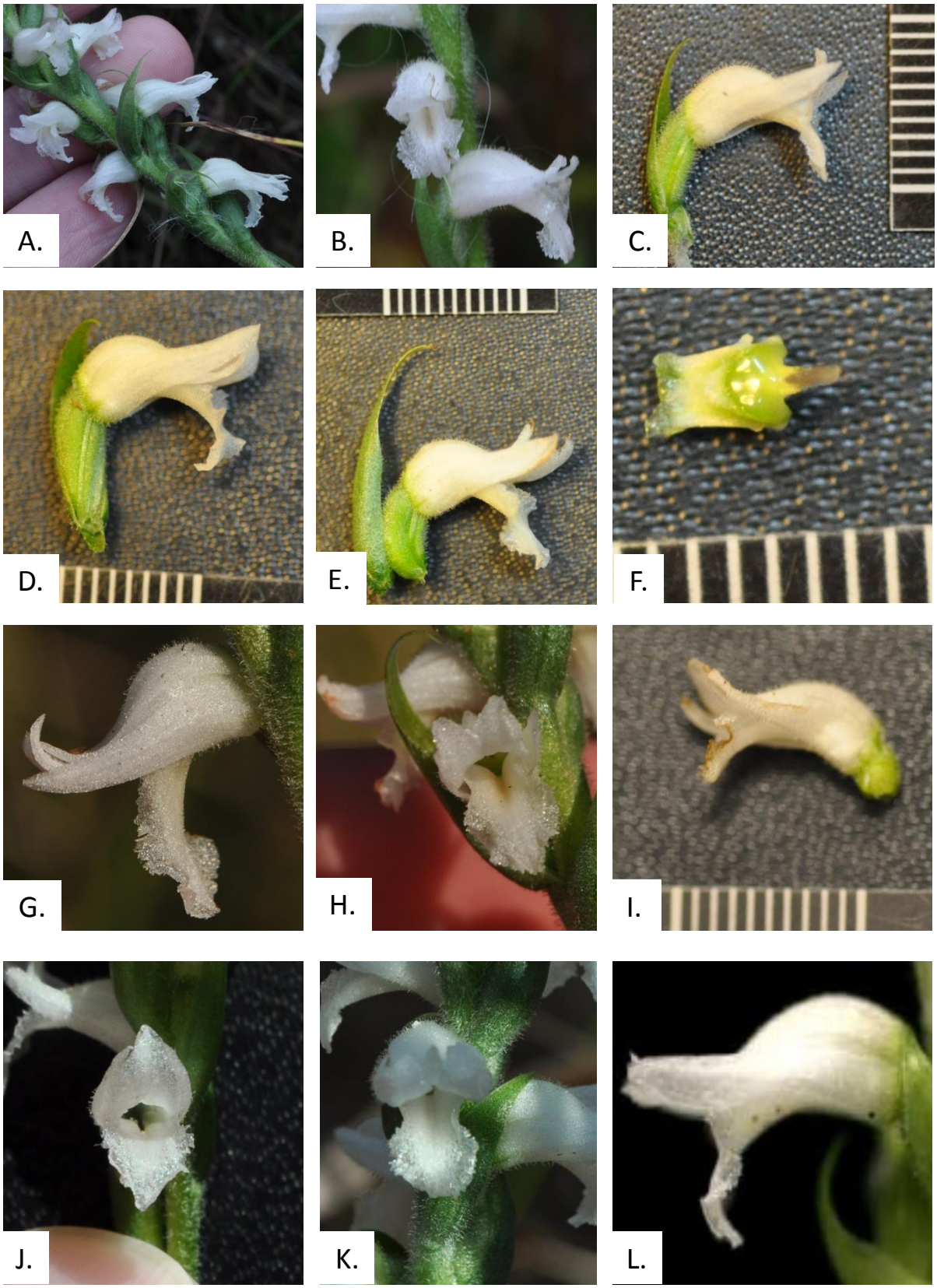


Fig. 1. *Spiranthes cernua* s.s. **A.** Lexington Co., SC. **B.** Worcester Co., MD, sample location of sc8. **C.** Sussex Co., DE. **D.** Towns Co., GA. Photo: J. Fowler. **E.** James City Co., VA, sample location of sc9. **F.** Wayne Co., GA, sample location sc15. **G.** Pike Co., AL. **H.** *Spiranthes odorata* (L), Richland Co., SC; *S. cernua* (R), Lexington Co., SC.

Fig. 2. [Legend to following page of pictures] *Spiranthes cernua* s.s. Scale bars, where present, denote mm. **A–C.** Bear, New Castle Co., DE, sample location sc1. Near the original location of the horticultural cultivar *S*. *cernua* 'Chadds Ford'. **D.** Black Bird, New Castle Co., DE. **E–F**. Kent Co., DE. **F.** Column with stigmatic surface. **G–H.** Sussex Co., DE. **I.** Cape May Co., NJ., sample location for sc6. **J.** James City Co., VA, sample location for sc9. **K.** Lexington Co., SC. **L.** Liberty Co., FL. Photo S. Stewart, sample site of 4m.




A.
B.
C.
D.
E.
F.
G.
H.
I.
J.
K.
L.

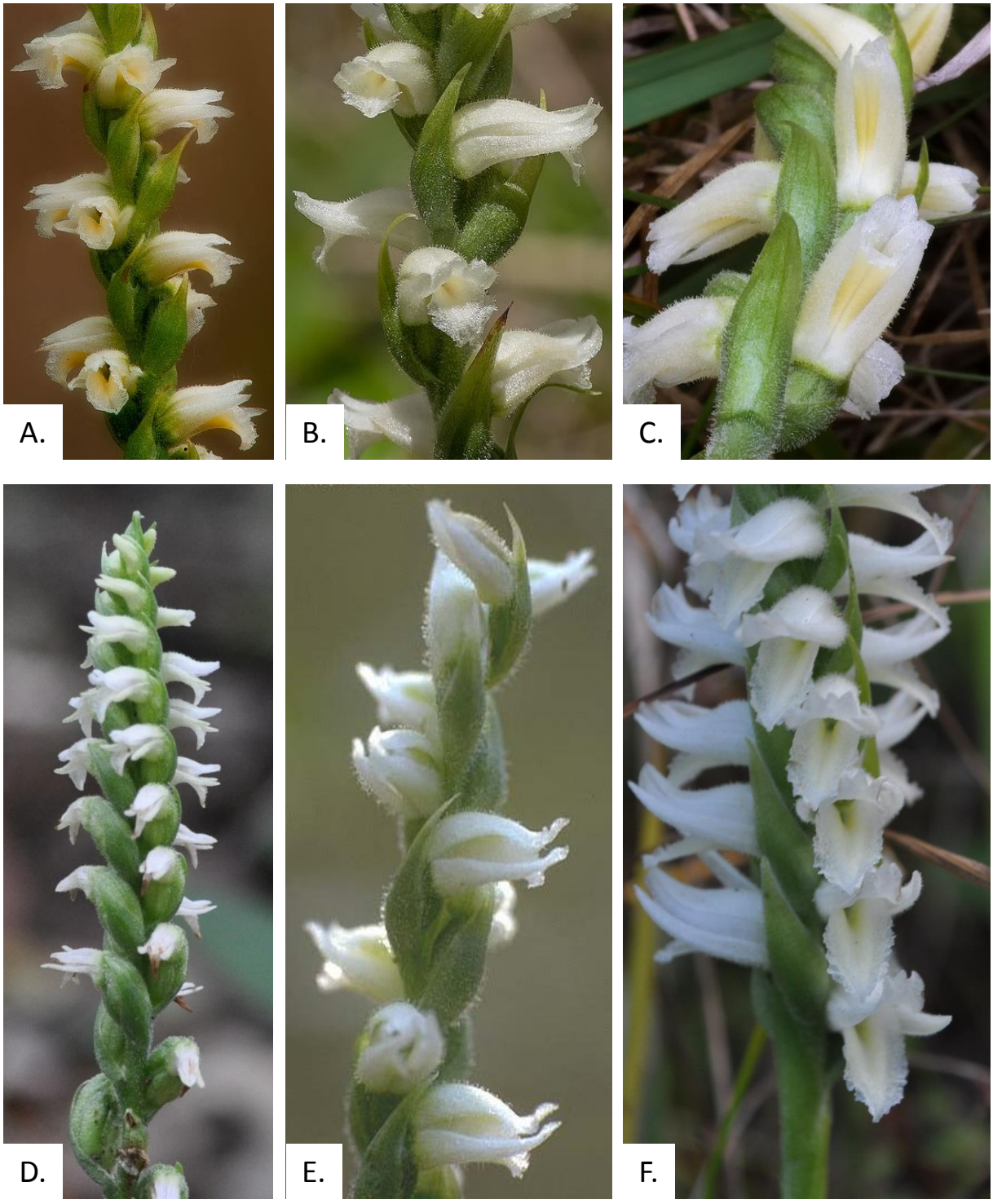


Fig. 3. A. *Spiranthes casei*. Photo: A. Strouse. **B–C.** *Spiranthes ochroleuca*. Photos: J. Fowler. **C.** Characteristic ochroleucous-yellow coloration on abaxial labellum surface. **D.** *Spiranthes ovalis* var. *erostellata*, Waukesha Co., WI. **E.** *Spiranthes parksii* (= *S*. *cernua* s.s.). Photo H. Wilson. **F.** *Spiranthes odorata*, Taylor Co., FL, sample location of so10.

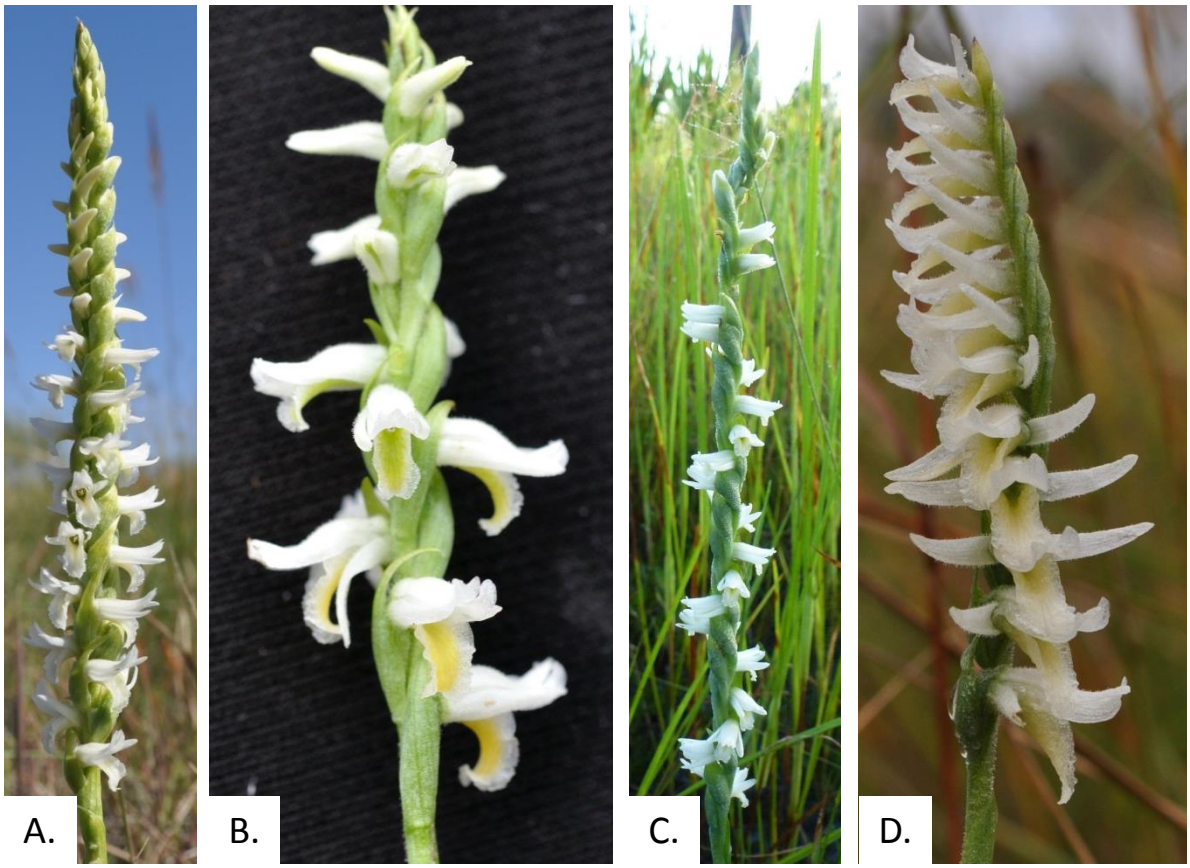


Fig. 4. A. *Spiranthes magnicamporum*, Guadalupe Co., NM, sample sm7h. **B.** *Spiranthes triloba*, Collier Co., FL36. **C.** *Spiranthes igniorchis*, Polk Co., FL, sample 26734B. Photo: S. Orzell. **D.** *Spiranthes longilabris*, Brunswick Co., NC, sample location of 13b.

Fig. 5. [Next four pages] Chart of molecular phylogenetic samples used in our phylogenetic reconstructions. An ‘x’ indicates successful amplification.



ID #	Species/Variety	Collection Site	Collector	Coll.Date	ACO	ITS	matK	ndhj	trnLF	trnSM	Xdh	ycf1 3'	Herbarium specimen	Photo Voucher
	arcisepalum. Northeast/Appalachian													
4c	S. arcisepalum	Giles Co., VA	Stan Bentley	9/6/03			x	x	EU384709	x		x		
4e	S. arcisepalum	Armstrong Co., PA (ne-Pit)	Scott Shriver	9/20/03		x	x	x	EU384711	x		x		
4f	S. arcisepalum	Somerset Co., PA (se-Pit)	Scott Shriver	9/20/03		x	x	x	x	x		x		
4g	S. arcisepalum	Westmoreland Co., PA (e-Pit)	Scott Shriver	9/20/03		x	x	x	x	x		x		
4s	S. arcisepalum	Arookstook Co., ME	PMB	9/13/06			x	x	x	x		x		
4u	S. arcisepalum	Seneca Co., OH	David McCann	9/25/06	x	x	x	x	x	x	x	x		yes
4y	S. arcisepalum	Oneida Co., NY (Oriskany)	Charles Ufford	10/3/06	x	x	x	x	x	x	x	x	82220	yes
4z	S. arcisepalum	Singer Bog, OH	Tim M. Jones	10/1/06	x	x	x	x	x	x	x	x	82216-7*	yes
NY1	S. arcisepalum	Delaware Co., NY	M.C. Pace	_2015		x	x	x	x		x		Pace s.n.	yes
sc30a	S. arcisepalum	Seneca Co., OH	M.C. Pace	_2015		x	x	x	x		x		Pace s.n.	yes
	ochroleuca/casei													
2a	S. casei var. casei	Montmorency Co., MI	Fred Case	~9/04	x	x	x	x	x	x	x	x	80510	
2b	S. casei var. casei (atyp?)	Newaygo Co., MI	Fred Case	9/3/04	x	x	x	x	x	x		x		
2d	S. casei var. casei	Marinette Co. (Pond 518), WI	Kip Knudson	9/15/06	x	x	x	x	x	x	x	x		yes
2e	S. casei var. casei	Hamilton Co., NY (Speculator)	Charles Ufford	9/16/06	x	x	x	x	x	x	x	x		
4h	S. ochroleuca	Randolph Co., WV	Scott Shriver	10/19/03			x	x	EU384712	x		x		
4n	S. ochroleuca	Essex Co., NY (Muller Pond)	Ken Cameron	9/4/06	x	x	x	x	x	x	x	x	82209	
4p	S. ochroleuca	Washington Co., ME	Lucy Dueck	8/26/06		x	x	x	x	x	x	x		yes
4wx	S. ochroleuca	Lucas Co., OH	Lucy Dueck	10/4/06	x	x	x	x	x	x		x	82206, 82208	yes
16a	S. ochroleuca	Oconee Co., SC	Jim Fowler	10/25/02		x	x	x	x	x		x	80494	yes
16b	S. ochroleuca	Craig Co., VA	Stan Bentley	9/13/03	x	x	x	x	x	x		x		
16c	S. ochroleuca	NH	PMBrown	9/14/03	x	x	x	x	x	x			80498	yes
16e	S. ochroleuca	Allegany Co., MD	Scott Shriver	10/4/03		x	x	x	x	x		x		
16f	S. ochroleuca	Candia, NH	Jean Stefanik	9/13/04	x	x	x	x	x	x		x	80505-6	
16g	S. ochroleuca	Saginaw Co., MI	Fred Case	9/8/04	x	x	x	x	x	x		x		
16h	S. ochroleuca	Oneida Co., NY (Camden)	Charles Ufford	10/3/06	x	x	x	x	x	x		x	82219	yes
ci1a	S. casei	Ontario, Canada	Nancy Van Alstine	9/1/14	x	x	x							
	S. ×kapnosperia (cernua x ochroleuca)													
16i	S. ochroleuca (was 4d)	Oconee Co., SC (Blue Hole Falls)	Lucy Dueck	11/1/03		x	x	x	EU384710	x		x		yes
sc20a	S.cernua	Macon Co., NC	Wen	9/27/07	x	x		x	x	x		x	Wen 10003 (US)	
sh2	S. ochroleuca	Transylvania Co., NC	Jim Fowler	10/8/13	x	x	x	x	x	x		x		
	cernua/parksii /Coastal Plain/Piedmont													
4b	S. cernua (BCDEFGH)	Greenville Co., SC	Jim Fowler	10/25/02		x	x		EU384708	x		x	80495 (7)	yes
4j	S. cernua (scw21)	Brazos Co., TX	Cathy Walters (DNA)	10/20/03		x	x		EU384713	x			TAMU	
4k	S. cernua (scp61)	Brazos Co., TX	Cathy Walters (DNA)	10/20/03		x	x		EU384714	x			TAMU	
4L	S. cernua (green)	Henderson Co., TX	Lucy Dueck	11/6/04	x	x	x	x	EU384715	x			80491	yes
4m	S. cernua (deep south race)	Liberty Co., FL (via UFL)	Scott Stewart	7/3/06	x	x	x	x	x	x	x	x		yes
4cc	S. cernua (JF-B1)	Marlboro Co., SC. (Bennettsvl.)	Jim Fowler	10/21/06	x	x	x	x	x	x		x		yes
4dd	S. cernua (JF-B3)	Brunswick Co., NC (BoilSprLk)	Jim Fowler	10/29/06	x	x	x	x	x	x		x		
4ee	S. cernua - Chadd's Ford (17k)	cultivar - White Flwr. Farm, CN	Lucy Dueck/KC	8/19/06	x	x	x	x	x	x	x	x		
4ff	S. cernua -Chadd's Ford (17L)	cultivar - Sunshine Grdns., WV	Jeff Patton	10/5/06	x	x	x		x	x	x	x		
sc1b	S. cernua	New Castle Co., DE	M.C. Pace	9/29/13	x	x		x	x	x		x		
sc6d	S. cernua	Cape May, NJ	M.C. Pace	10/19/13	x	x	x	x	x	x	x	x	yes	yes

sc8d	S. cernua	MD	M.C. Pace	10/23/13	x	x	x	x	x	x	x	x		
sc9a	S. cernua	Little Creek Reservoir, James City, VA	M.C. Pace	10/25/13	x	x		x	x	x		x		
sc15b	S. cernua	Wayne Co, GA	M.C. Pace	11/6/13	x	x	x	x	x	x		x		
sc22	S. cernua	Brasstown Seeps, Towns Co., GA	Jim Fowler			x	x			x				yes
sc28	S. cernua	Aberdeen Proving Grounds, Monks Island, Harford Co., MD		10/13/13	x	x			x	x		x		
AR29	S. cernua	Ouachita Mountains, AR	M.C. Pace		x	x	x						Pace s.n.	yes
TX1	S. parksii	Brazos Co., TX	M.C. Pace		x	x	x	x	x			x		yes
20a	S. parksii - a (sp5)	Brazos Co., TX	Cathy Walters (DNA)	10/8/03		x	x	x	x	x		x	TAMU	yes
20b	S. parksii - b (sp137)	Brazos Co., TX	Cathy Walters (DNA)	10/22/03		x	x	x	x	x		x	TAMU	
20c	S. parksii - c (sp175)	Brazos Co., TX	Cathy Walters (DNA)	11/22/03		x	x		x	x			TAMU	
sp70	S. parksii	Brazos Co., TX	Cathy Walters (DNA)	10-11/03		x	x		x	x			TAMU	
sp146	S. parksii	Brazos Co., TX	Cathy Walters (DNA)	10-11/03		x	x		x	x			TAMU	
sp167	S. parksii	Brazos Co., TX	Cathy Walters (DNA)	10-11/03		x	x		x	x			TAMU	
sp267	S. parksii	Brazos Co., TX	Cathy Walters (DNA)	10-11/03		x	x		x	x			TAMU	
	S. cernua x S. magnicamporum	Upper Plains and Great Lakes Basin												
4a	S. cernua (S. cernua x S. magni)	NE	R.Bischof/NGPC (DNA)	<2001		x	gone?		EU384707	x				
4t	S. cernua (S. cernua x S. magni)	Marinette Co. (Spur Lake), WI	Kip Knudson	9/15/06	x	x	x	x	x	x	x	x		
4v	S. cernua (S. cernua x S. magni)	Seneca Co., OH	David McCann	9/25/06	x	x	x	x	x	x	x	x		yes
4aa	S. cernu (S. cernua x S. magni)	Kalamazoo Co., MI	Nick Stoynoff/MSU herb.	8/26/82		x	x		x	x			MSC283303	
4bb	S. cernua (S. cernua x S. magni)	Huron Co., MI	Julian Donahue/MSU herb.	9/20/63		x	x		x	x			MSC190339	
15a1	S. magnicamporum (SM1) [S. cernua x magni]	IA	R.Bischof/NGPC (DNA)	<2001		x	x (14)		x	x			NEB	
sm1c	S. cernua x S. magni	Arboretum, Dane Co., WI	M.C.Pace	_2013	x	x	x	x	x	x	x	x	Pace s.n.	yes
sc31a		Portage Co., OH	M.C.Pace	_2015		x	x	x	x		x		Pace s.n.	yes
sc32a		Clark Co., OH	M.C.Pace	_2015		x	x	x	x		x		Pace s.n.	yes
sc33a	S. cernua	Ontario, Canada	M.C.Pace	_2015	x	x	x	x	x		x		Pace s.n.	yes
sc35a	S. cernua	Ontario, Canada	M.C.Pace	_2015		x	x	x	x				Pace s.n.	yes
MI1		MI		_2015		x		x	x			x		yes
sm21r		Chiwaukee Prairie, Kenosha Co., WI	M.C.Pace	_2015	x		x	x	x		x	x	Pace s.n.	yes
sm23b		Illinois Beach State Park, Lake Co., IL	M.C.Pace	_2015	x		x	x	x			x	Pace s.n.	yes
sm27d	cleistogomous	Marquette Park, Lake Co., IN	M.C.Pace	_2015			x					x	Pace s.n.	yes
soch10a		Orleans Co., VT	M.C. Pace	_2015	x	x	x	x	x				Pace s.n.	yes
	S. lacera var. gracilis													
11a	S. lacera var. gracilis	Pickens Co., SC	Jim Fowler	7/12/03	x	x	x	x	x	x	x	x	80497	
	S. igniorchis													
slt2a	S. laciniata calcareous wet prairie	APARF, Polk Co., FL	Steve Orzell	8/22/14	x	x	x	x		x		x	Orzell 26733	
slt2c	S. laciniata calcareous wet prairie	APARF, Polk Co., FL	Steve Orzell	8/22/14	x	x		x	x	x		x	Orzell 26733	
slt3a	S. laciniata acidic wet prairie	APARF, Polk Co., FL	Steve Orzell	8/25/14	x	x	x	x	x	x		x	Orzell 26735	
slt3b	S. laciniata acidic wet prairie	APARF, Polk Co., FL	Steve Orzell	8/25/14	x	x		x		x		x	Orzell 26735	
slt4a	S. laciniata calcareous wet prairie	APARF, Polk Co., FL	Steve Orzell	8/25/14	x	x		x	x	x		x	Orzell 26734	
slt4b	S. laciniata calcareous wet prairie	APARF, Polk Co., FL	Steve Orzell	8/25/14	x	x		x	x				Orzell 26734	
26734B	S. laciniata calcareous wet prairie	APARF, Polk Co., FL	Steve Orzell		x		x	x		x		x		
26733A	S. laciniata calcareous wet prairie	APARF, Polk Co., FL	Steve Orzell		x			x	x	x		x		

	S. longilabris													
13a	S. longilabris	Brunswick Co., NC	Frank Galloway	10/31/03	x	x	x	x	x	x	x	x		
13b	S. longilabris	(Little Is.) Brunswick Co., NC	Jim Fowler	10/24/04	x	x	x	x	x	x	x	x		
13c	S. longilabris	Levy Co., FL	Scott Stewart	11/20/04	x	x	x	x	x	x	x	x		
13f	S. longilabris	Harden Co., TX	Joe Liggio	11/21/04		x	x	x	x	x		x	82222	
sloAvon	S. longilabris	Polk Co, FL	Steve Orzell		x		x							
	S. lucida													
SL13d	S. lucida	Ozark National Forest, AR	M.C. Pace	19-May-13	x	x	x	x	x	x	x	x	M.C. Pace 576	yes
	S. magnicamporum													
15b	S. magnicamporum (sm7)	Blanco Co., TX	Cathy Walters (DNA)	10/17/04		x	x (24)		x	x			TAMU	yes
15c	S. magnicamporum	Ozark Co., MO	Bill Summers	10/18/04		x (poor)	x	x	x	x	x		80501	
15e	S. magnicamporum	Grimes Co., TX	Joe Liggio	10/31/04	x	x	x	x	x	x			80489	
15f	S. magnicamporum - (sm)	Walworth Co., WI	Jeff Hapeman	9/16/06	x	x	x	x	x	x	x	x	82207	
15g	S. magnicamporum - (lg)	Walworth Co., WI	Jeff Hapeman	9/16/06	x	x	-	x	x	x	x	x		
sm7h	S.magnicamporum	NM	M.C.Pace	9/21/13	x	x	x	x	x	x	x	x		
sm8a	S.magnicamporum	Russell Co., VA		10/15/13	x	x	x		x	x	x	x		
sm11a	S.magnicamporum	Russell Co., VA		10/17/13	x	x	x	x	x	x	x	x		
sm12a	S.magnicamporum	GA	Jim Fowler	11/3/13	x	x	x	x	x	x	x	x		yes
sm13a	S.magnicamporum	Lanark Co., Ontario, Canada	Joyce Reddoch	9/25/13	x	x	x	x	x	x	x	x		
sm15a	S.magnicamporum	Marthaville, Lambton Co, Ontario, Canada	Oldham	9/7/11	x	x	x	x	x	x	x	x	Oldham 39307 (NYS)	
sm16a	S.magnicamporum	Black Belt Prairie, Starkville, MS	McCabe	10/29/91		x	x		x	x			McCabe s.n. (NYS)	
sm17a	S.magnicamporum	Cowan, Franklin co., TN	Sheviak	11/8/06	x	x		x	x	x	x	x	Sheviak 7064 (NYS)	
sm18a	S.magnicamporum	Honey Creek, Pottawattamie Co., IA	Rahel	9/1/98					x	x		x	Rahel s.n. (NYS)	
sm19a	S.magnicamporum	Chandlerville, Cass Co., IL	Sheviak	9/25/81	x	x	x		x	x		x	Sheviak 2142 (NYS)	
sm22c	S.magnicamporum	North Beach Park, Racine Co., WI	M.C.Pace		x		x					x	Pace s.n.	yes
sm23e	S.magnicamporum	Illinois Beach State Park, Lake Co., IL	M.C.Pace		x		x						Pace s.n.	yes
sm25g	S.magnicamporum	Van Vlissingen Prairie, Cook Co., IL	M.C.Pace		x		x					x	Pace s.n.	yes
sm26k	S.magnicamporum	Van Vlissingen Prairie, Cook Co., IL	M.C.Pace		x		x					x	Pace s.n.	yes
sm28h	S.magnicamporum	Spinn Prairie, White Co., IN	M.C.Pace		x		x					x	Pace s.n.	yes
	S. odorata													
17c	S. odorata (non-flwr'g)	Rice's Crk Br., Brunswick Co., NC	Lucy Dueck	8/8/04			x	x	x	x				
17d	S. odorata	Clark's Bridge, Brunswick Co., NC	Frank Galloway	10/31/03		x	x	x	x	x	x	x		
17e	S. odorata	Gilbert's Bridge, Brunswick Co., NC	Frank Galloway	10/31/03		x	x	x	x	x				
17g	S. odorata	Levy Co., FL	Scott Stewart	11/20/04		x	x	x	x	x		x		
17i	S. odorata	San Jacinto Co., TX	Joe Liggio	10/24/04		x	x	x	x	x	x	x	80488	
17opq	S. odorata	Effingham Co., GA	Darlene (L) Vincent	12/2/06	x	x	x	x	x	x	x	x	82211	yes
17z	S. odorata	Levy Co. (Goethe St.For.), FL	Scott Stewart	11/20/04		x	x	x	x	x	x	x		
So1d	S. odorata	Piney Island, NC	M.C. Pace	10/26/2013	x	x	x	x	x	x	x	x	Pace s.n.	yes
So5h	S. odorata	Bridge, Brunswick Co., NC	M.C. Pace	11/4/2013	x	x	x		x	x	x	x	Pace s.n.	yes
So10i	S. odorata	Roadside, Taylor Co., FL	M.C. Pace	11/9/2013	x	x	x		x	x	x	x	Pace s.n.	yes
so11	S. odorata	Kentucky	Sheviak			x	x		x	x	x	x	Sheviak 2408	
so12	S. odorata	Tennessee	Durr	10/1/2001	x	x	x	x	x	x	x	x	Durr s.n.	
so13	S. odorata	Back Bay NWR, Princess Anne Co., VA	Statler	11/4/2006	x	x			x	x		x	Statler s.n. (NYS)	

	S. ovalis var. ovalis													
18b	S. ovalis var. ovalis	Columbia Co., FL	PMBrown	11/11/06		x	x		x	x		x		
	S. ovalis var. erostellata													
19a	S. ovalis var. erostellata - a	Giles Co., VA	Stan Bentley	9/28/03		x	x	x	x	x		x		
19b	S. ovalis var. erostellata - b	Barnwell Co., SC (SRS)	Lucy Dueck	9/30/03		x	x		x	x			80493	yes
19c	S. ovalis var. erostellata - c	Rutherford Co., NC	Jim Fowler	10/11/03		x	x	x	x	x		x		
19d	S. ovalis var. erostellata/hybrid?	McCormick Co., SC (F659D)	Lucy Dueck	10/11/06		x	x	x	x	x		x	82210	yes
19e	S. ovalisvar. erostellata - e	Columbia Co., FL	PMBrown	11/11/06		x	x		x	x		x		
sov2	S. ovalis var. erostelalta	Waukesha Co., WI	M.C. Pace		x		x					x	Pace s.n.	yes
sov3	S. ovalis var. erostelalta	Orange Co., NC	Jim Fowler		x		x					x		yes
	S. triloba													
FL36	S. triloba	Florida Panther NWF, FL	M.C. Pace	11-Jan-13	x	x	x		x			x	Pace s.n.	yes
FL130	S. triloba	Fakahatchee Strand, FL	M.C. Pace	16-Jan-13	x	x	x	x	x	x			Pace s.n.	yes
FL135	S. triloba	Corkscrew Swamp Sanctuary, FL	M.C. Pace	17-Jan-13	x	x	x	x	x	x	x	x	Pace s.n.	yes
FL146	S. triloba	Wakulla Co., FL	M.C. Pace	9-Nov-13		x	x			x	x	x	Pace s.n.	yes
Flpolk	S. triloba	Polk Co., FL	Steve Orzell			x	x	x		x		x	Orzell 26717	yes
FL164	S. triloba	Citis Co., FL	M.C. Pace				x			x		x	Pace s.n.	yes
FL165	S. triloba	Jesserson Co., FL	M.C. Pace				x			x		x	Pace s.n.	yes
17h	S. odorata	Collier Co. (Naples), FL	Scott Stewart	5/25/05			x-h2		x	x		x		
17m	S. odorata	Columbia Co.,FL (Ich.Spr.S.P.)	PMBrown	11/11/06			x (14,1-4)	x	x	x		x		
17n	S. odorata (dwarf)	Levy Co., FL	PMBrown	11/11/06	x	x	x	x	x	x	x	x		
Ich2	S. x ichetuckneensis	Columbia Co., FL	PMBrown	11/11/06		x	x	x	x	x	x			
si2	S. triloba	Ichetucknee Springs, Columbia Co., FL	M.C. Pace	11/13/13	x	x	x	x		x	x	x	Pace s.n.	yes
	S. sylvatica													
25d	S. sylvatica	Alachua Co., FL	Scott Stewart	5/20/05	x	x	x	x	x	x	x	x		
	S. torta													
28b	S. torta (sm flwr & stem)	Miami-Dade Co., FL	PMB/RH	6/20/06	x	x	x		x	x	x	x	82221	yes
28g	S. torta	Puerto Rico	Jim Ackerman	4/1/07	x	x	x (1-4)		x	x	x	x	82230	
	S. tuberosa													
26b	S. tuberosa (late blm.)	S. Aiken Co., SC	Lucy Dueck	8/19/03	x	x	x	x	x	x		x	82218	yes
	Outgroup taxa													
30	Sacoila lanceolata var. lanc.	Levy Co., FL	Scott Stewart	6/1/04	x	x	x		x	x		x		
Dici	Dichromanthus cinnabara		G. Salazar		x	x	x	x	x	x	x	x		
Mepo	Mesadenella polyantha		G. Salazar		x	x	x		x	x	x	x		
Dedi	Deiregyne diaphana		G. Salazar			x	x		x	x	x	x		
Scll	Schiedeella llaveana		G. Salazar			x	x		x		x	x		

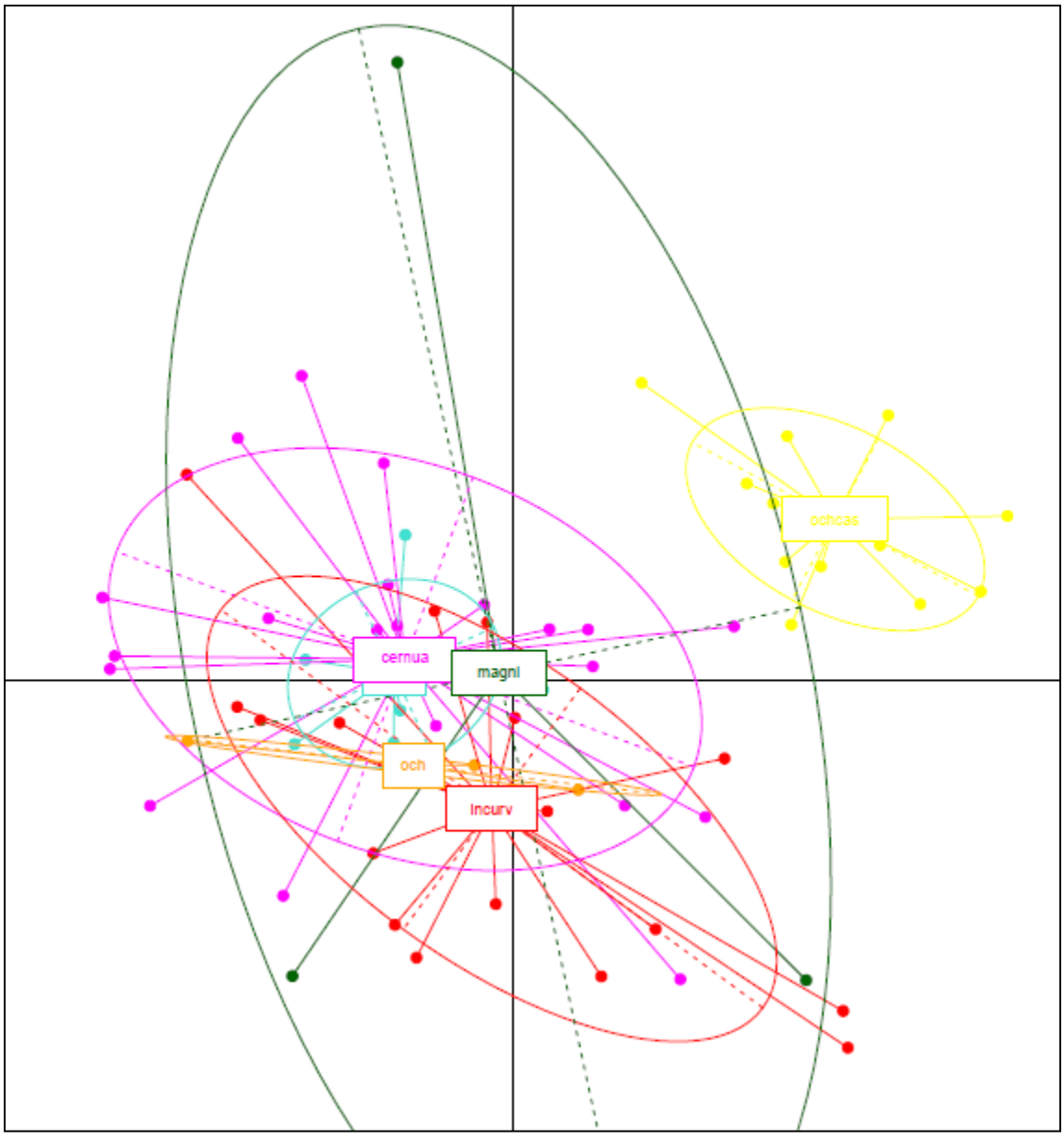


Fig. 6. Morphometric analysis based on labellum morphology. Dark-green = *S*. *magnicamporum*; pink = *S*. *cernua* s.s.; red = *S*. ×*incurvum*; bright blue = *S*. *arcisepalum*; orange = *S*. *ochroleuca*; yellow = *S*. *casei*.

Fig. 7.

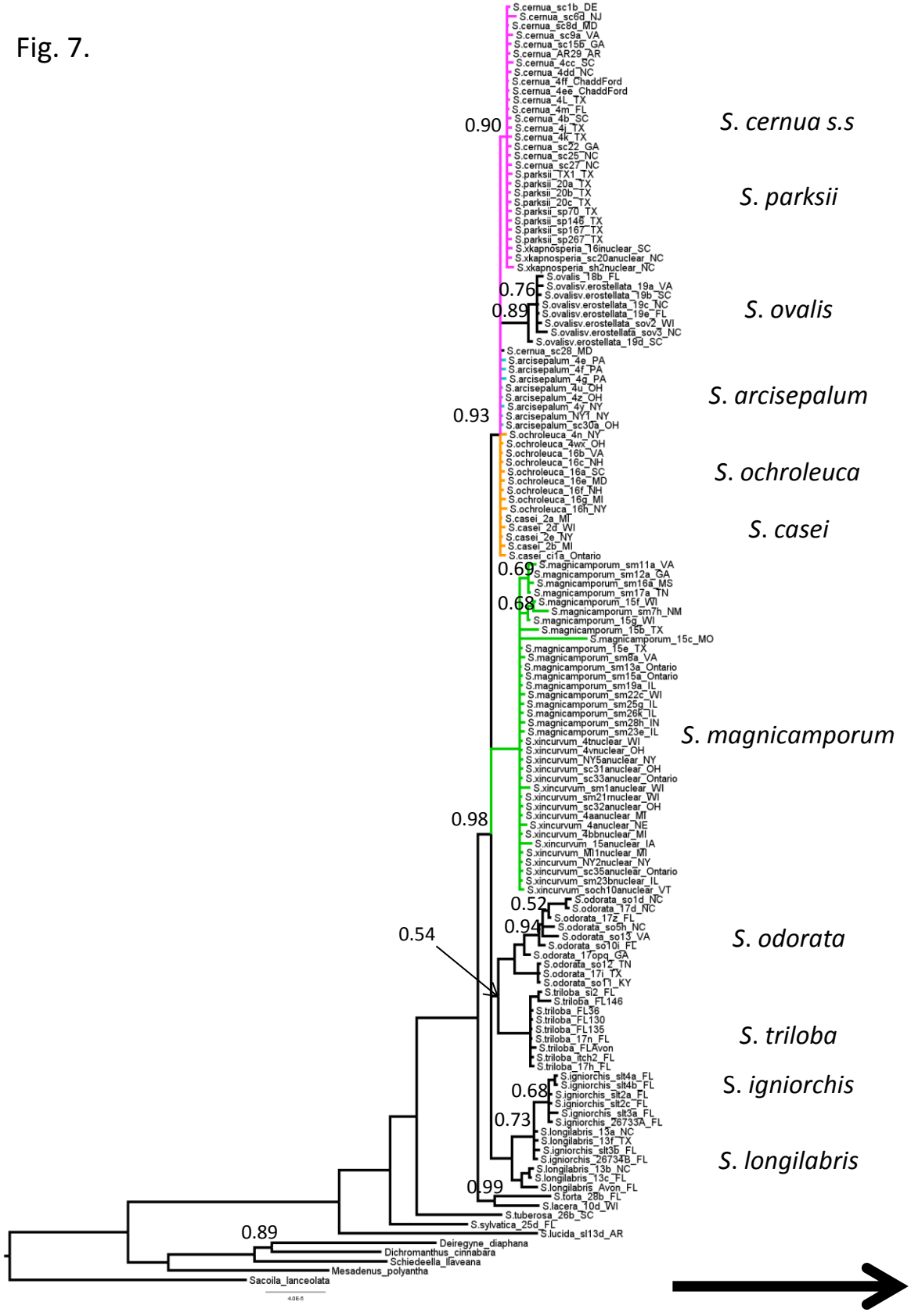

S.cernua_sc1b_DE
S.cernua_sc6d_NJ
S.cernua_sc8d_MD
S.cernua_sc9a_VA
S.cernua_sc15b_GA
S.cernua_AR29_AR
S.cernua_4cc_SC
S.cernua_4dd_NC
S.cernua_4ff_ChaddFord
S.cernua_4ee_ChaddFord
S.cernua_4L_TX
S.cernua_4m_FL
S.cernua_4b_SC
S.cernua_4j_TX
S.cernua_4k_TX
S.cernua_sc22_GA
S.cernua_sc25_NC
S.cernua_sc27_NC
S.parksii_TX1_TX
S.parksii_20a_TX
S.parksii_20b_TX
S.parksii_20c_TX
S.parksii_sp70_TX
S.parksii_sp145_TX
S.parksii_sp167_TX
S.parksii_sp267_TX
S.xkapnosperia_16inuclear_SC
S.xkapnosperia_sc20anuclear_NC
S.xkapnosperia_sh2nuclear_NC
S.ovalis_18b_FL
S.ovalisv.erostellata_19a_VA
S.ovalisv.erostellata_19b_SC
S.ovalisv.erostellata_19c_NC
S.ovalisv.erostellata_19e_FL
S.ovalisv.erostellata_sov2_WI
S.ovalisv.erostellata_sov3_NC
S.ovalisv.erostellata_19d_SC
S.cernua_sc28_MD
S.arcisepalum_4e_PA
S.arcisepalum_4f_PA
S.arcisepalum_4g_PA
S.arcisepalum_4u_OH
S.arcisepalum_4z_OH
S.arcisepalum_4y_NY
S.arcisepalum_NY1_NY
S.arcisepalum_sc30a_OH
S.ochroleuca_4n_NY
S.ochroleuca_4wx_OH
S.ochroleuca_16b_VA
S.ochroleuca_16c_NH
S.ochroleuca_16a_SC
S.ochroleuca_16e_MD
S.ochroleuca_16f_NH
S.ochroleuca_16g_MI
S.ochroleuca_16h_NY
S.casei_2a_MI
S.casei_2d_WI
S.casei_2e_NY
S.casei_2b_MI
S.casei_ci1a_Ontario
S.magnicamporum_sm11a_VA
S.magnicamporum_sm12a_GA
S.magnicamporum_sm16a_MS
S.magnicamporum_sm17a_TN
S.magnicamporum_15f_WI
S.magnicamporum_sm7h_NM
S.magnicamporum_15g_WI
S.magnicamporum_15b_TX
S.magnicamporum_15c_MO
S.magnicamporum_15e_TX
S.magnicamporum_sm8a_VA
S.magnicamporum_sm13a_Ontario
S.magnicamporum_sm15a_Ontario
S.magnicamporum_sm19a_IL
S.magnicamporum_sm22c_WI
S.magnicamporum_sm25g_IL
S.magnicamporum_sm26k_IL
S.magnicamporum_sm28h_IN
S.magnicamporum_sm23e_IL
S.xincurvum_4tnuclear_WI
S.xincurvum_4vnuclear_OH
S.xincurvum_NY5anuclear_NY
S.xincurvum_sc31anuclear_OH
S.xincurvum_sc33anuclear_Ontario
S.xincurvum_sm1anuclear_WI
S.xincurvum_sm21rnuclear_WI
S.xincurvum_sc32anuclear_OH
S.xincurvum_4aanuclear_MI
S.xincurvum_4anuclear_NE
S.xincurvum_4bbnuclear_MI
S.xincurvum_15anuclear_IA
S.xincurvum_MI1nuclear_MI
S.xincurvum_NY2nuclear_NY
S.xincurvum_sc35anuclear_Ontario
S.xincurvum_sm23bnuclear_IL
S.xincurvum_soch10anuclear_VT
S.odorata_so1d_NC
S.odorata_17d_NC
S.odorata_17z_FL
S.odorata_so5h_NC
S.odorata_so13_VA
S.odorata_so10i_FL
S.odorata_17opq_GA
S.odorata_so12_TN
S.odorata_17i_TX
S.odorata_so11_KY
S.triloba_sl2_FL
S.triloba_FL146
S.triloba_FL36
S.triloba_FL130
S.triloba_FL135
S.triloba_17n_FL
S.triloba_FLAvon
S.triloba_itch2_FL
S.triloba_17h_FL
S.igniorchis_slt4a_FL
S.igniorchis_slt4b_FL
S.igniorchis_slt2a_FL
S.igniorchis_slt2c_FL
S.igniorchis_slt3a_FL
S.igniorchis_26733A_FL
S.longilabris_13a_NC
S.longilabris_13f_TX
S.igniorchis_slt3b_FL
S.igniorchis_26734B_FL
S.longilabris_13b_NC
S.longilabris_13c_FL
S.longilabris_Avon_FL
S.forta_28b_FL
S.lacera_10d_WI
S.tuberosa_26b_SC
S.sylvatica_25d_FL
S.lucida_sl13d_AR
Deiregyne_diaphana
Dichromanthus_cinnabara
Schiedeella_flaveana
Mesadenus_polyantha
Sacoila_lanceolata
4.0E-6
0.90
0.76
0.89
0.93
0.69
0.68
0.98
0.52
0.94
0.54
0.68
0.73
0.99
0.89
S. cernua s.s
S. parksii
S. ovalis
S. arcisepalum
S. ochroleuca
S. casei
S. magnicamporum
S. odorata
S. triloba
S. igniorchis
S. longilabris



Fig 7. [Legend to preceding page/tree] Combined nuclear (low copy *ACO* copy A & *Xdh* copy A, and nrITS copy A) Bayesian Majority Rule tree with branch lengths of the *Spiranthes cernua* species complex s.l. Except where noted, pp = 1. The positions of included taxa are indicated at right. Only copies of suspected hybrids which are incongruent when compared to the plastid reconstruction are included in this tree. The topological positions of select taxa are highlighted by color: *S*. *arcisepalum* (bright blue), *S*. *cernua* s.s. + *S*. *parksii* (pink), *S*. *magnicamporum* (green), & *S*. *ochroleuca* + *S*. *casei* (orange).

Fig. 8.

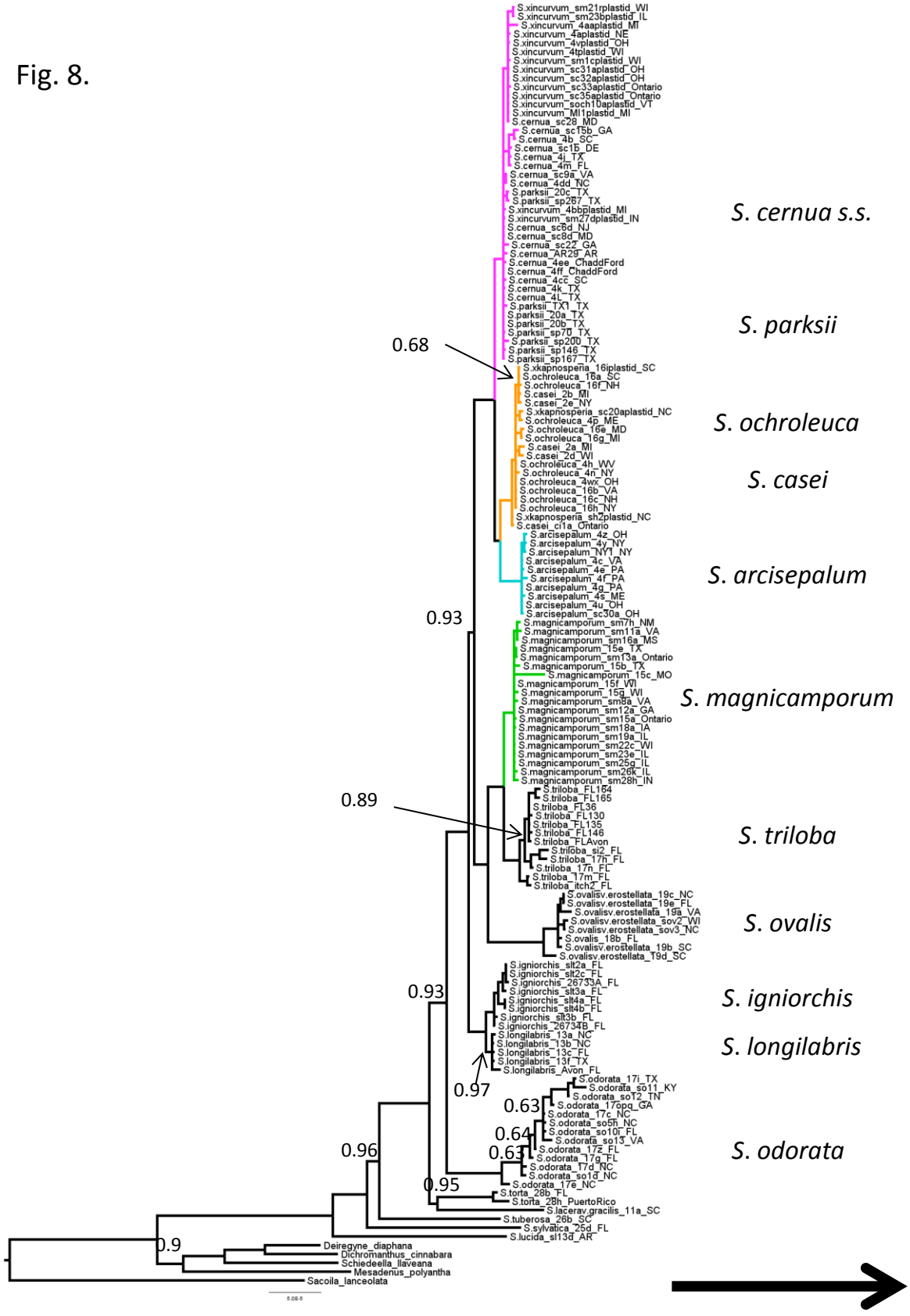

S.xincurvum_sm21rplastid_WI
S.xincurvum_sm23bplastid_IL
S.xincurvum_4aaplastid_MI
S.xincurvum_4aplastid_NE
S.xincurvum_4vplastid_OH
S.xincurvum_4tplastid_WI
S.xincurvum_sm1cplastid_WI
S.xincurvum_sc31aplastid_OH
S.xincurvum_sc32aplastid_OH
S.xincurvum_sc33aplastid_Ontario
S.xincurvum_sc35aplastid_Ontario
S.xincurvum_soch1aplastid_VT
S.xincurvum_MI1plastid_MI
S.cernua_sc28_MD
S.cernua_sc15b_GA
S.cernua_4b_SC
S.cernua_sc15_DE
S.cernua_4j_TX
S.cernua_4m_FL
S.cernua_sc9a_VA
S.cernua_4dd_NC
S.parksii_20c_TX
S.parksii_sp287_TX
S.xincurvum_4bbplastid_MI
S.xincurvum_sm27dplastid_IN
S.cernua_sc6d_NJ
S.cernua_sc8d_MD
S.cernua_sc22_GA
S.cernua_AR29_AR
S.cernua_4ee_ChaddFord
S.cernua_4ff_ChaddFord
S.cernua_4c2_SC
S.cernua_4k_TX
S.cernua_4L_TX
S.parksii_TX1_TX
S.parksii_20a_TX
S.parksii_20b_TX
S.parksii_sp70_TX
S.parksii_sp200_TX
S.parksii_sp146_TX
S.parksii_sp167_TX
S. cernua s.s.
S. parksii
0.68
S.xkapnosperia_16iplastid_SC
S.ochroleuca_16a_SC
S.ochroleuca_16f_NH
S.casei_2b_MI
S.casei_2e_NY
S.xkapnosperia_sc20aplastid_NC
S.ochroleuca_4p_ME
S.ochroleuca_16e_MD
S.ochroleuca_16g_MI
S.casei_2a_MI
S.casei_2d_WI
S.ochroleuca_4h_WV
S.ochroleuca_4n_NY
S.ochroleuca_4wx_OH
S.ochroleuca_16b_VA
S.ochroleuca_16c_NH
S.ochroleuca_16h_NY
S.xkapnosperia_sh2plastid_NC
S.casei_ci1a_Ontario
S. ochroleuca
S. casei
S.arcisepalum_4z_OH
S.arcisepalum_4y_NY
S.arcisepalum_NY1_NY
S.arcisepalum_4c_VA
S.arcisepalum_4e_PA
S.arcisepalum_4f_PA
S.arcisepalum_4g_PA
S.arcisepalum_4s_ME
S.arcisepalum_4u_OH
S.arcisepalum_sc30a_OH
S. arcisepalum
0.93
S.magnicamporum_sm7h_NM
S.magnicamporum_sm11a_VA
S.magnicamporum_sm16a_MS
S.magnicamporum_15e_TX
S.magnicamporum_sm13a_Ontario
S.magnicamporum_15b_TX
S.magnicamporum_15c_MO
S.magnicamporum_15f_WI
S.magnicamporum_15g_WI
S.magnicamporum_sm8a_VA
S.magnicamporum_sm12a_GA
S.magnicamporum_sm15a_Ontario
S.magnicamporum_sm18a_IA
S.magnicamporum_sm19a_IL
S.magnicamporum_sm22c_WI
S.magnicamporum_sm23e_IL
S.magnicamporum_sm25g_IL
S.magnicamporum_sm26k_IL
S.magnicamporum_sm28h_IN
S. magnicamporum
0.89
S.triloba_FL164
S.triloba_FL165
S.triloba_FL36
S.triloba_FL130
S.triloba_FL135
S.triloba_FL146
S.triloba_FLAvon
S.triloba_sl2_FL
S.triloba_17h_FL
S.triloba_17n_FL
S.triloba_17m_FL
S.triloba_itch2_FL
S. triloba
S.ovalisv.erostellata_19c_NC
S.ovalisv.erostellata_19e_FL
S.ovalisv.erostellata_19a_VA
S.ovalisv.erostellata_sov2_WI
S.ovalisv.erostellata_sov3_NC
S.ovalis_18b_FL
S.ovalisv.erostellata_19b_SC
S.ovalisv.erostellata_T9d_SC
S. ovalis
S.igniorchis_slt2a_FL
S.igniorchis_slt2c_FL
S.igniorchis_26733A_FL
S.igniorchis_slt3a_FL
S.igniorchis_slt4a_FL
S.igniorchis_slt4b_FL
S.igniorchis_slt3b_FL
S.igniorchis_26734B_FL
S.longilabris_13a_NC
S.longilabris_13b_NC
S.longilabris_13c_FL
S.longilabris_13f_TX
S.longilabris_Avon_FL
0.93
S. igniorchis
S. longilabris
0.97
S.odorata_17i_TX
S.odorata_so11_KY
S.odorata_soT2_TN
S.odorata_17opq_GA
S.odorata_17c_NC
S.odorata_so5h_NC
S.odorata_so10f_FL
S.odorata_so13_VA
S.odorata_17z_FL
S.odorata_17g_FL
S.odorata_17d_NC
S.odorata_so1d_NC
S.odorata_17e_NC
0.63
0.64
0.63
0.96
0.95
S. odorata
S.torta_28b_FL
S.torta_28h_PuertoRico
S.lacerav.gracilis_11a_SC
S.tuberosa_26b_SC
S.sylvatica_25d_FL
S.lucida_sl13d_AR
0.9
Deiregyne_diaphana
Dichromanthus_cinnabara
Schiedeella_llaveana
Mesadenus_polyantha
Sacoila_lanceolata
5.0E-6



Fig. 8. [Legend to preceding page/tree] Combined plastid (*mat*K, *ndh*J, *trn*L-F, *trn*S, *ycf*1 3') and nuclear putative maternal copy (*Xdh* copy B, and nrITS copy B) Bayesian Majority Rule tree with branch lengths of the *Spiranthes cernua* species complex s.l. Except where noted, pp = 1. The positions of included taxa are indicated at right. The topological positions of select taxa are highlighted by color: *S*. *arcisepalum* (bright blue), *S*. *cernua* s.s. + *S*. *parksii* (pink), *S*. *magnicamporum* (green), & *S*. *ochroleuca* + *S*. *casei* (orange).

Fig. 9.

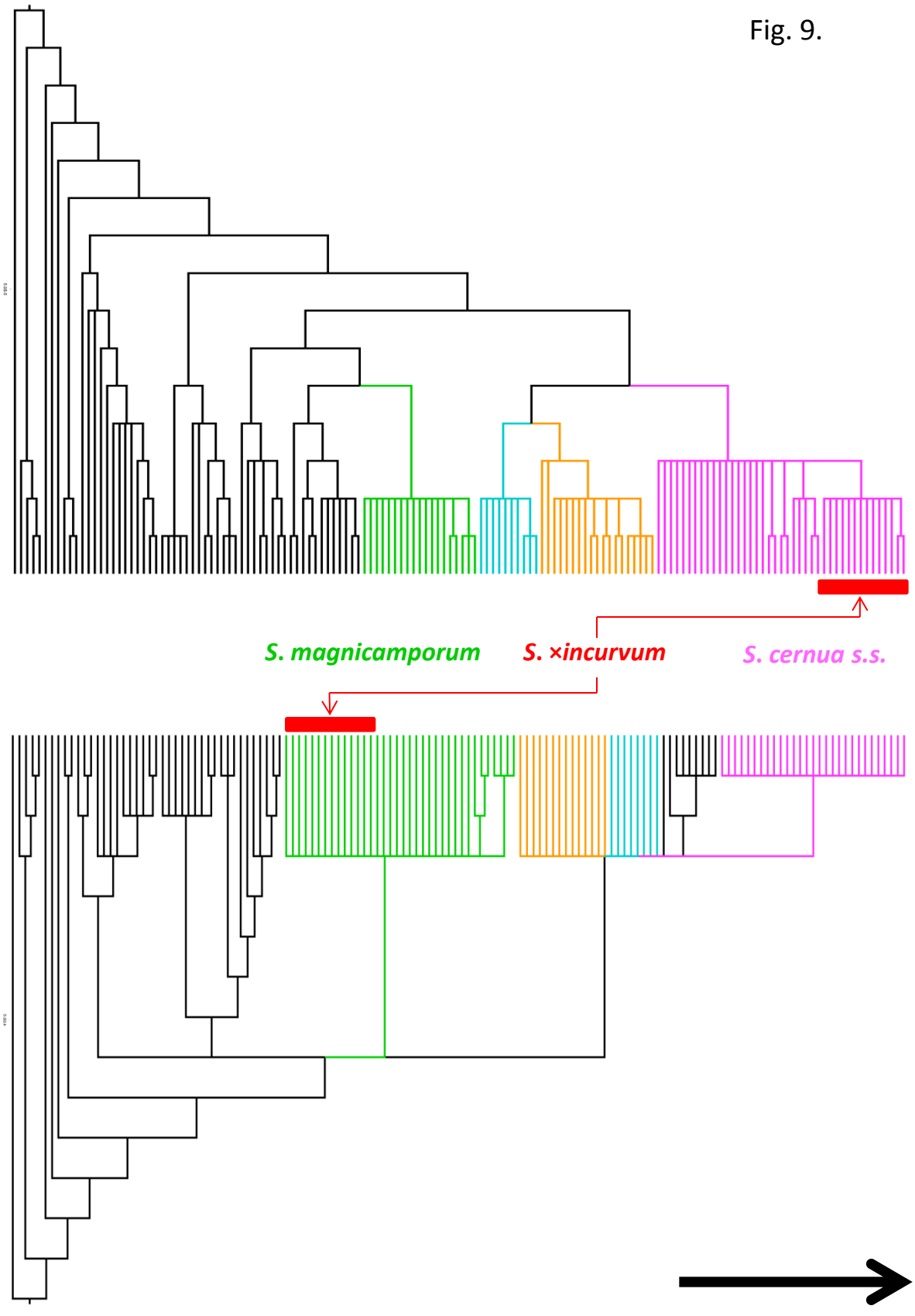




Fig. 9. [Legend to preceding page/tree] Comparison of Bayesian Majority Rule cladograms illustrating the incongruent topological position of hybrid *S*. ×*incurvum* (red) between *S*. *cernua* s.s. (pink; plastid *mat*K, *ndh*J*, trn*L-F*, trn*S*, ycf*1 3' and nuclear ACO & putative maternal copy *Xdh* copy B and nrITS copy B) & *S*. *magnicamporum* (green; nuclear putative paternal copy *Xdh* copy A and nrITS copy A). Posterior probabilities are the same as in Fig 7 & 8. The topological positions of select taxa are highlighted by color: *S*. *arcisepalum* (bright blue), *S*. *cernua* s.s. + *S*. *parksii* (pink), *S*. *magnicamporum* (green), & *S*. *ochroleuca* + *S*. *casei* (orange).

Fig. 10

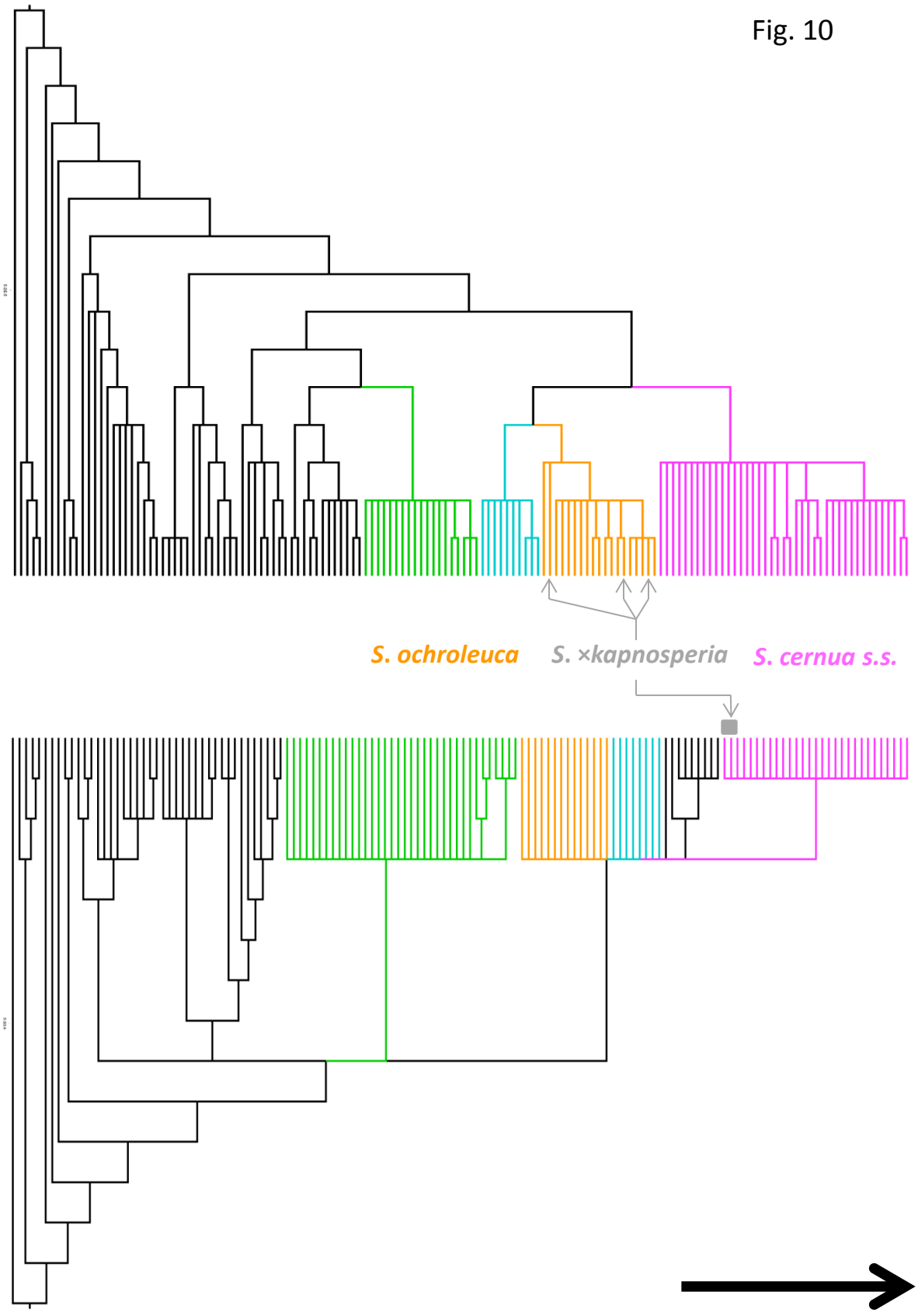




Fig. 10. [Legend to preceding page/tree] Comparison of Bayesian Majority Rule cladograhms illustrating the incongruent topological position of hybrid *S*. ×*kapnosperia* (gray) between *S*. *cernua* s.s. (pink; nuclear putative paternal copy *Xdh* copy A and nrITS copy A) & *S*. *ochroleuca* (orange; plastid *mat*K, *ndh*J, *trn*L-F, *trn*S, *ycf*1 3' and nuclear ACO & putative maternal copy *Xdh* copy B and nrITS copy B). The topological positions of select taxa are highlighted by color: *S*. *arcisepalum* (bright blue), *S*. *cernua* s.s. + *S*. *parksii* (pink), *S*. *magnicamporum* (green), & *S*. *ochroleuca* + *S*. *casei* (orange).

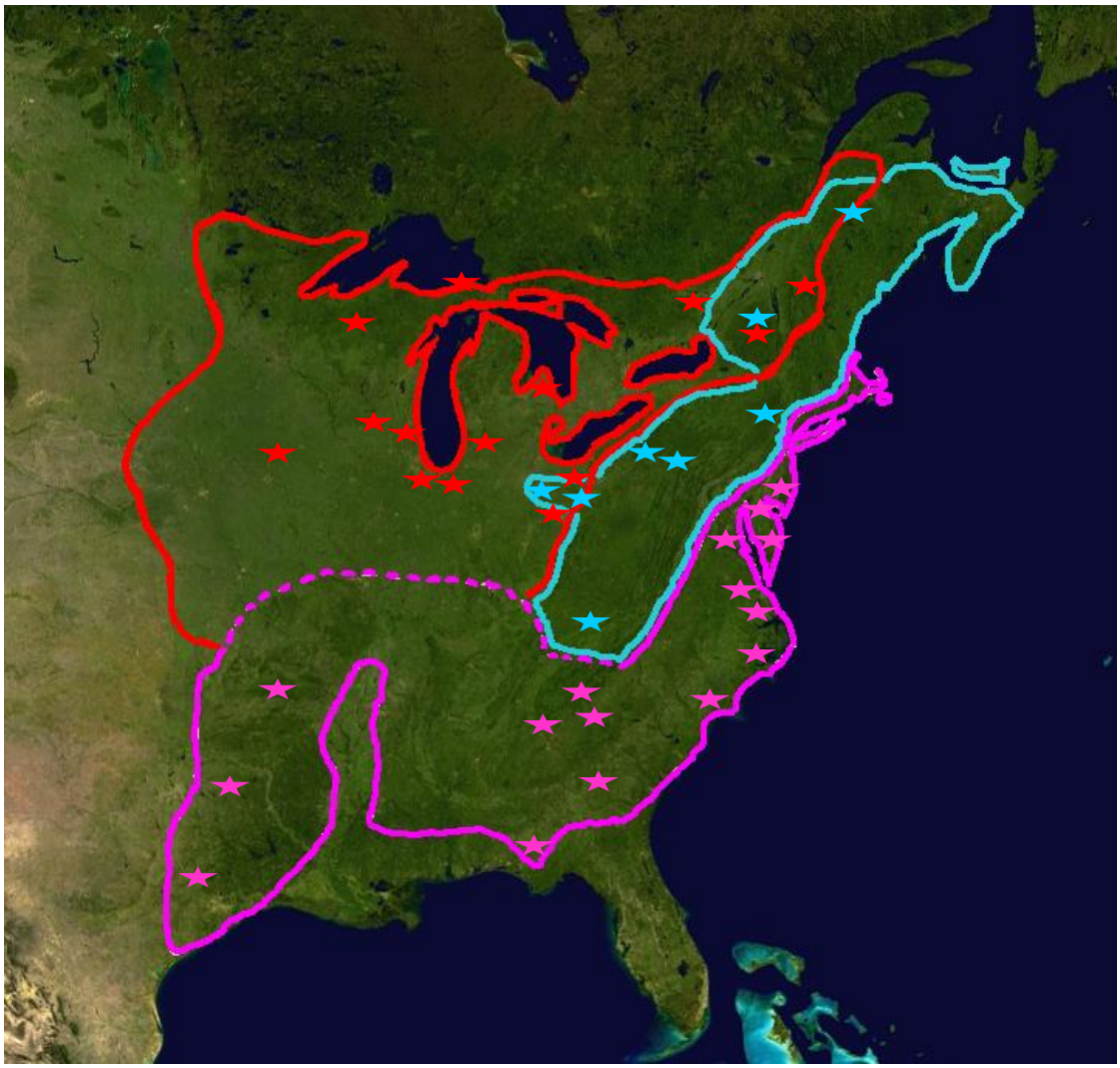


Fig. 11. Potential distribution map of *S*. *arcisepalum* (blue), *S*. *cernua* s.s. (including S. *parksii*) & *S*. ×*incurvum* (red) based on field, herbarium, and molecular phylogenetic study. These three taxa encompass the former *S*. *cernua* s.l. Stars denote the locations of molecular phylogenetic samples. The dashed line indicates the hypothesized northwestern distributional limit of *S*. *cernua* s.s. Further field, morphological, and molecular phylogenetic work may necessitate refinement of species potential geographic boundaries.

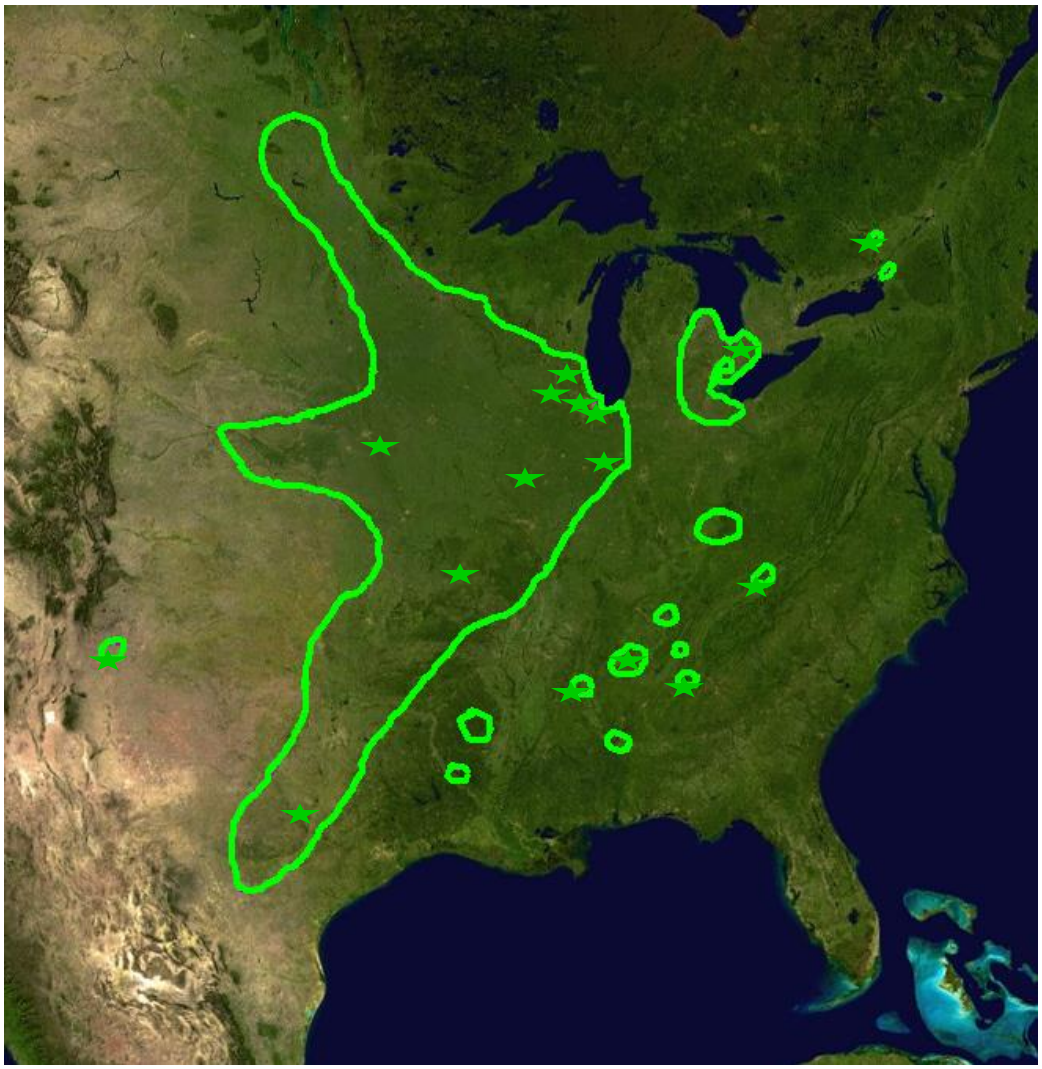


Fig. 12. Distribution map of *S*. *magnicamporum* (green) based on field, herbarium, and molecular phylogenetic study. Stars indicate molecular phylogenetic sample locations. Note the many disjunct populations spread throughout the southern and southeastern United States, in addition to disjunct populations in New Mexico, northern New York, and eastern Ontario.

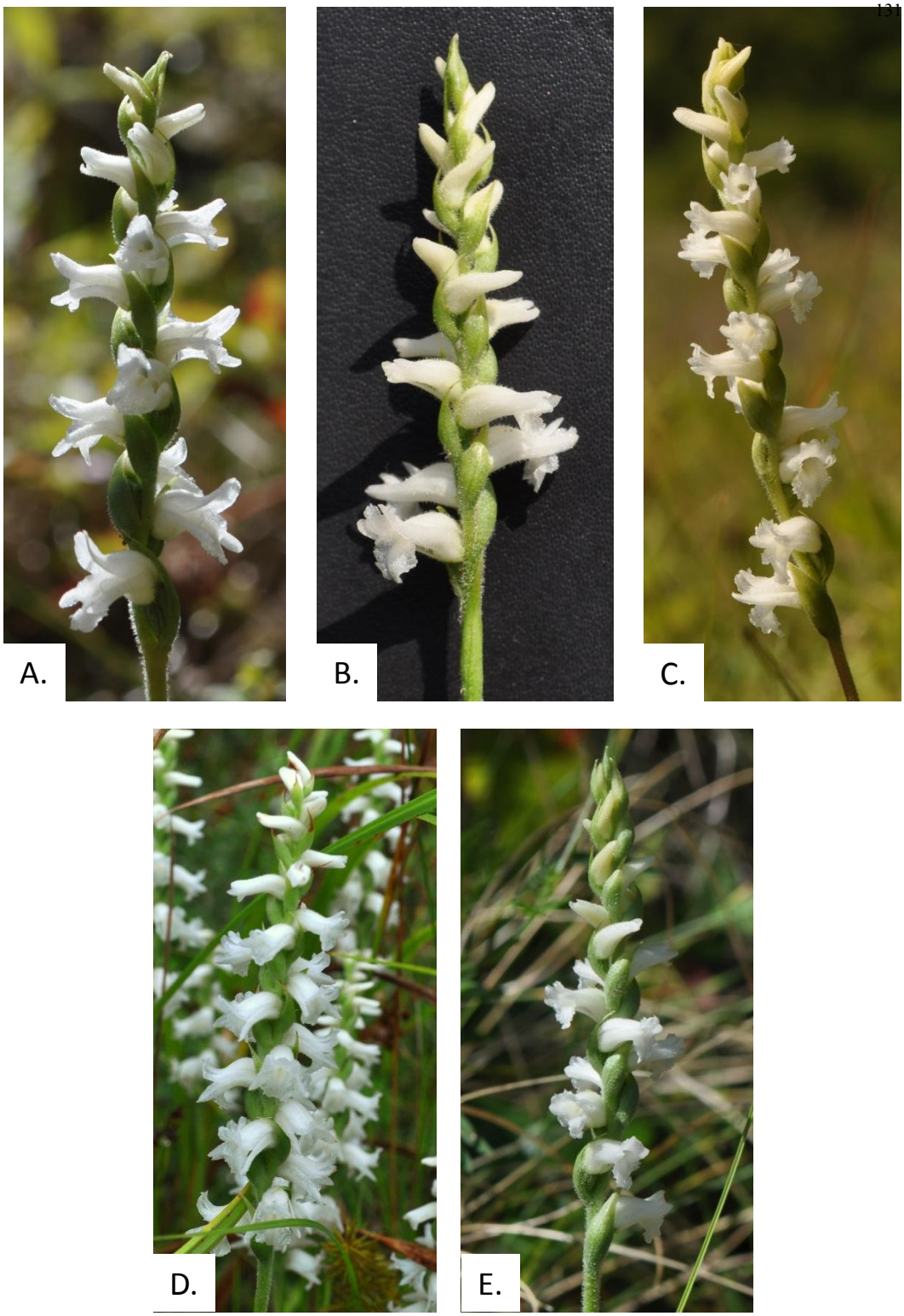


Fig. 13. *Spiranthes arcisepalum* sp. nov. **A.** Holotype location, Long Lake, Hamilton Co., NY. **B.** Delaware Co., NY, sample NY1. **C.** Lake Durant, Hamilton Co., NY. **D.** Wells, Hamilton Co., NY. **E.** Indian Lake, Hamilton Co., NY.

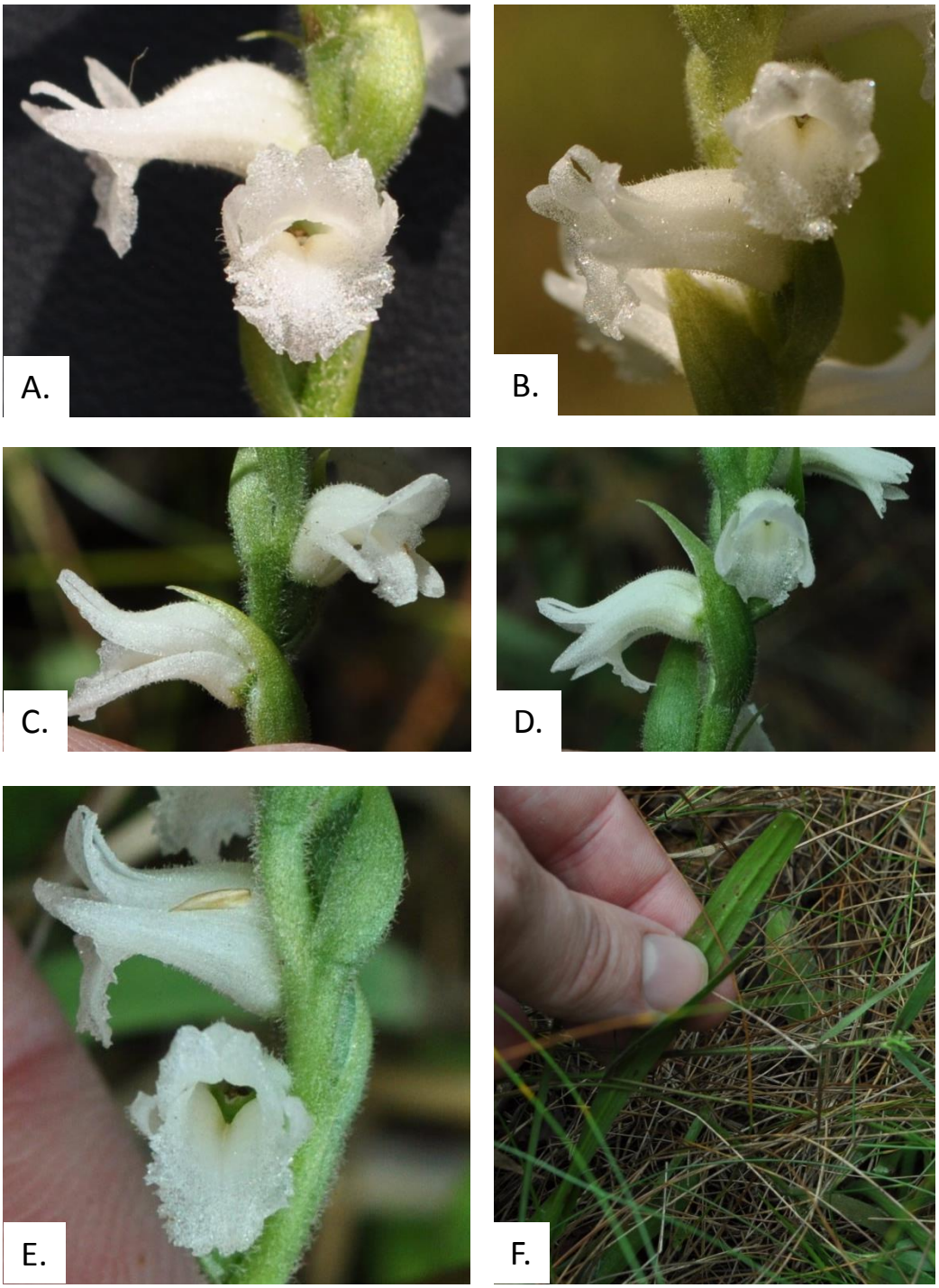


Fig. 14. Floral and foliar morphology of *Spiranthes arcisepalum* sp. nov. **A.** Delaware Co., NY, sample NY1. **B.** Lake Durant, Hamilton Co., NY. **C.** Holotype location, Long Lake, Hamilton Co., NY. **D.** Seneca Co., OH, sample sc30a. **E.** Holotype location, Long Lake, Hamilton Co., NY. **F.** basal leaf, Seneca Co., OH.

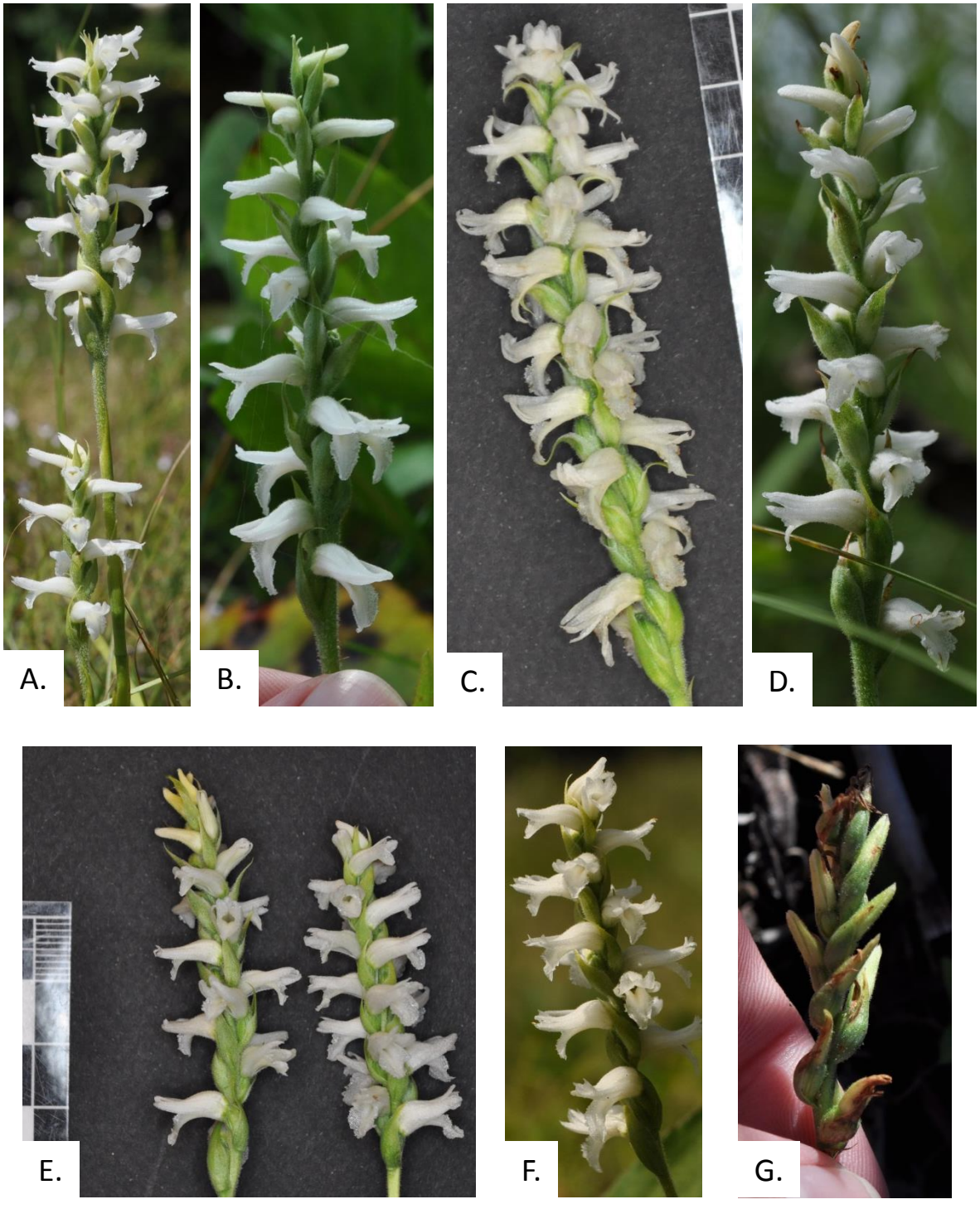


Fig. 15. *Spiranthes* ×*incurvum* [S. cernua × S. magnicamporum]. **A.** Hamilton Co., NY. **B.** Portage Co., OH, sample sc31. **C.** Clark Co., OH, sample sc32. **D.** Dane Co., WI, sample sm1a. **E.** Orleans Co., VT (scale in metric). **F.** Hamilton Co., NY. **G.** Cleistogamous, entirely apomictic population, Lake Co., IL.

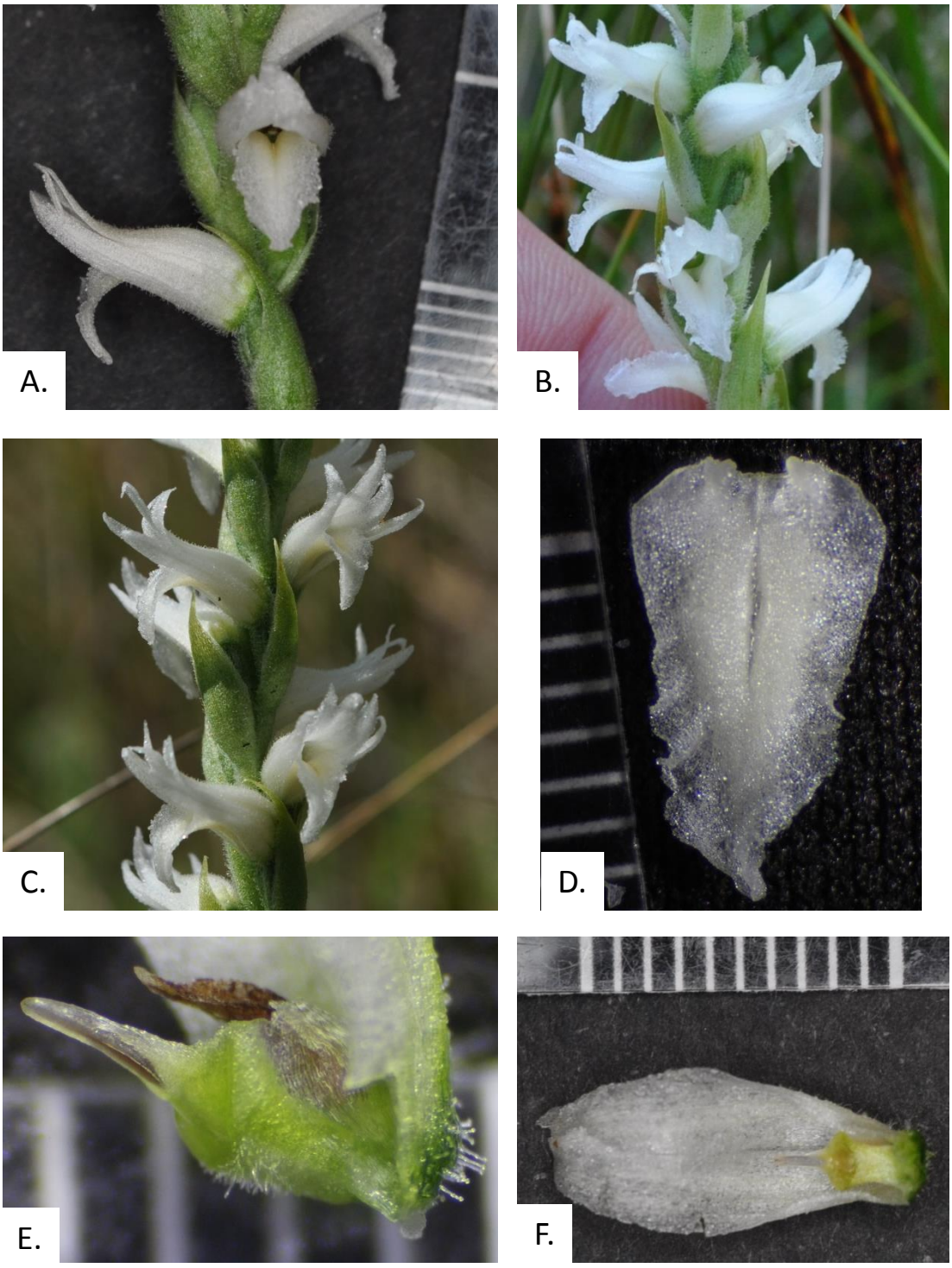


Fig. 16. Floral morphology of *Spiranthes* ×*incurvum* [*S. cernua* × *S. magnicamporum*]. **A.** Orleans Co., VT, sample location of soch10. **B.** Hamilton Co., NY. **C.** Orleans Co., VT, sample soch10a. **D–E.** Dane Co., WI, sample sm1a. **D.** Labellum, flattened. **E.** Column, side view. **F.** Clark Co., OH, column, stigmatic surface/ventral view, attached to dorsal petals and sepal, sample location of sc32.



Fig. 17. *Spiranthes* ×*ikapnosperia* notho. nov. [*S. cernua* × *S. ochroleuca*]. **A.** Holotype sheet, Macon Co., NC, *Wen 10003* (US), sample sc20a. **B.** Transylvania Co., NC, sample sh2. Photo: J. Fowler.



A.



B.



C.



D.



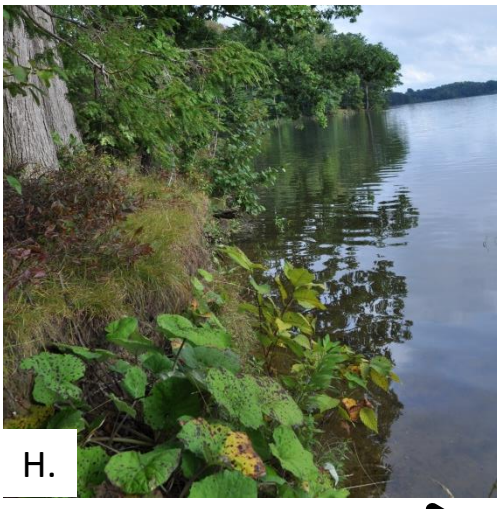
E.



F.



G.



H.



Fig. 18. A–B. Habitat of *S. cernua* s.s. **A.** Marshy pond edge, with *Nyssa sylvatica*, *Chamaecyparis thyoides*, *Sarracenia* spp., &*Eriocaulon* spp., Lexington Co., SC. **B.** Seeping old-field slope (with Mr. A. Hemme, Longwood Gardens), New Castle Co., DE, sample site of sc1. **C–H.** Habitat of *S.* ×*incurvum*. **C.** Hot & dry roadside slope, with *Solidago nemoralis* & *Andropogon gerardii*, Orleans Co., VT. **D.** High diversity fen, Clark Co., OH, sample location of sc32a. **E.** Mowed roadside, with *Daucus carota*, Hamilton Co., NY. **F.** Sandy, dry dune swales and back dunes, co-occurring with *S. magnicamporum* (with Mr. S. Martella), Lake Co., IL. **G.** Wet calcareous dune swale with open standing water, Lake Co., IN. **H.** Lake edge, just above water level, Portage Co., OH, sample location of sc31a.

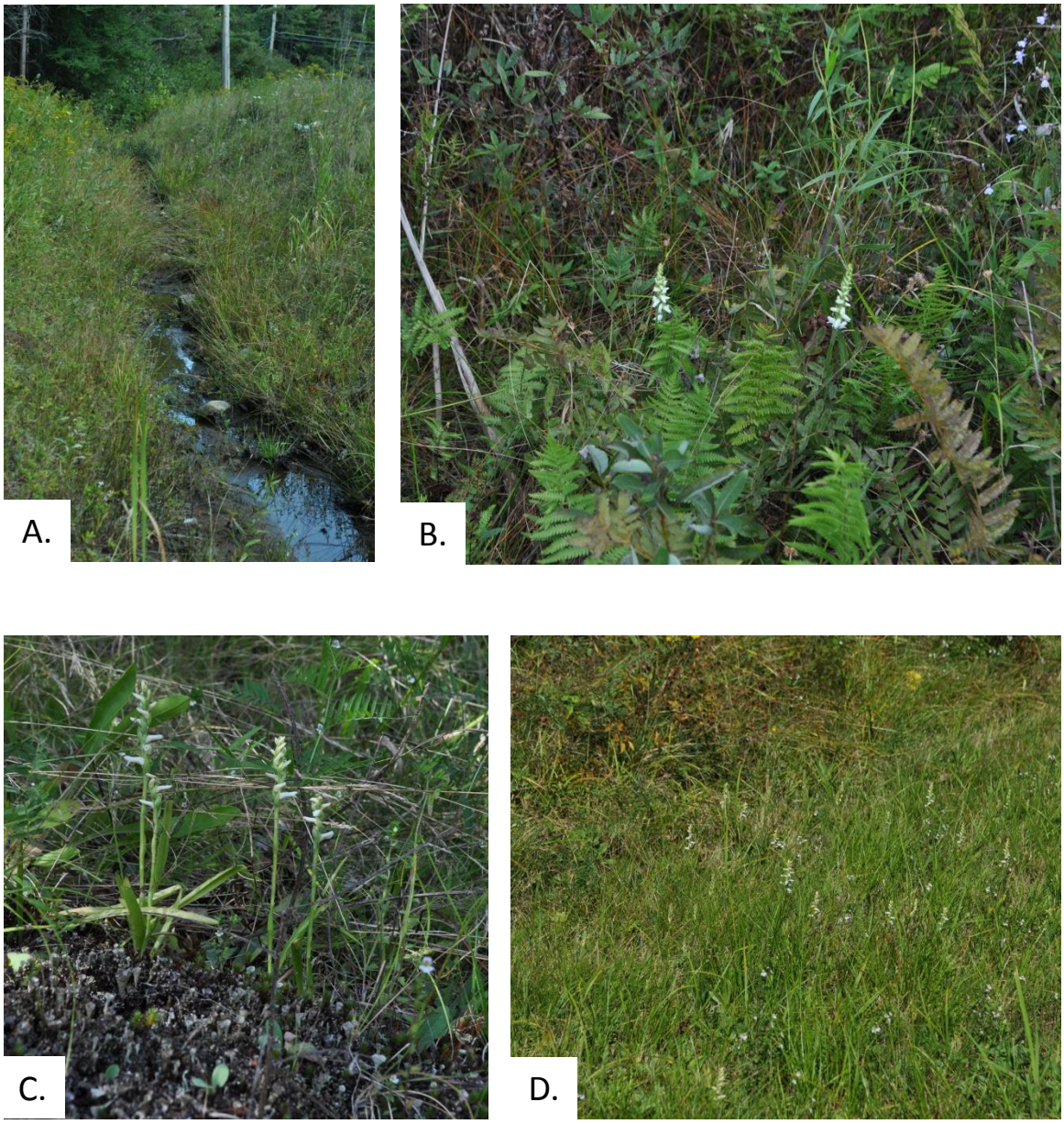


Fig. 19. A–C. Habitat of *Spiranthes arcisepalum*. **A.** Holotype location, wet, seeping roadside ditch, Long Lake, Hamilton Co., NY. **B.** *Spiranthes arcisepalum* in mossy, peat fen/wet prairie complex, Seneca Co., OH. **C.** Seeping, dry, lichen and moss hummock, Indian Lake, Hamilton Co., NY. **D.** *Spiranthes* ×*incurvum* & *S. arcisepalum* co-occurring in a wet roadside, just down slope from C., Indian Lake, Hamilton Co., NY.



Fig. 20. Mixed sheet of *S*. *arcisepalum* (blue) & *S*. ×*incurvum* (red), *Sheviak 1644* (NYS).

CHAPTER 4

Intrinsic nature or sporadic individuals?

Assessment of the frequency and evolutionary significance of hybridization in the Western North American Cordillera *Spiranthes* clade (Orchidaceae).

Matthew C. Pace, Kenneth M. Cameron

***Abstract*—**Hybridization is a key driver of plant diversity. However despite its predominance in the horticulture industry, naturally occurring hybridization has not traditionally been considered a major driver of speciation in the largest plant family, Orchidaceae. The species of *Spiranthes* found in the western mountain systems of the United States have long been a source of much taxonomic confusion. Much of this confusion has been attributed to hybridization between species. In an attempt to clarify the evolutionary origins of the Western North American Cordillera *Spiranthes* clade, we used molecular phylogenetics incorporating low copy nuclear, nuclear ribosomal, and plastid DNA sources. Our results indicate that 1) *Spiranthes delitescens* is a hybrid between *Spiranthes romanzoffiana* × *Spiranthes vernalis*, 2) *Spiranthes diluvialis* is of hybrid origin resulting from *Spiranthes magnicamporum* × *Spiranthes romanzoffiana*, and 3) hybridization between *Spiranthes porrifolia* and *Spiranthes romanzoffiana* may be rare. Furthermore, a realignment of taxon identity is necessary for *Spiranthes stellata* subsp. *stellata* and *Spiranthes stellata* subsp. *perexilis*, with subsp. *perexilis* elevated to full species status of *Spiranthes perexilis*, and subsp. *stellata* of hybrid origin between the sister species *Spiranthes perexilis* and *Spiranthes romanzoffiana*.

Key words—California Sierra Nevada Mountains; hybridization; Intermountain Basin & Range, morphometrics; sister species; taxonomic confusion

> "A major component of the complex is *Spiranthes stellata*: intrinsically variable subsp. *stellata* merges with the background tetraploid variation and through it with *S. romanzoffiana*. Might it also provide a conduit for genes of subsp. *perexilis* into the tetraploid complex such that the lip pubescence that suggests gene flow from *S. porrifolia* might in fact derive from *S. stellata*? The characteristics of the intermediate plants and *S. stellata* itself ... suggest hybridization with *S. porrifolia*."
> –C. Sheviak, 2014

Hybridization is a key driver of angiosperm diversity (see Abbott et al. 2013, Soltis and Soltis 2009 for reviews), but hybridization has traditionally not been considered a major driver of speciation in the largest plant family, Orchidaceae. This is counterintuitive given that manmade orchid hybrids form the basis for a multi-billion dollar horticultural trade. Instead, epiphytism, montane vicariance, and floral specialization driven by the evolution of pollinator-specific deception are hypothesized to have led to the spectacular diversification of orchids (Cozzolino and Widmer 2010, Gravendeel et al. 2004, Peakall et al. 2010, Silvera et al. 2009, Vereecken et al. 2010). Yet as molecular phylogenetic studies continue to expand across the orchid family, hybridization is increasingly found to play a role in the evolution of species within genera such as *Epidendrum* L. (Marques et al. 2014; Moraes et al. 2013; Pinheiro et al. 2010; Vega 2013), *Polystachya* Hook. (Russell et al. 2010), and *Goodyera* R.Br. in W.T. Aiton (Lee et al. 2012). Molecular evidence suggests that hybridization may also play a role in *Calopogon* R.Br. (Goldman et al. 2004), however further study is needed. In North America, *Platanthera* Rich. is hypothesized to widely and frequently hybridize –48% of named North American *Platanthera* taxa are hybrids– however most proposed hybrids have not been included in phylogenetic or population genetic analyses, so the true evolutionary role of hybridization within *Platanthera* may not be as pervasive as is currently thought (e.g., Paul et al. 2013; Wallace 2003, 2006). Additional orchid genera in which hybridization may play an important role in speciation –but which lack genetic data– include *Sobralia* Ruiz & Pav. (resulting from the creation of human-mediated hybrid swarms; e.g. Dressler and Pupulin 2014), and *Laelia* Lindl. (Halbinger and Soto

1997, Salazar et al. 2014). Hybridization as a potential driver for orchid speciation and diversity is perhaps most pronounced in Europe and the Mediterranean Basin where a large number of named orchids are of hybrid origin, particularly in the genera *Dactylorhiza* Neck. ex Nevski (De Hert et al. 2011; Hedren 1996; Pedersen 2006; Pillon et al. 2007), *Ophrys* L. (Devey et al. 2008; Gulyas et al. 2005; Soliva and Widmer 2003; Stokl et al. 2009), and *Orchis* Tourn. ex L. The latter, in fact, has more named hybrid than non-hybrid 'species' (37 vs. 21; Jacquemyn et al. 2012; Luca et al. 2012; Govaerts et al. 2014). It is noteworthy that hybridization appears to be common in genera such as *Ophrys*, which have evolved highly specialized sexually deceptive flowers, since this adaptation has traditionally been hypothesized to serve as a reproductive barrier that insures pollinator fidelity, and thereby reducing the potential for hybridization.

The nearly cosmopolitan, terrestrial, and primarily temperate orchid genus *Spiranthes* Rich. (Orchidoideae: Cranichideae: Spiranthinae) is another orchid genus in which hybridization is hypothesized to be nearly pervasive, despite the lack of molecular phylogenetic data to support this claim. This idea is a response to the conflicting inter-species similarity and/or intra-species variability of many hypothesized species, their often sympatric or commingling occurrence, and pollination relationships with many species of the generalist pollinator *Bombus* Latreille. To date 11 hybrids or species of hybrid origin have been formally described and several more putative natural crossings have not received official taxonomic recognition. As a result, North American *Spiranthes* would appear to mirror European *Ophrys* as a temperate terrestrial genus with porous reproductive barriers. Over time, however, many of these putative hybrid taxa have been shown not to be derived from a hybrid origin, but instead represent 'good' species or morphological extremes within species. For example, *Spiranthes* ×*laciniata* was elevated to non-nothospecies status by Correll (1950) (a status that appears to be supported by molecular phylogenetic data

(Dueck et al. 2014)); the ambiguously described *Spiranthes* ×*folsomii* P.M. Br. and *Spiranthes* ×*itchetuckneensis* P.M. Br. were revealed to be synonyms of the non-hybrid *Spiranthes triloba* (Small) Kuntz. emend M.C. Pace (Chapter 1); *Spiranthes* ×*meridionalis* P.M. Br. is partially based on mixed paratypes and is likely a synonym of *Spiranthes praecox* (Walter) S. Watson; and *Spiranthes* ×*simpsonii* Catling & Sheviak, *Spiranthes* ×*borealis* P.M. Br., and *Spiranthes* ×*eamesii* P.M. Br. likely represent extremes in the normal variation of one of their supposed parents. The hybrid origins of only two species in the genus are confirmed by genetic data: *Spiranthes hongkongensis* S.Y. Hu & Barretto (Sun 1996) and *Spirnthes diluvials* Sheviak (Arft and Ranker 1998).

The Western North American Cordillera Spiranthes clade. One natural, monophyletic group of *Spiranthes* in which hybridization has been hypothesized to be especially frequent is the Western North American Cordillera clade. Five of the six species in this clade are thought to either be derived from, or engage in hybridization: *Spiranthes delitescens* Sheviak, *S. diluvialis*, *Spiranthes porrifolia* Lindl., *Spiranthes romanzoffiana* Cham., and *Spiranthes stellata* P.M. Br., Dueck, & K.M. Cameron. Only *Spiranthes infernalis* Sheviak is excluded from this list and its species status is questionable – it may be a unique, localized, disjunct phenotype of *S. porrifolia*.

Spiranthes delitescens is one of the rarest orchids on Earth, found in only four cienegas in the Sky Island region of extreme southern Cochise and Santa Cruz Counties, Arizona. At the time of its description Sheviak (1990) hypothesized that *S. delitescens* is of hybrid origin, possibly derived from a cross between *S. porrifolia* and *Spiranthes vernalis* Engl. & A. Gray. In their molecular phylogenetic study of this genus, however, Dueck and Cameron (2007) and Dueck et al. (2014) found no evidence to support or reject the hybrid nature of this species.

Instead, they found it to share a common ancestor with *S. romanzoffiana*, but in an unresolved position.

Conversely, *S. diluvialis*, is one of the two genetically confirmed hybrids in the genus (Arft and Ranker 1998; Dueck and Cameron 2007; Dueck et al. 2014; Szalanski 2001), resulting from crosses between *S. romanzoffiana* and *S. magnicamporum*. This species is composed of several widely scattered disjunct populations in British Columbia, Colorado, Idaho, Montana, Nebraska, Nevada, Washington, and Wyoming. It is unknown how many hybridization events have occurred, when or where they happened, if the extant range of *S. diluvialis* represents an ancient hybrid zone between its parents, or if the extant discontiguous populations are the result of repeated independent hybridization or dispersal. Arft and Ranker (1998) hypothesized that at least two separate hybridization events between *S. magnicamporum* and *S. romanzoffiana* led to the formation of *S. diluvialis*, with subsequent localized dispersal in Utah and Colorado (at the time of their study *S. diluvialis* was known from Colorado, Montana, Nevada, Utah, and Wyoming, but their study only included samples from Colorado and Utah).

Species delimitation becomes very difficult when dealing with the remaining three species in the Western Cordillera clade, particularly between plants growing within the California Sierra Nevada. *Spiranthes romanzoffiana* is the most widely dispersed orchid in North America, spanning approximately 36° latitude and 120° longitude, from the Arctic Circle down the Western Cordillera to Mount Graham in southern Arizona, and across boreal and montane North America from the western Aleutian Islands and California to New England and Labrador. Within this vast expanse *S. romanzoffiana* is composed of both diploids and tetraploids (2*n*=44, 66, 88; Sheviak 2013), ranges in size from minute arctic and montane plants 5 cm tall to large robust coastal dune plants 60 cm tall, the leaves of some populations are fugacious,

inflorescences can be loosely or very tightly spiraled, and the characteristic floral 'hood' – formed by the dorsal petals and dorsal and lateral sepals– can range from highly to poorly developed. Ongoing hybridization involving *S. romanzoffiana* is believed to be contained to montane California, Oregon, and Washington where its range overlaps with *S. porrifolia* and *S. stellata*. *Spiranthes porrifolia* is restricted to wet meadows and seeps in the Sierra Nevada, Cascades, and southern Coastal Range of California, Oregon, and Washington. Unlike many species in the genus, *S. porrifolia* is relatively uniform in its habitat preferences and morphology, with tubular ochroleucous flowers, slightly spreading lateral sepals, and a narrow, papillate lower labellum. The same cannot be said for *S. stellata*; although it is restricted to wet meadows and seeps in the California Sierra Nevada and a small disjunct population in the southern Cascades of Oregon, this species (as it is currently understood) presents a wide variety of flora forms, from typical outward swept lateral sepals, to populations which approach the hood development of *S. romanzoffiana*, to others with flowers that approximate the more slender and tubular profile of *S. porrifolia*, and the labellum spans the morphological spectrum of both *S. romanzoffiana* and *S. porrifolia* in shape and texture. The morphological variability of *S. stellata* is such that this species is not accepted by the Jepson Flora Project (2015), the standard floristic work of California. Instead, the Jepson Flora Project treats *S. stellata* as representing tentative hybrids between *S. romanzoffiana* and *S. porrifolia*. Although it is predominantly tetraploid, *Spiranthes stellata* is also composed of rare diploid (three known populations) and triploid (one known individual) plants. Based primarily on their ploidy, more openly spiraled inflorescences, and slightly smaller flowers, Sheviak (2012) segregated the diploid and triploid individuals under *S. stellata* subsp. *perexilis*. Sheviak (2012, 2013) proposes that *S. romanzoffiana*, *S. porriffolia*, *S. stellata* subsp. *stellata*, and *S. stellata* subsp. *perexilis* all engage in frequent bidirectional gene

flow and introgression such that any slight variation in size, shape, sepal orientation, or labellum texture is indicative of a hybrid origin.

The collective work of Sheviak, and Dueck and Cameron, have done much to influence and improve scientific understanding of this challenging genus, however some limitations should be discussed. Working primarily before the widespread implementation of molecular phylogenetic techniques and reconstructions, Sheviak was almost wholly reliant on cytology and morphology as tools for inferring species-status and hybridization. Though these tools are undoubtedly useful and powerful lines of evidence, the lack of a molecular phylogenetic perspective gives way to a degree of hopelessness: "*Spiranthes romanzoffiana* [is] an abstraction" (Sheviak 2013). Although the philosophical concept of 'species' can be argued as a largely human-implemented construct that attempts to approximate the collective products of evolution, this quote denotes more than a hint of exasperation with a very challenging situation. The collective work of Dueck and Cameron injected a much needed molecular component to the study of *Spiranthes*, however they lacked low-copy nuclear markers, and often included individuals that are 'classic' representatives of their proposed species, or were limited in sample size or geographic distribution. The continent-wide distribution of the genus and the rarity of some species also posed a challenge to previous authors: Sheviak (2013) cites the limits of working on a Californian group from his New York base as one reason for delaying his publication of *S. stellata* subsp. *perexilis*.

To address these long standing issues regarding the extent and evolutionary significance of hybridization, we undertook a phylogenetic examination of hybridization within the Western North American Cordillera *Spiranthes* clade using plastid, nuclear ribosomal, and low-copy nuclear loci. This group of taxa was selected because it represents a historically challenging

taxonomic group that includes species of conservation concern at the Federal and State level. Gaining an understanding of the evolutionary significance of hybridization, the hybrid origins of species, and locating potential hybrid zones is beneficial to conservation efforts (Allendorf et al. 2001; Cozzolino et al. 2006; López-Pujol et al. 2012; Nolte and Tautz. 2010; Sanders et al. 2014; Stronen and Paquet 2013). We hypothesize hybrids to be rare, and if hybrid individuals are present, they will present incongruent tree topologies based on the dataset in question. Such clade-switching was the main method employed to detect the hybrid origin of both *S. diluvialis* and *S. hongkongensis*.

METHODS

Taxonomic Sampling—Herbarium specimens of species in the Western North American Cordillera *Spiranthes* clade were carefully reviewed from AMES, BH, CHICO, CLEM, F, JEPS/UC, MO, NY, NYS, RSA, and US (acronyms follow Thiers [continuously updated]). Individual flowers from the lowermost quarter of the inflorescence were rehydrated for morphological examination from select individual specimens. Our molecular phylogenetic data set incorporates and expands upon the dataset of Dueck et al. (2014). For areas we could not visit for fieldwork, 1–10 year old herbarium specimens was judiciously sampled for inclusion in the molecular phylogenetic analysis.

Morphometrics—Data analysis performed and PCA generated in RStudio (2012) using rehydrated labella from herbarium samples (fig. 2). Measurements of labella were also generated from scaled line drawings available in the literature (Sheviak 1989). In total 50 individual flowers were measured: 11 *S. stellata* subsp. *stellata* (including individual stems from the holotype sheet), 4 *S. stellata* subsp. *perexilis*, 32 *S. porrifolia*, and 3 *S. romanzoffiana*.

Measurements included labellum length, width at widest point, width at isthmus/narrowest point, width of dilated tip, length of dilated tip, length from ovary to dilated tip, and callosity height.

Phylogenetic Methods—Phylogenetic analyses incorporated and expanded upon the dataset of Dueck et al. (2014). *Deiregyne diaphana* (Lindl.) Garay, *Dichromanthus cinnabarinus* (Lex.) Garay, and *Schiedeella llaveana* (Lindl.) Schltr. were included as additional outgroups (Salazar et al. 2003). Sixteen new samples were included for molecular phylogenetic analysis, for a total of 3 samples for *S. delitescens*, 3 samples of *S. diluvialis*, 3 samples of *S. infernalis*, 11 samples of *S. magnicamporum*, 12 samples of *S. porrifolia*, 19 samples of *S. romanzoffiana*, 13 samples of *S. stellata*, and 8 samples of *S. vernalis* (fig 1.). For new accessions, 3–4 unopened buds or ca. 1 cm sq^2 of leaf tissue were collected and silica dried for later extraction of Total gDNA. IBI Plant Isolate Kits (Peosta, IA) and Maxwell® 16 LEV Plant DNA Kits (Madison, WI) were used for all newly collected samples. All accessions were amplified for the plastid gene regions *mat*K, *ndh*J, *trn*L intron, *trn*S-fM, and *ycf*1 3', nuclear ribosomal ITS, and the low-copy nuclear regions *ACO* and *Xdh* (fig. 1). Amplification of loci and cycle sequencing followed standard protocols based on annealing temperatures of the associated primers. PCR products were purified using ExoSap-It (Cleveland, OH), and cycle sequencing products were cleaned using Agencourt CleanSeq (Beverly, MA) magnetic beads. Direct sequencing of cleaned cycle sequencing products was performed at the University of Wisconsin – Madison Biotechnology Center.

Resulting chromotograms were edited and aligned using software modules available in Geneious 7.1 including MUSCLE. Ambiguities in plastid datasets were coded with standard IUPAC-IUB Symbols for Nucleotide Nomenclature (Cornish-Bowden 1985). Conversely, in an attempt to tease apart putative parental copies, nuclear ribosomal and low-copy nuclear

sequences with ambiguities were duplicated as putative homoeologous gene copies (‘copy A’ and ‘copy B’) (as graphically depicted in Chapter 3). Nucleotide ambiguities were then coded such that ‘copy A’ matched the nucleotides of potential parental species A, and ‘copy B’ matched the nucleotides of potential parental species B. This coding approach was used in lieu of cloning in an attempt to retrieve both parental copies of potential hybrids. If samples failed to amplify after repeated attempts for a given locus they were coded as missing data. Relationships among accessions and taxa were visualized in FigTree (Rambaut 2014). Analyses were performed under Bayesian inference (MrBayes on XSEDE (3.1.2)) implemented through CIPRES Portal V. 3.3 (Miller et al. 2010) with the following parameters: rates = invgamma, 5,000,000 number of generations, sample frequency of 100,000, nruns = 2, nchains = 4, temp = 0.2, and a burnin of 500,000.

RESULTS

Phylogenetic Analyses— Gene amplification of all samples from all taxa proved difficult for some loci. Whereas nearly every sample amplified for nrITS (92 samples) and trnS-M (88 samples), amplification of *ACO* was particularly difficult for many members of the Western North American Cordillera *Spiranthes* clade (only 25 samples). Furthermore, although *Xdh* provides species-specific mutations that allow for the recovery of most taxa within species-specific clades, resolution among these clades is lacking for some of the taxa of interest, making interpretation of relationships based on single gene-trees challenging. Combining all non-plastid loci into a ‘combined nuclear’ tree increased resolution between taxa, aiding interpretation of the topology. Identification of potential parental species was facilitated by comparing the combined

plastid to the combined nuclear trees to each other and looking for incongruences in the topological position of individual terminals/samples among otherwise stable clades.

Xdh—*Xdh* recovered a polytomy between individually moderately to strongly supported clades of *S. romanzoffiana* + *S. stellata*, and *S. porrifolia*. Individual *Xdh* copies for *S. stellata* did not segregate into different species clades, with both copies recovered within the *S. romanzoffiana* + *S. stellata* polytomy. Copy A of *S. delitescens* was recovered along this polytomy as well, whereas copy B was recovered within *S. vernalis*. *Xdh* did not amplify for *S. diluvialis*.

ACO—ACO did not amplify for the majority of the western species. For the accessions that did amplify, *S. infernalis* was fully supported as distinct from *S. romanzoffiana*. *Spiranthes diluvialis* was recovered within the *S. magnicamporum* clade. *Spiranthes delitescens* was recovered as sister to *S. vernalis*.

nrITS—*Spiranthes infernalis* was recovered within a polytomy with *S. porrifolia*; together, this clade was sister to all other taxa. Spiranthes romanzoffiana was recovered as a strongly supported subclade (pp = 0.91) within an unresolved polytomy of individual *Spiranthes stellata* samples. *Spiranthes delitescens* was embedded as a fully supported subclade within the *S. romanzoffiana* clade. Sample ss8a (a priori *S. stellata* subsp. *stellata*) was the only accession of potential hybrid samples in which individual copies of nrITS were recovered in their distinct putative parental clades. In all other cases of potential hybrid individuals, both putative Copy A and putative copy B were recovered within a single clade. *Spiranthes diluvialis* was placed within a weakly supported (pp = 0.72) subclade of *S. magnicamporum* primarily from the southeastern United States (but also including samples from New Mexico and the Great Lakes Basin).

Combined plastid—The western taxa were recovered as three distinct and fully supported (pp = 1) clades: 1) *S. porrifolia* + *S. infernalis*; 2) *S. stellata* subsp. *perexilis*; 3) *S. romanzoffiana* + *S. stellata* subsp. *stellata* + *S. delitescens* + *S. diluvialis* (fig. 5). *Spiranthes magnicamporum* and *S. vernalis* were recovered as fully supported and distinct species.

Combined nuclear—The combined nuclear tree produced a topology of a fully supported (pp = 1) *S. porrifolia* + *S. infernalis* as sister to a fully supported unresolved clade of *S. romanzoffiana* and *S. stellata* subsp. *stellata* and subsp. *perexilis* (fig. 5). However, *S. stellata* subsp. *stellata* and subsp. *perexilis* were strongly supported (pp = 0.96) as a distinct clade within this wider unresolved *S. romanzoffiana* polytomy. *Spiranthes delitescens*, based on different putative parental gene copies, is recovered within unresolved polytomies of *S. romanzoffiana* and *S. vernalis*. The combined nuclear data recovers *S. diluvialis* as embedded within a grade of *S. magnicamporum* subclades, with strong support (pp = 0.99) for a shared most recent common ancestor between the samples of *S. diluvialis* from NE, ID, and WA and the accession of *S. magnicamporum* from NM.

Morphometrics—Visualization morphological variation via principal components analyses indicate four major groups based on characteristics of the labellum (fig. 3): 1) a variable and wide-ranging *S. porrifolia* group, 2) *S. stellata* subsp. *stellata*, 3) *S. stellata* subsp. *perexilis*, and 4) *S. romanzoffiana*. The placement of the *S. stellata* subsp. *stellata* group is intermediate between *S. stellata* subsp. *perexilis* and *S. romanzoffiana*. The intermediate *S. stellata* subsp. *stellata* group includes the lectotype of *S. stellata* subsp. *stellata* designated by Sheviak (2013), in addition to several specimens Sheviak (1989) previously identified as *S. porrifolia*. One individual phylogenetic sample (ss1; which may represent a hybrid) was not placed as being

intermediate between tentative parental species or near its a priori species (*S. stellata* subsp. *stellata*), instead expressing very characteristic features of *S. porrifolia*.

DISCUSSION

Overall, identification of potential hybrid individuals was facilitated by comparisons of the combined plastid vs. combined nuclear topologies (fig. 4, fig. 5). Our phylogenetic hypotheses indicate that most of the samples identified a priori as '*S. stellata* subsp. *stellata*' appear to be of hybrid origin between *S. romanzoffiana* and *S. stellata* subsp. *perexilis* (fig. 5). For most taxa, putative copies of nuclear genes or nrITS did not segregate into potential parental clades; instead both copies were recovered within a single clade of one potential parent. The exceptions are: 1) sample ss8a nrITS putative homeologs were recovered in distinct *S. stellata* subsp. *perexilis* and *S. romanzoffiana* clades; and 2) individual *Xdh* copies, representing potential parental copies, of *S. delitescens* were recovered in *S. romanzoffiana* and *S. vernalis*. In the case of *S. stellata* subsp. *stellata*, the intermediate nature of labellum morphology (fig. 3) supports the clade switching identified via comparisons of phylogenetic topologies (fig. 5), further suggesting a hybrid origin for this taxon.

Hybridization within the Western Cordillera Clade

Elucidating the hybrid origin of Spiranthes delitescens—For the first time, we present molecular genetic evidence for the parental identities of Federally Endangered *S. delitescens*, long suspected to be of ancient hybrid origin. Both low copy nuclear genes indicate that *S. vernalis* (2n = 30; *ACO, Xdh* putative homeologous gene copy B) is a parent (presumably paternal). In contrast, the plastid loci together with nrITS and *Xdh* putative homeologous gene copy A indicate *S. romanzoffiana* is a parent (2n = 44, 66, 88; presumably maternal). The

combination of n = 15 and n = 22 lineage parentage for amphiploid *S. delitescens* (2n = 74) is supported by the cytological evidence initially presented by Sheviak (1990). However, the specific parental species suggested by our molecular phylogenetic –*S. romanzoffiana* and *S. vernalis*– data do not fully correspond to the parental species originally proposed by Sheviak (1990): *S. porrifolia* and *S. vernalis*. Though he was admittedly unsatisfied with this putative crossing due to differences in overall morphology, the tapering of *S. delitescens*' glandular trichomes suggested the acute-tipped non-glandular trichomes of *S. vernalis*, whereas the floral shape and sepal orientation of *S. delitescens* were evocative of *S. porrifolia*. *Spiranthes graminea* Lindl. ex Benth. was also considered by Sheviak as a possible parent based on morphological similarities, but ultimately discarded due to cytological evidence and the presence of free lateral sepals. *Spiranthes graminea* is in the n =15 lineage, as is *S. vernalis*; one parental species needs to be in the n = 22 lineage –which at the time of the description of *S. delitescens* included *S. lucida*, *S. porrifolia*, and *S. romanzoffiana*– in order to give rise to amphiploid 2n = 74 *S. delitescens*. *Spiranthes romanzoffiana* was not seriously considered as a possible parent because it does not possess any features, aside from connate lateral sepals, found in *S. delitescens*.

Dueck and Cameron (2007, 2008b) and Dueck et al. (2014), based on nrITS and fewer plastid loci, recovered *S. delitescens* within an unresolved *S. romanzoffiana* polytomy, with no evidence for the second parental origins with *S. vernalis*. Thus *S. delitescens* serves as a case-study for how phylogenetic reconstructions incorporating low copy nuclear markers are valuable sources of information for discovering the evolutionary parents of proposed ancient hybrid species were other traditional phylogenetic regions –in addition to traditional morphological analysis– fall short.

The hybridization of *S. vernalis* and *S. romanzoffiana* leading to the evolution of *S. delitescens* is quite fascinating, and likely represents an example of ancient hybridization. Although *S. delitescens* grows within ca. 135 km of the southernmost extant population of *S. romanzoffiana* in Mt. Graham, AZ, it is separated by ca. 1400 km from the nearest extant population of *S. vernalis* along the Gulf Coast of TX. Furthermore, although *S. vernalis* and *S. romanzoffiana* both prefer moist, low-statured graminoid habitats, the two species grow in mostly distinct ecosystems: *S. vernalis* in prairies and pine flatwoods; *S. romanzoffiana* in cool mountain bogs, lake edges, fens, and arctic tundra. The one habitat both parental species share is coastal dune swales, however this habitat is absent in the current distribution of *S. delitescens*, and is not the habitat in which *S. delitescens* occurs. Both parental species do not currently overlap in bloom period. It is only along the northern limit of its extant range that *S. vernalis* approaches the same bloom period of *S. romanzoffiana* in mid- to late summer. It is speculative but it appears past glacial cycles may have led to the southwestward migration of *S. vernalis* (or a now extinct common ancestor of *S. delitescens* and *S. vernalis*) toward the southern Rocky Mountains, and may have also pushed its bloom period later. Curiously, *S. vernalis* and *S. romanzoffiana* are not known to share any extant pollinators (they do share *Apis mellifera*, however this species did not exist in North America prior to European colonization). Three explanative scenarios are possible: 1) a shared extant pollinator (likely a species of *Bombus*) exists but has yet to be observed pollinating one or both parental species, 2) an extant species of pollinator shared both species during the time period of hybridization but has since shifted to other hosts or no longer co-occurs with one or both species, or 3) a pollinator existed during the time of hybridization but has since gone extinct.

Further evidence on the hybrid origin of Spiranthes diluvialis—Comparisons of our nuclear vs. plastid data support the parental species of *S. diluvialis* as *S. magnicamporum* (likely paternal) and *S. romanzoffiana* (likely maternal). In our combined plastid + nuclear phylogenetic hypothesis (fig. 4), these samples are fully supported as sharing a most recent common ancestor with the sample of *S. magnicamporum* from NM (sm7h). The maternal lineage of *S. diluvialis* is weakly supported (pp = 0.76) as a tripartite polytomy within a fully supported by widely unresolved polytomy of *S. romanzoffiana* from across North American and Scotland, indicating that the included samples of *S. diluvialis* may have shared a most recent common ancestor with each other (their relationship to any one sample or regional sampling of *S. romanzoffiana* remains unresolved). Further research (particularly population genetic analyses) is clearly needed before the multiple hybridization events hypothesis put forward by Arft and Ranker (1998) could be rejected or accepted. Given the widespread and disjunct distribution of *S. diluvialis*, we originally anticipated that multiple hybridization events/origins of *S. diluvialis* would be supported. An analogous case was examined by Wallace (2003), who found evidence for multiple origins for the widespread hybrid species *Platanthera huronensis* (Nutt.) Lindl., estimating at least five separate hybridization events.

It is speculative, but *S. magnicamporum* appears to have occurred throughout the middle and southern Rocky Mountains and Great Basin, far to the west of its current distribution, an area where *S. romanzoffiana* is extant. As local western and montane plant communities migrated in response to climatic shifts and glacial expansions and contractions, *S. magnicamporum* and *S. romanzoffiana* –both of which prefer moist graminoid habitats– likely became sympatric and co-occurred, and may have shifted their bloom times. Most of the middle North American Western Cordillera –including areas where *S. diluvialis* is extant in Colorado, Idaho, Montana, and Utah–

was ice-free during the Pleistocene Epoch (Dyke et al. 2003) –the likely period in which this species originated– and mountain lake pollen records indicate an apparent increase in cool-moist plant taxa (Jiménez-Moreno et al. 2007). Although the geographic heterogeneity of the region and cyclical nature of glacial expansion and retreat makes any broad-scale statements regarding environmental conditions in the western mountain systems of North America difficult, additional lake pollen and rodent midden records indicate a fairy wet climate for many portions of the Rocky Mountains, the Sonoran and Chihuahuan Deserts, and Great Salt Lake region into the Younger Dryas Period (Cole et al. 2013, Davis 1998, Holmgren et al. 2010). This indicates that suitable habitat for *S. magnicamporum* may have occurred throughout the Cordillera for tens of thousands of years after a possible initial period of hybridization with *S. romanzoffiana*.

Spiranthes romanzoffiana, *S. magnicamporum*, and *S. diluvialis* share *Bombus fervidus* Fabricius as a pollinator (Catling 1983, Sheviak 1982, Sipes and Tepedino 1995); additionally *S. romanzoffiana* and *S. magnicamporum* also share *Bombus vagans* Smith (Catling 1983, Catling 2014, Godfery 1933). Extant populations of *S. magnicamporum* and *S. romanzoffiana* rarely grow within pollination range of each other in western North America (the closest populations in New Mexico are separated by a distance of ca. 53–80 km and ca. 600–900 m in elevation), thus hybridization is unlikely to be ongoing. Curiously, in Eastern North America –where *S. diluvialis* is absent– *S. magnicamporum* and *S. romanzoffiana* grow within pollinator range of each other in places such as Michigan, Minnesota, New York, Ontario, and Wisconsin. Yet even in these geographically close populations, the species are ecologically and phenologically separated: *S. romanzoffiana* in bogs, fens, and wet roadsides, blooming from mid-July to mid-August; *S. magnicamporum* in dune swales, moist to dry prairies, and alvars, blooming from early-September to mid-October.

Lack of evidence for hybridization between S. romanzoffiana and S. porrifolia—Although it must be stressed that this is a broad-scale and non-population genetic study, we did not recover any hybrid individuals between *S. romanzoffiana* and *S. porrifolia*, despite their hypothesized frequency in the literature based on observations in the field (Coleman 1995; Sheviak 2012, 2013). Future fine-scale population genetic investigations may reveal instances of hybridization or introgression not found in this study. However given our geographically expansive sampling, examples of introgression/chloroplast capture between these two species of the type exhibited by other hybrids in the Western Cordillera clade appear to either be rare or absent. Thus is may be tentatively suggested that any intermediate characters found in individual plants or populations of *S. romanzoffiana* that approximate *S. porrifolia* (and vice versa) are not the result of hybridization, but are due to other evolutionary or environmental forces.

Illuminating and realigning the identity of cf. S. stellata and cf. S. stellata subsp. perexilis: taxonomic changes supported by molecular and morphometric analyses—Continued research into the molecular phylogenetic relationships and morphology of North American orchids has cast an increasingly and justifiably critical light on the taxa described by P.M. Brown, especially within the journal that he self publishes (e.g., *S.* ×*itchetuckneensis*; Chapter 1). The description of *S. stellata* was initially a welcome exception as it included a detailed descriptive treatment and accompanying molecular data to support is species status; however *S. stellata* has been a controversial taxon, a status highlighted by its rejection by the Jepson Flora which considers it to represent putative hybrids between *S. romanzoffiana* and *S. porrifolia* (Coleman et al. 2014). The data we present here (fig. 5) indicates putative *S. romanzoffiana* × *S. porrifolia* may not exist (or is exceptionally rare), likely ruling out this long standing hypothesis concerning the identity of *S. stellata* as a hybrid. However our molecular phylogenetic and

morphological data (fig. 3, fig. 5, fig. 6-9) shows many plants identified as *S. stellata* are actually hybrid in nature, whereas others are not, based on their change in topological position between plastid and non-plastid datasets and morphometric placement –this later stable group mostly being identified *a priori* as cf. *S. stellata* subsp. *perexilis*.

In the course of our analyses, it became apparent that the confusion in the literature over the identity of *S. stellata, S. stellata* subsp. *perexilis*, putative hybrids, and morphs of *S. romanzoffiana* was the result of a mixed holotype sheet (originally intended to show morphological variation of its representative species, fig. 10), poor selection of paratypes, later lectotypification, narrow inclusion of material for phylogenetic analyses, and a miss-reading of what cf. *S. stellata* and cf. *S. stellata* subsp. *perexilis* actually represent in evolutionary terms.

Although some of our included loci did not provide desired levels of resolution between putative species, our overall phylogenetic data support a distinct *S. romanzoffiana* clade sister to a distinct cf. *S. stellata* clade. Between these two clades, some individual accessions will switch clade position based on plastid vs. non-plastid data (fig. 5); on the whole, comparisons of the nrITS copies against the combined plastid reconstructions proved most valuable when detecting potential hybrids. These hybrid 'clade jumpers' represent plants that are relatively robust in habit, with densely spiraled inflorescences composed of flowers with wide-spreading ovate to subovate or widely-elliptical upper labella (fig. 7-11). The stable component of the *S. stellata* clade is composed of gracile, open spiraled, narrow-labellaed plants matching the description of cf. *S. stellata* subsp. *perexilis* (fig. 12 & 13). As noted by Sheviak (2012), the original description of *S. stellata* designated *Colwell 07-279* as the holotype (Brown et al. 2008; fig. 10), a sheet which presents 3 individuals, 2 of which are gracile in habit and approximate *S. stellata* subsp.

perexilis. As such, Sheviak lectotypified the central robust, densely spiraled, and wide-spreading ovate upper labellumed individual as the lectotype of *S. stellata* subsp. *stellata*.

Our morphometric analysis (fig. 3) depicts well defined groupings of *S. romanzoffiana* and cf. *S. stellata* subsp. *perexilis* (fig. 3). A third grouping, composed of phylogenetically confirmed hybrid individuals and other densely spiraled and wide-spreading ovate upper labellumed individuals (e.g., fig. 8), is placed in-between *S. romanzoffiana* and cf. *S. stellata* subsp. *perexilis*, lending support to their hybrid ancestry and thus approximately intermediate morphology. Crucially, this hybrid grouping includes the lectotype of *S. stellata* subsp. *stellata*.

Therefore, because plants matching the morphology of the *S. stellata* subsp. *stellata* lectotype are recovered as hybrids in our phylogenetic reconstructions and placed in an intermediate morphometric position –whereas cf. *S. stellata* subsp. *perexilis* is distinct in all datasets– we feel it is prudent and necessary to realign the identities of these taxa: cf. *S. stellata* subsp. *perexilis* actually represents a non-hybrid valid species, here elevated to *Spiranthes perexilis* (Sheviak) M.C. Pace; cf. *S. stellata* subsp. *stellata* embodies plants of hybrid origin between *S. perexilis* × *S. romanzoffiana*, here transferred to *Spiranthes* ×*stellata* P.M. Br., Dueck & K.M. Cameron (pro sp.). The lectotypification by Sheviak (2012) was the deciding factor in determining accurate taxonomies and ranks/categories for these taxa; the holotype and lectotype specimens of the respective basionyms have been annotated to reflect their correct taxonomy. For the rest of our discussion we will apply the new concepts of *S. perexilis* and *S.* ×*stellata*.

Brown et al. (2008) made much of the fact that *S. romanzoffiana*, *S. perexilis*, and *S.* ×*stellata* rarely co-occur, essentially ruling out the likelihood of cross-pollination and hybridization. We agree that the extent of hybridization within *Spiranthes* has been overemphasized, however the interpretation of Brown et al. (2008) represents a rather naïve view

of hybridization and the distribution of hybrid species; the reader need only consider the distribution of *S. diluvialis*, *S.* ×*incurvum* (Chapter 3), or *Typha* ×*glauca* Godr, species which due to certain characteristics or geologic history frequently occur without one or either parent co-occurring. Additionally, pollinators have the ability to move pollen over several km; in this case *Bombus* –the likely pollinator for the three taxa– though usually restricted to foraging within several hundred m of its home nest (Wolf and Moritz 2008), have been documented as travelling up to 11.6 km (Kraus et al. 2009, Hagen et al. 2011, Walther-Hellwig and Frankl 2000). Lastly, although most terrestrial orchid seeds fall within a few m of the mother plant (Jersáková and Malinová 2007), their dust-like seeds have to potential to be transported several or hundreds of km (Arditti and Ghani 2000, Vanden Broeck et al. 2014). The combination of pollen movement and high potential disperseability makes it very likely that even chance hybridization events could disperse to areas well-removed and allopatric with respect to their parental species. Moreover, it is well known that many populations of *Spiranthes* do not bloom every year, possibly relating to moisture availability, and are thus easily overlooked. It is entirely possible that the lack of co-occurrence observed by Brown et al. (2008) reflects the shifting and unreliable nature of population blooming patterns.

The description of *S. perexilis* (basionym *S. stellata* subsp. *perexilis*; Sheviak 2012) did not incorporate any genetic data outside of cytology –indeed, *S. perexilis* seems to have been described primarily on differences in ploidy: *S. perexilis* as 2n = 44 and 66, *S.* ×*stellata* as 2n = 88. Morphologically, the scatterplots of *S. perexilis* and *S.* ×*stellata* show some clustering of the taxa (Sheviak 2012) –with *S. perexilis* being very slightly smaller– but they are continuums; Sheviak (2012, 2013) even states that some of the characters used to distinguish the taxa overlap and encompass one another, and was unsure as to whether these issues were due to hybridization

or were the direct result of polyploidy. Curiously, if the reader approaches Sheviak (2012) through the implementation of the taxonomy we present here, uncertainty is lessened and it becomes clear Sheviak understood *S. perexilis* was hybridizing with *S. romanzoffiana*, but misunderstood its extent and interaction with *S. porrifolia*, in addition to a misunderstanding of identity of *S.* ×*stellata*. Furthermore, our morphometric analysis of the labellum (fig. 3, fig. 9) improves upon the work of Sheviak (2012), showing clear differences due to the narrower labellum and highly reduced subpandurate labellum apex of *S. perexilis* vs. *S.* ×*stellata*. Similarly, our work on the identity of *S. perexilis* and *S.* ×*stellata* is an example of how phylogenetic reconstructions, coupled with rigorous morphological examination, are increasingly essential complimentary paths to properly illuminate the evolutionary history and correct taxonomy of long challenging groups.

One confusing individual –ss5 (*Sheviak 2925* plant "d", NYS)– matches the description of *S. perexilis* in its slightly small flowers (lateral sepal length 5.7 mm), open spiral, and more gracile appearance, yet is a phylogenetically confirmed hybrid. This contradictory individual is from a population of plants that includes another confirmed hybrid (ss4), represented by plants of moderately densely spiraled inflorescences and larger sized flowers (lateral sepal length 6.4 mm). Sample ss5 does make difficult the identification of *S. perexilis* vs. *S.* ×*stellata*, but it may also represent a stunted individual or a plant of hybrid origin that expresses more of the characters derived from its partial *S. perexilis* parentage. Furthermore, the majority of individuals and populations identified as S. ×*stellata* are readily distinguishable upon close examination.

It must be noted that the phylogenetic work of Dueck and Cameron (2008) and Dueck et al. (2014) did not recover any discordance in their reconstruction of the Western Cordillera Clade. This is because the majority of their *S. perexilis* samples originated from southwestern

Oregon –where *S.* ×*stellata* does not occur– representing gracile, open spiraled plants; it is unknown if their sampling bias for open spiraled plants was deliberate, coincidental, or simply due to a lack of available material. Unfortunately, physical herbarium vouchers are lacking for their DNA samples (here represented by accessions 31a, 31e, and 31f), but photographic vouchers depict open spiraled plants with narrow subpandurate labella –plants that match the description of *S. perexilis*.

Distribution and frequency of S.* ×*stellata—The hybridization we found between individuals of *S. perexilis* (nrITS copy B, *Xdh*) and *S. romanzoffiana* (plastid, nrITS copy A) supports some of the ideas put forward by Sheviak (2012, 2013). However, whereas Sheviak (2013) hypothesized this pairing to occur throughout their shared range, our data suggest *S.* ×*stellata* may be restricted to the mid- and southern California Sierra Nevada (fig. 9). We will discuss the distributions and habitats of the hybrid individuals we recovered in our phylogenetic analyses: El Dorado, Fresno, Inyo, and Nevada counties. Pollinator observations and data are lacking for *S. perexilis* and *S.* ×*stellata*, so identification of insects that may be facilitating hybridization cannot be made.

Inyo Co. Despite its large geographic size (26,487.8 km^2), *Spiranthes* are restricted to the northwestern edge of Inyo Co. This general scarcity of *Spiranthes* may be due to the counties' position at the eastern foot of the California Sierra Nevada and the dry rain shadow these mountains cast (Death Valley is also located in Inyo Co.). Notwithstanding their rarity, *S. romanzoffiana*, *S. porrifolia,* and *S.* ×*stellata* are all found growing along streams, wet roadsides, and meadows around Mount Morgan and the Palisade Group. It is within the valley just to the north-northwest of Mount Morgan near Rock Creek Lake that *S.* ×*stellata* is found. These plants present many of the features that approximate *S. perexilis*, including a subpandurate labellum

with reduced apex dilation, papillate labellum apex surface, and free wide-spreading lateral sepals. Yet the back portion of the labellum is greatly expanded and the callosities are reduced and not upright, approaching the minimal and mounded callosities of *S. romanzoffiana*. Additionally, an herbarium specimen of plants that fall well outside the morphology of *S.* ×*stellata* and which can be confidently identified as *S. romanzoffiana* occur within a few hundred meters of the included hybrid sample. Though the sampled plant of *S.* ×*stellata* is grossly similar to *S. perexilis* in many morphological features, there appear to be a few characters which indicate its hybrid origin (e.g., the highly reduced callosities). Yet these plants illustrate the difficultly in separating potential hybrid individuals/populations from their parental species in the Sierra Nevada.

Nevada Co. This population was originally sampled because it presents a wide array of morphological forms (fig. 8, fig. 11), from plants approaching *S. romanzoffiana* and *S. perexilis*, to plants suspected of being of hybrid origin involving *S. porrifolia*. Flower size, inflorescence spiral tightness, and labellum shape vary, and the leaves range from linear-lanceolate to oblanceolate. Surprisingly, even though they exhibit marked morphological variation, all three individual plants were recovered as hybrids (= *S.* ×*stellata*) between *S. perexilis* and *S. romanzoffiana*. Interestingly, one individual (ss8a) was the only sample of *S.* ×*stellata* in which the different putative copies of nrITS proved valuable in identifying hybrid plants; in all other instances both nrITS copies clustered in the *S. perexilis* clade.

Fresno Co. Of all the plants recovered as *S.* ×*stellata*, this is the only population to occur in a 'natural', non-directly human created or impacted site, with the included sample (ss7) growing in the wet mossy seeps and cracks of a secluded south-facing open granitic slab with glacial polish and erratics. This outcrop also supports a rich microflora of additional native Sierra

Nevada species. The other populations we found to be of hybrid origin occur along road embankments or powerline cuts. Thus although human impacted environments may facilitate hybridization between *S. perexilis* and *S. romanzoffiana*, and may have increased their frequency, *S.* ×*stellata* is not limited to such environments and can be considered to be a naturally occurring phenomenon.

El Dorado Co. El Dorado County contains two sampled individuals of *S.* ×*stellata* from a single population. One of these individuals, sample sst4, was hypothesized by Sheviak (2013, picture p. 22; *Sheviak 2925* plant "a" (NYS)) to be a putative hybrid between *S. romanzoffiana* and *S. perexilis* (as *S. stellata sensu* Brown et al. 2008); thus our data confirms the original hypothesis put forth by Sheviak in this particular instance. This individual has relatively large flowers (to 6.6 mm) in a moderately open spiral, with widely spreading, slightly recurved lateral sepals typical of *S. perexilis*. Both *S. perexilis* and *S. romanzoffiana* are known from El Dorado Co.; herbarium specimens from NYS and RSA, in addition to verified citizen scientist observations on iNaturalist, place *S. romanzoffiana* within 6.5 km, and *S. pereilis* within 3.5 km of the hybrid population. Other un-sampled individuals from the sampled herbarium sheet match the morphology of sst4; it is likely that all individuals in this population are *S.* ×*stellata*.

In the research we present here, two *S.* ×*stellata* plants (ss4, 5) are 2n = 66, and one other (ss2) is 2n = 88 (as determined by Sheviak 2013). Across its vast range *S. romanzoffiana* is composed of 2n = 44 and 88 (a single individual from Trinidad, Humbolt Co., CA –counted by Sheviak (2013) as 2n = 66– may in fact represent an individual of *S.* ×*stellata*, although it closely matches *S. romanzoffiana* in hood development, and labellum surface texture and apex expansion).

Evidence for extremely rare hybridization between S. perexilis and S. porrifolia—We recovered a single individual of *Spiranthes perexilis* × *Spiranthes porrifolia* (sample ss1;*Sheviak 6995*) . This individual has a chromosome count of 2n = 88 (herbarium annotation on the herbarium sheet by Sheviak). This specimen was collected in El Dorado Co., CA, just southwest of Loon Lake and ca. 8 km northeast of the El Dorado population of *S. perexilis* × *S. romanzoffiana*. The plant was originally identified by Sheviak as *S. romanzoffiana* and then annotated to *S. stellata sensu* Brown et al. (2008; =*S.* ×*stellata*). The labellum, which is slightly ovatly subpandurate and displays large callosities, is evocative of *S. perexilis* though the dilation is larger than typically expressed in that species. Similarly, the inflorescence is not very densely spiraled and the lateral sepals are free, though not spreading. Confusingly, the overall appearance of the plant largely resembles *S.* ×*stellata*; only the prominent callosities and non-spreading sepals suggest the parentage of *S. porrifolia* (both parental species and this hybrid individual have papillate labella).

As is the case with *S.* ×*stellata*, the example of *S. perexilis* × *S. porrifolia* exemplifies the challenge in species delimitation between parental species and hybrid populations and individuals in some areas of the California Sierra Nevada. Sheviak (2013) also recognized these difficulties, specifically with the populations from El Dorado County, labeling the situation "both informative and intractable." However our molecular data offer a first step toward a path out of this maze, allowing for the assessment of morphological characters for species delimitation. Additionally, it must be stressed that our preliminary molecular data indicate hybridization events are somewhat infrequent, restricted to the Sierra Nevada of California, specifically the region in-between Yosemite and Sequoia/Kings Canyon National Parks, and the Tahoe and El Dorado National Forest regions. Further population-level genetic and morphological studies are

critical to understanding the full extent of hybridization within the Californian Sierra Nevada. If the hybrid between *S. perexilis* × *S. porrifolia* is found to be more widespread than presented here, it may warrant formal taxonomic recognition.

Concluding remarks—Taxon identification of *Spiranthes* in the California Sierra Nevada and Western Cordillera system of North America has long been a problem for botanists working in the region and on North American orchids. The data were present here helps to clarify the phylogenetic, morphological, and taxonomic position, relationships, standing, and identities of *S. diluvialis*, *S. delitescens*, *S. perexilis*, *S. porrifolia*, *S. romanzoffiana*, *S.* ×*stellata*, and *S. perexilis* × *S. romanzoffiana*. Given the wet meadow, streamside, and cienegas habitats of these species, and the rarity of some species (particularly *S. delitescens* and *S. perexilis*), we hope our work can be used to help construct meaningful conservation strategies for these species in times of global climate change. As indicated by Sheviak (2012; 2013) many sites in the California Sierra Nevada may have already changed such that they no longer present suitable habitat for *Spiranthes*. Additionally, research into the pollinator networks of these orchids is needed, in addition to Next Generation Sequencing facilitated population genetic analyses. Better understanding of these relationships may help clarify patterns of diversity and to construct strategies to ensure their existence into the future.

Abbott, R., D. Albach, S. Ansell, J.W. Arntzen, S.J.E. Baird, N. Bierne, J. Boughman, et al. 2013. Hybridization and speciation. Journal of Evolutionary Biology, 28: 229–246.

Allendorf, F.W., R.F. Leary, P. Spruell, J.K. Wenburg. 2001. The problems with hybrids: setting conservation guidelines. Trends in Ecology and Evolution, 16(11): 613–622.

Arditti, J., and A.K.A. Ghani. Tansley Review No. 110. Numerical and physical properties of orchid seeds and their biological implications. New Phytologist, 145: 367–421.

Arft, A. M., and T. A. Ranker. 1998. Allopolyploid origin and population genetics of the rare orchid *Spiranthes diluvialis*. American Journal of Botany, 85: 110–122.

Jepson Flora Project (eds. B.G. Baldwin, D.J. Keil, S. Markos, B.D. Mishler, R.Patterson, T.J. Rosath, D.H. Wilken). 2015. Jepson eFlora, http://ucjeps.berkeley.edu/IJM.html [accessed on May 6, 2015]

Brown, P.M., L.A. Dueck, and K.M. Cameron. 2008. *Spiranthes stellata* (Orchidaceae), a new species of ladies'-tresses from the western United States. North American Native Orchid Journal, 14(1): 3-11.

Bullini, L., R. Cianchi, P. Arduino, L. De Bonis, M.C. Mosco, A. Verardi, D. Porretta, B. Corrias, W. Rossi. 2001. Molecular evidence for allopolyploid speciation and a single origin of the western Mediterranean orchid *Dactylorhiza insularis* (Orchidaceae). Biological Journal of the Linnean Society, 72: 193–201.

Catling, P.M. 1982. Breeding systems of northeastern North American *Spiranthes* (Orchidaceae). Canadian Journal of Botany, 60(12): 3017–3039.

Catling, P.M. 1983. Pollination of northeastern North American *Spiranthes* (Orchidaceae). Canadian Journal of Botany, 61: 1080–1093.

Catling, P.M. 2014. Pollination of Great Plains Ladies'-tresses (*Spiranthes magnicamporum*) near Ottawa, Ontario. The Native Orchid Conference Journal, 11(2): 8–10.

Catling, P.M., and J.R. Brown. 1983. Morphometrics and ecological isolation in sympatric *Spiranthes* (Orchidaceae) in southwestern Ontario. Canadian Journal of Botany, 61: 2747–2759.

Cole, K.L., J.F. Fisher, K. Ironside, J.I. Mead, and P. Koehler. 2013. The biogeographic histories of *Pinus edulis* and *Pinus monophylla* over the last 50,000 years. Quaternary International, 310: 96–110.

Coleman, R.A. 1995. The Wild Orchids of California. Comstock Publishing Associates (Cornell University Press), Ithaca, N.Y. & London.

Coleman, R.A., D.H. Wilken, and W.F. Jennings. 2014. *Spiranthes*. *In* Jepson Flora Project (eds.) Jepson eFlora, http://ucjeps.berkeley.edu/cgi-bin/get_IJM.pl?tid=10976, accessed on February 06, 2015

Cornish-Bowden, A. 1985. Nomenclature for incompletely specified bases in nucleic acid sequences: recommendations 1984. Nucleic Acids Research, 13(9): 3021–3030.

Correll, D.S. 1950. Native Orchids of North American north of Mexico. Stanford University Press, Standford, USA.

Cozzolino, S., and A. Widmer. 2005. Orchid diversity: an evolutionary consequence of deception? Trends in Ecology and Evolution, 20(9): 487–494.

Cozzolino, S., A.M. Nardella, S. Impagliazzo, A. Widmer, and C. Lexer. 2006. Hybridization and conservation of Mediterranean orchids: Should we protect the orchid hybrids or the orchid hybrid zones? Biological Conservation, 129(1): 14-23.

Davis, O.K. 1998. Palynological ecidence for vegetation cycles in a 1.5 million year pollen record from the Great Salt Lake, Utah, USA. Palaeogeography, Palaeoclimatology, Palaeoecology, 138: 175–185.

De Hert, K., H. Jacquemyn, S. Van Glabeke, I. Roldán-Ruiz, K. Vandepitte, L. Leus, and O. Honnay. 2011. Patterns of hybridization between diploid and derived allotetraploid species of *Dactylorhiza* (Orchidaceae) co-occurring in Belgium. American Journal of Botany, 98(6): 946–955.

Devey, D.S., R.M. Bateman, M.F. Fay, and J.A. Hawkins. 2008. Friends or relatives? Phylogenetics and species delimitation in the controversial European orchid genus *Ophrys*. Annals of Botany, 101(3): 385–402.

Dressler, R.L., and F. Pupulin. 2014. Two new white-flowered species of *Sobralia* (Orchidaceae) from Costa Rica. Harvard Papers in Botany, 19(1): 117–122.

Dueck, L.A., and K.M. Cameron. 2007. Sequencing re-defines *Spiranthes* relationships with implications for rare and endangered taxa. Lankesteriana, 7(1-2): 190–195.

Dueck, L.A., and K.M. Cameron. 2008a. Molecular evidence on the species status and phylogenetic relationships of *Spiranthes parksii*, an endangered orchid from Texas. Conservation Genetics, 9:1617–1631.

Dueck, L.A., and K.M. Cameron. 2008b. Molecular evidence for new species – *Spiranthes stellata*. North American Orchid Journal, 14(1): 12–21.

Dueck, L.A., D. Aygoren, and K.M. Cameron. 2014. A molecular framework for understanding the phylogeny of *Spiranthes* (Orchidaceae), a cosmopolitan genus with a North American center of diversity. American Journal of Botany, 101(9): 1551–1571.

Dyke, A.S., A. Moore, and L. Robertonson. 2003. Deglaciation of North America, Open file 1574. Geological Survey of Canada.

Fraser-Jenkins, C.R. 2001. *Dryopteris stanley-walkeri* Fras.-Jenk., the missing diploid common-ancestor of *D. carthusiana* and *D. cristata*. *In* Plant Diversity of the Himalaya. Eds. P.C. Pande, and S.S. Samant. Nainital, India: Gyanodaya Prakashan; 119–152.

Godfery, M.J. 1933. Monograph and iconograph of native British Orchidaceae. Cambridge University Press, Cambridge, UK.

Goldman, D.H., RK. Jansen, C. van den Berg, L.J. Leitch, M.F. Fay, and M.W. Chase. 2004. Molecular and cytological examination of *Calopogon* (Orchidaceae, Epidendroideae): circumscription, phylogeny, polyploidy, and possible hybrid speciation. American Journal of Botany, 91(5): 707–723.

Górniak, M., D.L. Szlachetko, A.K. Kowalkowska, J. Bohdanowicz, and C.X. Canh. 2014. Taxonomic placement of *Paphiopedilum canhii* (Cypripedioideae; Orchidaceae) based on cytological, molecular and micromorphological evidence. Molecular Phylogenetics and Evolution, 70: 429–441.

Govaerts, R., P. Bernet, K. Kratochvil, G. Gerlach, G. Carr, P. Alrich, A.M.Pridgeon, J. Pfahl, M.A. Campacci, D. Holland Baptista, H. Tigges, J. Shaw, P. Cribb, A. George, K. Kreuz, J. Wood. 2014. World Checklist of Orchidaceae. Facilitated by the Royal Botanic Gardens, Kew. Published on the Internet; http://apps.kew.org/wcsp/ Retrieved 2014-12-12.

Gravendeel, B., A. Smithson, F.J.W Silk, and A. Schuiteman. 2004. Epiphytism and pollinator specialization: drivers for orchid diversity? Philosophical Transactions of the Royal Society London B, 359: 1523–1535.

Gulyas, G., G. Sramko, A. Molnar, S. Rudnoy, Z. Illyes, T. Balazs, and Z. Bratek. 2005. Nuclear ribosomal DNA its paralogs as evidence of recent interspecific hybridization in the genus *Ophrys* (Orchidaceae). Acta Biologica Cracoviensia Series Botanica, 47(2): 61–67.

Hagen, M., M. Wikelski, W.D. Kissling. 2011. Space Use of Bumblebees (*Bombus* spp.) Revealed by Radio-Tracking. PLoS ONE 6(5): e19997. doi:10.1371/journal.pone.0019997

Halbinger, F. & M.A. Soto. 1997. *Laelias* of Mexico. *Orquídea (Mexico City)*, n.s. 15: 1–160.

Hedren, M. 1996. Genetic differentiation, polyploidization and hybridization in northern European *Dactylorhiza* (Orchidaceae): Evidence from allozyme markers. Plant Systematics and Evolution, 201(1–4): 31–55.

Holmgren, C. A., J.L. Betancourt, and K.A. Rylander. 2010. A long-term vegetation history of the Mojave–Colorado Desert ecotone at Joshua Tree National Park. Journal of Quaternary Science, 25: 222–236.

Jacquemyn, H., R. Brys, O. Honnay, I. Roldán-Ruiz, B. Lievens, and T. Wiegand. 2012. Nonrandom spatial structuring of orchids in a hybrid zone of three *Orchis* species. New Phytologist, 193: 454–464.

Jersáková, J., and T. Malinová. 2007. Spatial aspects of seed dispersal and seedling recruitment in orchids. New Phytologist, 176(2): 237–241.

Jiménez-Moreno, G., R.S. Anderson, and P.J. Fawcett. 2007. Orbital- and millennial-scale vegetation and climate changes of the past 225 ka from Bear Lake, Utah–Idaho (USA). Quaternary Science Reviews, 26: 1713–1724.

Kainulainen, K., and B. Bremer. 2014. Phylogeny of *Euclinia* and allied genera of Gardenieae (Rubiaceae), and description of *Melanoxerus*, an endemic genus of Madagascar. Taxon, 63(4): 819–830.

Kislev, M.E. 1980. *Triticum parvicoccum* sp. nov., the oldest naked wheat. Israel Journal of Botany, 28: 95–107.

Kraus, F.B., S. Wolf, and R.F.A. Moritz. 2009. Male flight distance and population substructure in the bumblebee *Bombus terrestris*. Journal of Animal Ecology, 78(1): 247–252.

Lee, C.S., S. Kim, S.H. Yeau, and N.S. Lee. 2012. Nuclear and cpDNA Sequences Demonstrate Spontaneous Hybridization between *Goodyera schlechtendaliana* Rchb. f. and *G. velutina* Maxim. (Orchidaceae) in Jeju Island, Korea. Systematic Botany, 37(2): 356–364.

López-Pujol, J., N. Garcia-Jacas, A. Susanna, R. Vilatersana. 2012. Should we conserve pure species or hybrid species? Delimiting hybridization and introgression in the Iberian endemic *Centaurea podospermifolia*. Biological Conservation, 152: 271–279.

Luca, A., F. Bellusci, B. Menale, A. Musacchio, and G. Pellegrino. 2012. *Orchis* × *colemanii* hybridization: Molecular and morphological evidence, seed set success, and evolutionary importance. Flora, 207(10): 753–761.

Marques, I., D. Draper, L. Riofrio, and C. Naranjo. 2014. Multiple hybridization events, polyploidy and low postmating isolation entangle the evolution of neotropical species of *Epidendrum* (Orchidaceae). BMC Evolutionary Biology, 14(20), 10.1186/1471-2148-14-20.

Moraes, A.P., M. Chinaglia, C. Palma-Silva, and F. Pinheiro. 2013. Interploidy hybridization in sympatric zones: the formation of *Epidendrum fulgens* × *E. puniceoluteum* hybrids (Epidendroideae, Orchidaceae). Ecology and Evolution, 3(11): 3824–3837.

Nolte, A.W., and D. Tautz. 2010. Understanding the onset of hybrid speciation. Trends in Genetics, 26(2): 54–58.

Paul, J., C. Budd, and J.R. Freeland. 2013. Conservation genetics of an endangered orchid in eastern Canada. Conservation Genetics, 14: 195–204.

Peakall, R., D. Ebert, J. Poldy, R.A. Barrow, W. Francke, C.C. Bower, and F. P. Schiestl. 2010. Pollinator specificity, floral odour chemistry and the phylogeny of Australian sexually deceptive *Chiloglottis* orchids: implications for pollinator-driven speciation. New Phytologist, 18(2): 437–450.

Pedersen, H.Æ. 2006. Systematics and evolution of the *Dactylorhiza romana*/*sambucina* polyploid complex (Orchidaceae). Botanical Journal of the Linnean Society, 152: 405–434.

Pillon, Y., M.F. Fay, M. Hedren, R.M. Bateman, D.S. Devey, A.B. Shipunov, M. van der Bank and M.W. Chase. 2007. Evolution and temporal diversification of western European polyploid species complexes in *Dactylorhiza* (Orchidaceae). Taxon, 56(4): 1185–1208.

Pinheiro, F., D. De Barros, C. Palma-Silva, D. Meyer, M.F. Fay, R.M. Suzuki, C. Lexer, and S. Cozzolino. 2010. Hybridization and introgression across different ploidy levels in the Neotropical orchids *Epidendrum fulgens* and *E. puniceoluteum* (Orchidaceae). Molecular Ecology, 19: 3981–3994.

Rambaut. A. 2014. FigTree v1.4.2, released 2014-07-09. http://tree.bio.ed.ac.uk/software/figtree/

Russell, A., R. Sameul, V. Klejna, M.H.J. Barfuss, B. Rupp, and M.W. Chase. 2010. Reticulate evolution in diploid and tetraploid species of *Polystachya* (Orchidaceae) as shown by plastid DNA sequences and low-copy nuclear genes. Annals of Botany, 106: 37–56.

Salamini, F., H. Özkan, A. Brandolini, R. Schäfer-Pregl, and W. Martin. 2002. Genetics and geography of wild cereal domestication in the near east. Nature Reviews: Genetics, 3(6): 429–441.

Salazar, G.A., R. Jiménez-Machorro H.M. Huerta, and E. Hágsater. 2014. A new species and a new natural hybrid of *Laelia* (Orchidaceae) from Oaxaca, Mexico. Phytotaxa, 178(1): 161–170.

Sanders, K.L., A.R. Rasmussen, and M.L. Guinea. 2014. High rates of hybridisation reveal fragile reproductive barriers between endangered Australian sea snakes. Biological Conservation, 171: 200–208.

Sheviak, C.J. 1982. Biosystematic study of the *Spiranthes cernua* complex. Bulletin of the New York State Museum Science Service, Bulletin Number 448.

Sheviak, C.J. 1984. *Spiranthes diluvialis* (Orchidaceae), a new *Spiranthes* from the Western United States. Brittonia, 36(1): 8–14.

Sheviak, C.J. 1989. A new *Spiranthes* (Orchidaceae) from Ash Meadows, Nevada. Rhodora, 91(867): 225–234.

Sheviak, C.J. 1990. A new *Spiranthes* (Orhidaceae) from the cienegas of southernmost Arizona. Rhodora, 92(872): 215–230.

Sheviak, C.J. 1991. Morphological variation in the compliospecies *Spiranthes cernua* (L.) Rich.: ecologically-limited effects of gene flow. Lindleyana, 6(4): 228–234.

Sheviak, C.J. 2012. A new and rare *Spiranthes* from the Sierra Nevada. The Native Orchid Conference Journal, 9(4): 1–6.

Sheviak, C.J. 2013. A ctyogeographic survey of *Spiranthes romanzoffiana* Cham. and its taxonomic implications. The Native Orchid Conference Journal, 10(2): 1–31.

Sheviak, C.J., and P.M. Brown. 2002. *Spiranthes*. In *Flora of North America*, Volume 26. Oxford University Press, USA.

Sheviak, C.J., and P.M. Catling. 1980. The identity and status of *Spiranthes ochroleuca* (Rydberg) Rydberg. Rhodora, 82(832): 525–562.

Sipes, S.D., and V.J. Tepedino. 1995. Reproductive biology of the rare orchid, *Spiranthes diluvialis*: breeding system, pollination, and implications for conservation. Conservation Biology, 9: 929–938.

Silvera, K., L.S. Santiago, J.C. Cushman, K. Winter. 2009. Crassulacean Acid Metabolism and Epiphytism Linked to Adaptive Radiations in the Orchidaceae. Plant Physiology, 149(4): 1838–1847.

Soliva, M., and A. Widmer. 2003. Gene flow across species boundaries in sympatric, sexually deceptive *Ophrys* (Orchidaceae) species. Evolution, 57(10): 2252–2261.

Soltis, P.S., and D.E. Soltis. 2009. The Role of hybridization in plant speciation. Annual Review of Plant Biology, 60: 561–588.

Stein, D.B., C. Hutton, D.S. Conant, C.H. Haufler, and C.R. Werth. 2010. Reconstructing *Dryopteris* "*semicristata*" (Dryopteridaceae): Molecular profiles of tetraploids verify their undiscovered diploid ancestor. American Journal of Botany, 97(6): 998-1004.

Stokl, J., P.M. Schluter, T.F. Stuessy, H.F. Paulus, R. Fraberger, D. Erdmann, C. Schulz, et al. 2009. Speciation in sexually deceptive orchids: pollinator-driven selection maintains discrete odour phenotypes in hybridizing species. Biological Journal of the Linnean Society, 98(2): 439–451.

Stronen, A.V., and P.C. Paquet. 2013. Perspectives on the conservation of wild hybrids. Biological Conservation, 167: 390–395.

Sun, M. 1996. The Allopolyploid Origin of *Spiranthes hongkongensis* (Orchidaceae). American Journal of Botany, 83(2): 252–260.

Szalanski, A.L., G. Steinauer, R. Bischof, and J. Petersen. 2001. Origin and Conservation Genetics of the Threatened Ute Ladies'-Tresses, *Spiranthes diluvialis* (Orchidaceae). American Journal of Botany, 8(1): 177–180.

Vanden Broeck, A., W. Van Landuyt, K. Cox, L. De Bruyn, R. Gyselings, G. Oostermeijer, B. Valentin, G. Bozic, B. Dolinar, Z. Illyés, and J. Mergeay. 2014. High levels of effective long-distance dispersal may blur ecotypic divergence in a rare terrestrial orchid. BMC Ecology, 14(20): doi:10.1186/1472-6785-14-20.

Vega, Y., I. Marques, S. Castro, and J. Loureiro. 2013. Outcomes of Extensive Hybridization and Introgression in *Epidendrum* (Orchidaceae): Can We Rely on Species Boundaries? PLoS ONE 8(11): e80662. doi:10.1371/journal.pone.0080662

Vereecken, N.J., S. Cozzolino, and F.P. Schiestl. 2010. Hybrid floral scent novelty drives pollinator shift in sexually deceptive orchids. Evolutionary Biology, 10: 103.

Wallace, L.E. 2003. Molecular evidence for allopolyploid speciation and recurrent origins in *Platanthera huronensis* (Orchidaceae). International Journal of Plant Science, 164: 907–916.

Wallace, L.E. 2006. Spatial genetic structure and frequency of interspecific hybridization in *Platanthera aquilonis* and *P. dilatata* (Orchidaceae) occurring in sympatry. American Journal of Botany, 93(7): 1001–1009.

Walters, C. 2005. Genetic relationships among *Spiranthes parksii* and congeneric species. Master's Thesis. Texas A&M University

Walther-Hellwig, K., and R. Frankl. 2000. Foraging distances of *Bombus muscorum*, *Bombus lapidarius*, and *Bombus terrestris* (Hymenoptera, Apidae). Journal of Insect Behavior, 13(2): 239–246.

Werth, C.R., and D.B. Lellinger. 1992a. Genomically preserved plants and their nomenclature. Taxon, 41(3): 513–521.

Werth, C.R., and D.B. Lellinger. 1992b. (146–149) Four proposals to amend the code to provide for naming genomically preserved plants. Taxon, 41(3): 597–598.

Whittenmore, A.T., and R.T. Olsen. 2011. *Ulmus americana* (Ulmaceae) is a polyploid complex. American Journal of Botany, 98(4): 754–760.

Widmer A., and M. Baltisberger. 1999. Molecular evidence for allopolyploid speciation and a single origin of the narrow endemic *Draba ladina* (Brassicaceae). American Journal of Botany, 86: 1282–1289.

Wolf, S., and R.F.A. Moritz. 2008. Foraging distance in *Bombus terrestris* L. (Hymenoptera : Apidae). Apidologie, 39(4):419–427.

Fig. 1. [legend for following two pages] Sample amplification data matrix for molecular phylogenetic samples. An ‘X’ indicates successful amplification.



ID #	Species/Variety	Collection Site	Collector	Coll.Date	ACO	ITS	matK	ndhj	trnLF	trnSM	Xdh	ycf1 3'	Herbarium specimen	Photo Voucher
	S. delitescens													
5a	S. delitescens	Santa Cruz Co., AZ	Aaron Hicks	5/2005	x	x	x	x	x	x	x	x	in hort.	
5b	S. delitescens	Santa Cruz Co., AZ	Aaron Hicks	5/2005	x	x	x	x	x	x	x	x	in hort.	
5c	S. delitescens	Santa Cruz Co., AZ	Aaron Hicks	5/2005	x	x	x	x	x	x	x	x	in hort.	
	S. diluvialis													
6a1	S. diluvialis (SD1)	NE	R.Bischof/NGPC (DNA)	<2001		x	x	x	x	x		x	NEB	
sd1	S. diluvialis	Fort Hall Indian Reservation, Bannock Co., ID	C.J. Sheviak	8/10/2009	x	x	x	x	x	x		x	C.J. Sheviak 7087 (NYS)	
sd2	S. diluvialis	Chelan Co., WA	Kathryn A. Beck	8/3/2000		x	x	x	x	x		x	K.A Beck 200025 (NYS)	
	S. infernalis													
9a	S. infernalis	Nye Co., NV	Carol Siegel/Jody Fraser	6/19/04	x	x	x		x	x	x	x	80511*	yes
9b	S. infernalis	Nye Co.,NV	Carol Siegel/Jody Fraser	6/19/04	x	x	x		x	x	x	x	82229	yes
9c	S. infernalis	Nye Co.,NV	Carol Siegel/Jody Fraser	6/19/04	x	x	x		x	x	x	x		yes
	S. magnicamporum													
15c	S. magnicamporum	Ozark Co., MO	Bill Summers	10/18/04		x (poor)	x	x	x	x	x		80501	
15e	S. magnicamporum	Grimes Co., TX	Joe Liggio	10/31/04	x	x	x	x	x	x			80489	
15f	S. magnicamporum - (sm)	Walworth Co., WI	Jeff Hapeman	9/16/06	x	x	x	x	x	x	x	x	82207	
15g	S. magnicamporum - (lg)	Walworth Co., WI	Jeff Hapeman	9/16/06	x	x	-	x	x	x	x	x		
sm7h	S.magnicamporum	NM	M.C.Pace	9/21/13	x	x	x	x	x	x	x	x		
sm11a	S.magnicamporum	Russell Co., VA		10/17/13	x	x	x	x	x	x	x	x		
sm12a	S.magnicamporum	GA	Jim Fowler	11/3/13	x	x	x	x	x	x	x	x		yes
sm15a	S.magnicamporum	Marthaville, Lambton Co, Ontario, Canada	Oldham	9/7/11	x	x	x	x	x	x	x	x	Oldham 39307 (NYS)	
sm16a	S.magnicamporum	Black Belt Prairie, Starkville, MS	McCabe	10/29/91		x	x		x	x			McCabe s.n. (NYS)	
sm17a	S.magnicamporum	Cowan, Franklin co., TN	Sheviak	11/8/06	x	x		x	x	x	x	x	Sheviak 7064 (NYS)	
sm19a	S.magnicamporum	Chandlerville, Cass Co., IL	Sheviak	9/25/81	x	x	x		x	x		x	Sheviak 2142 (NYS)	
	S. porrifolia													
21d	S.porrifolia	Curry Co. (Oak Flat), OR	Veva Stansell	6/18/05		fungus	x (1-4)		x	x			USFS-OR	yes
21f	S.porrifolia	Butte Co.(Lk. Oroville), CA	Lucy Dueck	6/14/06		fungus	x-f2		x	x			82215	yes
21j	S.porrifolia	Sierra Co. (Dn'vl., mm17.86), CA	Lucy Dueck	6/15/06		x	x		x	x	x	x	82214	yes
21m	S.porrifolia	Sierra Co. (Dn'vl., mm10.37), CA	Lucy Dueck	6/15/06		x	x		x	x	x	x		yes
21n	S. porrifolia	Curry Co. (Agness), OR	Mike Parsons	~6/14/06		x	x		x	x	x	x		yes
21o	S. porrifolia	Sierra Co., (Downieville), CA	Mike Parsons	~6/18/06		x	x		x	x	x	x		yes
21q	S. porrifolia	Marin Co., CA	Brad Kelley	6/11/06		bad-dbl	x (14)		x	x	x	x		yes
21r	S. porrifolia	Curry Co., OR (Lobster Crk.)	Don Heller/Veva Stansell	6/26/06		fungus	x		x	x				
21s	S. porrifolia	Sierra Co. (Dn'vl., mm17.86), CA	Lucy Dueck	6/15/06		x	x		x	x	x	x	(presrvd)	yes
21t	S. porrifolia	Josephine Co., OR	William Gray	7/1/06		dbl	x (14)		x	x		x	82223	
21u	S. porrifolia	San Diego Co., CA	Nick Crawford	7/12/06		x	x		x	x	x	x	SD180698	yes
21x	S. porrifolia	Douglas Co., OR	Hope Stubbs	Aug-06		fungus	x (14)		x	x		x		yes
	S. roamanzoffiana													
24a	S. romanzoffiana(SR3)	WY	R.Bischof/NGPC (DNA)	≤2001		x		x	x	x		x		
24b	S. romanzoffiana	Presque Isle Co., MI	Fred Case	8/16/04		x	x	x	x	x	x	x		
24d	S. romanzoffiana	Fairbanks, ALASKA	Jason Grant/KCameron	7/0/00	x	x	x	x	x	x	x	x		
24e	S. romanzoffiana	Bunchgrass Co., WA	R. Bursik/U.ID. Herb.	7/26/87		x	x	x	x	x	x	x	ID-100680	yes
24f	S. romanzoffiana	Deschutes Co., OR	R&J Mastrogiuseppe/UID	7/6/92		x	x	x	x	x	x	x	ID-124784	yes
24j	S. romanzoffiana	Ravalli Co., Mont.	R. Bursik/U.ID. Herb.	8/4/88		x	x	x	x	x	x	x	ID-132379	yes

24m	S. romanzoffiana	Whiteboro, NY	Charles Ufford	8/26/05		x	x	x	x	x	x	x		
24n	S. romanzoffiana	Monterey Co., CA	Brad Kelley	6/30/06		x	x	x	x	x	x	x		yes
24o	S. romanzoffiana	Marinette Co., WI (Spur Lake)	Kip Knudson	7/24/06		x	x	x	x	x	x	x		yes
24pq	S. romanzoffiana	Marin Co., CA (Pt. Reyes)	Brad Kelley	7/30/06		x	x	x	xx	xx		x		yes
24r	S. romanzoffiana - Sr85	Scotland-Isle of Colonsay (southern)	R.Gulliver/A.Forrest-RBGE	2001	x	x	x		x	x	x	x	E-AM001143	
24s	S. romanzoffiana - Sr154	Scotland-Isle of Coll (northern)	E.Grant/A.Forrest-RBGE	2001		x	x	x	x	x	x	x	E-AM001212	
24t	S. romanzoffiana	Lane Co., OR (Patterson Mtn.)	Tanya Harvey	9/4/06		x	x	x	x	x	x			yes
24u	S. romanzoffiana (was PxR23)	Lane Co., OR (Patterson Mtn.)	Tanya Harvey	9/4/06		x	x	x	x	x	x	x		
24w	S. romanzoffiana	Unalaska Is. (Aleutians), AK	Robert Lauri	7/24/06	x	x	x		x	x	x	x		
sr2	S. romanzoffiana	Manitoba, Canada	C.J. Cheviak	7/15/03		x				x			Sheviak 6947 (NYS)	
sr3	S. romanzoffiana	Rio Arribo Co., NM	C.J. Cheviak	8/10/97		x				x			Sheviak 6089 (NYS)	
sr4	S. romanzoffiana	Mt. Graham, Grahama Co., AZ	C.J. Cheviak	8/5/84		x				x			Sheviak 2642 (NYS)	
sp1	S. romanzoffiana (as porriflia)	Craig Mountain, Nez Perce Co., ID	Karen L. Gray	Aug-98		x	x		x	x		x	K. Gray 1518 (NYS)	
	S.perexilis													
31a	S. stellata (PxR1)	Douglas Co., OR	Hope Stubbs	8/12/06		x	x	x	x	x	x	x		yes
31e	S. stellata (PxR7)	Lane Co., OR (Happy Lakes)	Tanya Harvey	9/7/06		x	x	x	x	x	x	x		yes
31f	S. stellata (21y = PxR9)	Tulare Co., CA	Sarah DeGroot (viaRobL)	7/25/06		x	x		x	x		x		yes
sst3	S. stellata (cf. perexilis)	Crater Lake NP, Klamath Co., OR	Peter F. Zika	8/7/94		x	x		x	x		x	Zika 15260 (NYS)	
sst6	S. stellata (open spiral)	Nobe Young Meadow, Tulare Co., CA	J. Stewart	7/25/07		x	x		x	x			Stewart 2690 (RSA)	
	S. xstellata													
sst1	S. stellata (stellata x porrifolia)	El Dorado Co., CA	Sheviak	0 0 2009		x	x		x	x	x	x	Sheviak 7084	
sst2	S. stellata (stellata x rom)	Tom's Place, Inyo Co. CA	Sheviak	8/5/04		x	x		x	x	x	x	Sheviak 7000	
sst4	S. stellata (stellata x rom)	Union Valley Reservoir A, El Dorado Co., CA	C.J Sheviak	6/30/87		x	x		x	x			Sheviak 2925a (NYS)	
sst5	S. stellata (stellata x rom)	Union Valley Reservoir D, El Dorado Co., CA	C.J Sheviak	6/30/87		x	x		x	x			Sheviak 2925d (NYS)	
sst7	S. stellata (stellata x rom)	Granite seeps, Fresno Co., CA	M. Honer	9/1/06		x	x		x	x			Honer 2414 (RSA)	
sst8a	S. stellata (stellata x rom) wide leaves)	Nevada Co., CA	L. Ahart	7/23/05		x	x		x	x			Ahart 12166 (RSA) a	
sst8b	S. stellata (stellata x rom) (linear leaves)	Nevada Co., CA	L. Ahart	7/23/05		x	x		x	x			Ahart 12166 (RSA) b	
sst8c	S. stellata (stellata x rom) (linear leaves)	Nevada Co., CA	L. Ahart	7/23/05		x	x		x	x			Ahart 12166 (RSA) c	
	S. vernalis													
27b	S. vernalis	Levy, FL	Paul Martin Brown	5/7/03		x	x		x	x		x		
27e	S. vernalis	Ritchie Co., WV	Scott Shriver	8?/17/03		x	x		x	x	x			
27g	S. vernalis	Levy, FL	Jim Fowler	4/2003		x	x		x	x		x		
27h	S. vernalis	Aiken Co., SC (w.SREL pkg.lot)	Lucy Dueck	5/28/03		x	x		x	x		x	80509	yes
27k	S. vervalis	Alachua Co., FL	Scott Stewart	5/20/05		x	x		x	x	x	x		
27m	S. vernalis	Bradford Co., FL	Scott Stewart	5/25/05	x	x	x		x	x	x	x		
27n	S. vernalis	Jefferson Co., TX	Joe Liggio	5/28/04		x	x		x	x		x	80952	
27o	S. vernalis	Jasper Co., TX	Joe Liggio	5/23/04		bad - dbl.	x (14)		x	x	x		80486	
	Outgroup taxa													
Sala	Sacoila lanceolata var. lanc.	Levy Co., FL	Scott Stewart	6/1/04	x	x	x		x	x		x		
Dici	Dichromanthus cinnabara		G. Salazar		x	x	x	x	x	x	x	x		
Mepo	Mesadenella polyantha		G. Salazar		x	x	x		x	x	x	x		
Dedi	Deiregyne diaphana		G. Salazar			x	x		x	x	x	x		
Scll	Schiedeella llaveana		G. Salazar			x	x		x		x	x		

Fig. 2. [legend for following chart] Samples used for morphometric analysis. Samples with double letter IDs (e.g. ba, bb), correspond to the figure numbers in Sheviak 1989, figure 2, page 230. "*stellata*" = *S*. *stellata* subsp. *stellata* (= *S*. x*stellata*), "stro" = S. *perexilis* x S. *romanzoffiana*; "*porrifolia*" = *S*. *porrifolia*; "px" = *S*. *perexilis*, "romanzoffiana" = *S*. *romanzoffiana*; "stpor" = *S*. *perexilis* x *S*. *porrifolia*. Measurements are in mm.

ID	taxon	Pace ID	labellum texture	labellum length	labellum widdest	labellum narrowist	dialaed tip width	dialaed tip length	distace from claw to expansion	calli length
lecto	stellata	stro	pap	7.7	2.9	1.9	2.4	2	5.7	
type	stellata	px	pap	4.8	1.9	0.9	1.6	1.1	3.7	
ss1	stpor	stpor	pap	8.2	3.8	2.2	2.8	2.3	5.8	1.2
ss2	stro	stro	glab	7.8	3.2	1.8	2.5	1.4	6.4	0.6
ss6	stellata	px	glab	6.2	2.3	0.7	1.3	1.2	5	0.3
ss7	stro	stro	glab	6.9	2.5	1	2	1.4	5.6	0.4
ss8a	stro	stro	glab	7.1	4.2	1.3	2.5	1.3	5.7	0.6
ss8b	stro	stro	glab	6.6	3.7	1.6	2.8	1.8	4.8	0.6
ss8c	stro	stro	glab	7	5	1.2	1.9	2.2	4.8	0.3
Sst2	stellata	px	pap	7.1	2.2	0.9	1.7	1.8	5.4	0.6
Sst3	stro	stro	glab	7.2	3.5	1.2	1.6	1.4	5.9	0.6
Sst4	porrifolia	porrifolia	pap	6.8	2.8	1.1	1.7	1.8	4.8	0.4
Sst5	stro	stro	glab	5.7	4.6	1.9	2.9	1.5	4.3	0.4
Sst6	porrifolia	porrifolia	glab	8.2	3.7	1.7	1.5	2.1	6	1
Sst7	stellata	px	glab	9	3.8	1	1.9	1.9	7.2	0.5
Spo1	porrifolia	porrifolia	pap	5	1.4	1	1.2	1.5	3.6	0.6
Spo3	porrifolia	porrifolia	pap	7.2	4.5	1.3	1.4	1.9	5.3	0.5
Spo4	porrifolia	porrifolia	pap	7.3	2	1.7	1.8	1.3	5.9	0.6
Spo5	porrifolia	porrifolia	pap	7.6	2.2	1.2	1.4	2.1	5.5	0.6
Spo6	porrifolia	porrifolia	pap	7.6	3.4	1.7	1.9	1.4	6.2	0.9
Spo7	porrifolia	porrifolia	pap	5.9	2	1	1.2	1.5	4.2	0.5
ba	porrifolia	porrifolia	pap	8.2	2.9	1.1	1.7	1.8	6.2	0.8
bb	porrifolia	porrifolia	pap	9.4	3.5	1.6	1.7	2.1	7	1.1
bc	porrifolia	porrifolia	pap	10	4.2	1.7	1.5	2.1	7.9	0.6
bd	porrifolia	porrifolia	pap	9	3.2	1.6	1.5	2.5	1.8	0.9
be	porrifolia	porrifolia	pap	10.3	4.2	2.5	2.8	3.1	7.1	1
bf	porrifolia	porrifolia	pap	7.4	3.4	1.6	1.7	1.8	5.6	0.4
bg	porrifolia	porrifolia	pap	7.2	3.3	1.2	1.6	1.6	5.4	0.9
da	porrifolia	porrifolia	pap	8	3.2	1.6	1.5	2	5.9	0.5
db	porrifolia	porrifolia	pap	8.5	4.5	2.4	2	1.9	6.4	0.8
dc	porrifolia	porrifolia	pap	8	3.9	1.8	1.8	2.5	6.3	0.8
dd	porrifolia	porrifolia	pap	8.4	4.5	1.4	1.5	2.3	6.2	0.7
de	porrifolia	porrifolia	pap	9.1	3.3	1.5	1.4	2.3	6.6	0.7
df	porrifolia	porrifolia	pap	7	3.7	1.5	1.7	1.8	5.4	0.8
dg	porrifolia	porrifolia	pap	8.6	3.3	1.3	1.6	1.9	6.4	0.7
ea	porrifolia	porrifolia	pap	7.1	2.8	1.2	1.5	1.6	5.3	0.6
eb	porrifolia	porrifolia	pap	7.1	3.1	1.8	2.1	1.9	5.3	0.8
ec	porrifolia	porrifolia	pap	8.8	2.8	1.1	1.2	2.2	6.5	1.2
ed	porrifolia	porrifolia	pap	9	3.9	1.8	1.5	3	5.7	1
ee	porrifolia	porrifolia	pap	7.9	3.6	1.5	1.7	2.6	5.4	0.6
eg	porrifolia	porrifolia	pap	9.6	3.7	1.5	1.7	1.9	5.1	1.2
sro1	romanzoffiana	romanzoffiana	glab	7.5	3.1	1.5	3.2	2.3	5.1	0.5
sro2	romanzoffiana	romanzoffiana	glab	7.7	4.2	2.1	3.5	2.1	5.7	0.5
sro3	romanzoffiana	romanzoffiana	glab	7.1	4.1	1.7	2.9	2	4.9	0.3
ca	porrifolia	porrifolia	pap	7.3	3.4	1.1	1.8	2	5.2	0.8
cb	porrifolia	stpor	pap	7.6	3.8	2	2.1	2.2	5.4	0.9
cc	porrifolia	porrifolia	pap	6.5	3.2	1.6	1.8	1.9	4.3	0.7
cd	porrifolia	porrifolia	pap	7.7	4	2.2	2.3	2.1	5.5	1.1
ce	porrifolia	porrifolia	pap	6.8	3.6	1.7	1.7	1.9	4.7	1
cf	porrifolia	stpor	pap	7.8	3.7	2.2	2.5	2.6	5	0.9

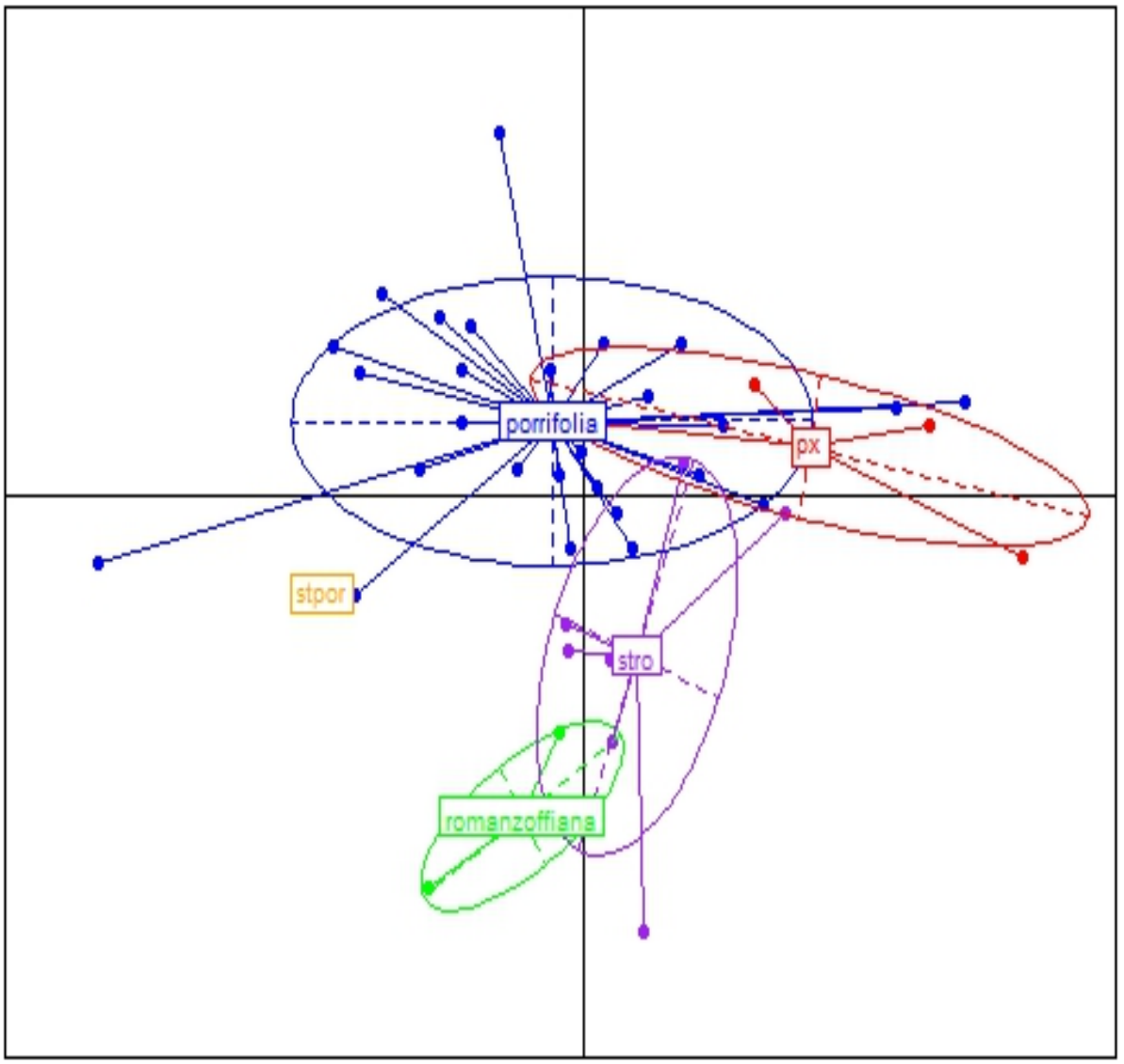


Fig. 3. Scatterplot visualization of the first two components of the labellum PCA. . “stro” = S. *perexilis* x *S. romanzoffiana* (purple); “*porrifolia*“ = *S*. *porrifolia* (blue); “px” = *S*. *perexilis* (red), “romanzoffiana” = *S*. *romanzoffiana* (green); “stpor” = *S*. *perexilis* x *S*. *porrifolia*; orange*)*

Fig. 4. [legend for following page] Bayesian Majority Rule phylogenetic reconstruction of combined plastid + nuclear data (*ACO*, nrITS, *matK*, *ndh*J, *trn*L-F, *trn*S-M, *Xdh, ycf*1 3'). Posterior probabilities = 1, unless otherwise noted.

Green: *Spiranthes romanzoffiana*

Orange: *Spiranthes magnicamporum*

Gray: *Spiranthes vernalis*

Pink : *Spiranthes delitescens* (embedded within *S*. *vernalis* (*ACO* copy A, *Xdh* copy A, and *S*. *romanzoffiana* (*ACO* copy B, *Xdh* copy B, nrITS, combined plastid).

Yellow: *Spiranthes diluvialis* (embedded within *S*. *magnicamporum* (*ACO*, nrITS), and *S*. *romanzoffiana* (combined plastid).

. >

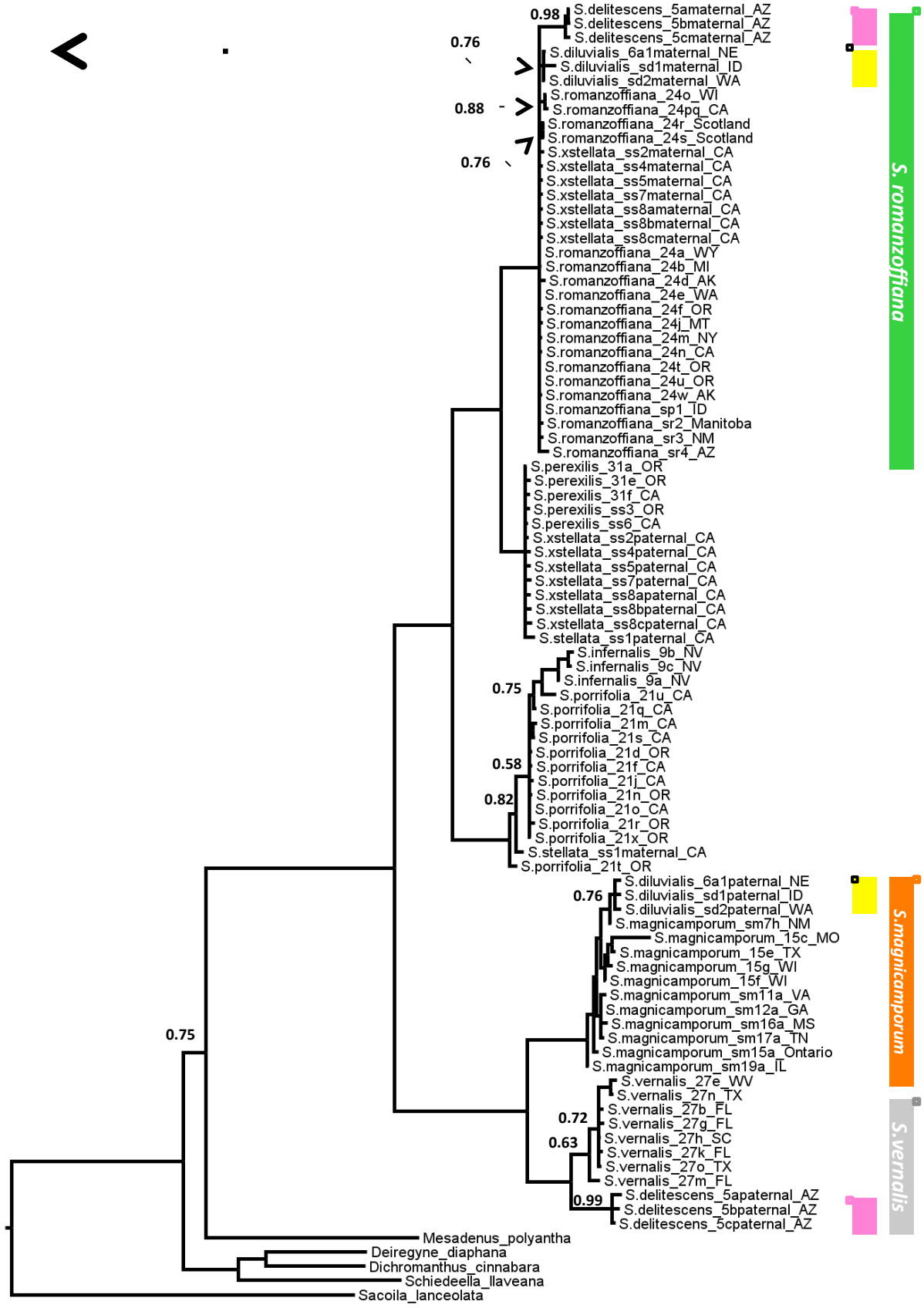

S.delitescens_5amaternal_AZ
S.delitescens_5bmaternal_AZ
S.delitescens_5cmaternal_AZ
S.diluvialis_6a1maternal_NE
S.diluvialis_sd1maternal_ID
S.diluvialis_sd2maternal_WA
S.romanzoffiana_24o_WI
S.romanzoffiana_24pq_CA
S.romanzoffiana_24r_Scotland
S.romanzoffiana_24s_Scotland
S.xstellata_ss2maternal_CA
S.xstellata_ss4maternal_CA
S.xstellata_ss5maternal_CA
S.xstellata_ss7maternal_CA
S.xstellata_ss8amaternal_CA
S.xstellata_ss8bmaternal_CA
S.xstellata_ss8cmaternal_CA
S.romanzoffiana_24a_WY
S.romanzoffiana_24b_MI
S.romanzoffiana_24d_AK
S.romanzoffiana_24e_WA
S.romanzoffiana_24f_OR
S.romanzoffiana_24j_MT
S.romanzoffiana_24m_NY
S.romanzoffiana_24n_CA
S.romanzoffiana_24t_OR
S.romanzoffiana_24u_OR
S.romanzoffiana_24w_AK
S.romanzoffiana_sp1_ID
S.romanzoffiana_sr2_Manitoba
S.romanzoffiana_sr3_NM
S.romanzoffiana_sr4_AZ
S.perexilis_31a_OR
S.perexilis_31e_OR
S.perexilis_31f_CA
S.perexilis_ss3_OR
S.perexilis_ss6_CA
S.xstellata_ss2paternal_CA
S.xstellata_ss4paternal_CA
S.xstellata_ss5paternal_CA
S.xstellata_ss7paternal_CA
S.xstellata_ss8apaternal_CA
S.xstellata_ss8bpaternal_CA
S.xstellata_ss8cpaternal_CA
S.stellata_ss1paternal_CA
S.infernalis_9b_NV
S.infernalis_9c_NV
S.infernalis_9a_NV
S.porrifolia_21u_CA
S.porrifolia_21q_CA
S.porrifolia_21m_CA
S.porrifolia_21s_CA
S.porrifolia_21d_OR
S.porrifolia_21f_CA
S.porrifolia_21j_CA
S.porrifolia_21n_OR
S.porrifolia_21o_CA
S.porrifolia_21r_OR
S.porrifolia_21x_OR
S.stellata_ss1maternal_CA
S.porrifolia_21t_OR
S.diluvialis_6a1paternal_NE
S.diluvialis_sd1paternal_ID
S.diluvialis_sd2paternal_WA
S.magnicamporum_sm7h_NM
S.magnicamporum_15c_MO
S.magnicamporum_15e_TX
S.magnicamporum_15g_WI
S.magnicamporum_15f_WI
S.magnicamporum_sm11a_VA
S.magnicamporum_sm12a_GA
S.magnicamporum_sm16a_MS
S.magnicamporum_sm17a_TN
S.magnicamporum_sm15a_Ontario
S.magnicamporum_sm19a_IL
S.vernalis_27e_WV
S.vernalis_27n_TX
S.vernalis_27b_FL
S.vernalis_27g_FL
S.vernalis_27h_SC
S.vernalis_27k_FL
S.vernalis_27o_TX
S.vernalis_27m_FL
S.delitescens_5apaternal_AZ
S.delitescens_5bpaternal_AZ
S.delitescens_5cpaternal_AZ
Mesadenus_polyantha
Deiregyne_diaphana
Dichromanthus_cinnabara
Schiedeella_llaveana
Sacoila_lanceolata
0.98
0.76
0.88
0.76
0.75
0.58
0.82
0.76
0.75
0.72
0.63
0.99
S. romanzoffiana
S.magnicamporum
S.vernalis

Fig. 5. [legend for following page] Bayesian Majority Rule phylogenetic reconstruction of maternally inherited pasted vs. n\bi-parentally inherited nuclear loci. ***Spiranthes romanzoffiana*** **(Green)**, ***Spiranthes porrifolia*** **(Blue)**, ***Spiranthes stellata*** **subsp.** ***stellata*** **(=** ***S.*** **×*stellata*) (Purple)**, and ***Spiranthes stellata*** **subsp.** ***perexilis*** **(=** ***S. perexilis*****) (Red)**. The orange arrow denotes the placement of the putative hybrid between *S*. *stellata* subsp. *perexilis* (= *S*. *perexilis*), and *S*. *porrifolia*. Posterior probabilities above 0.9 are presented.

.

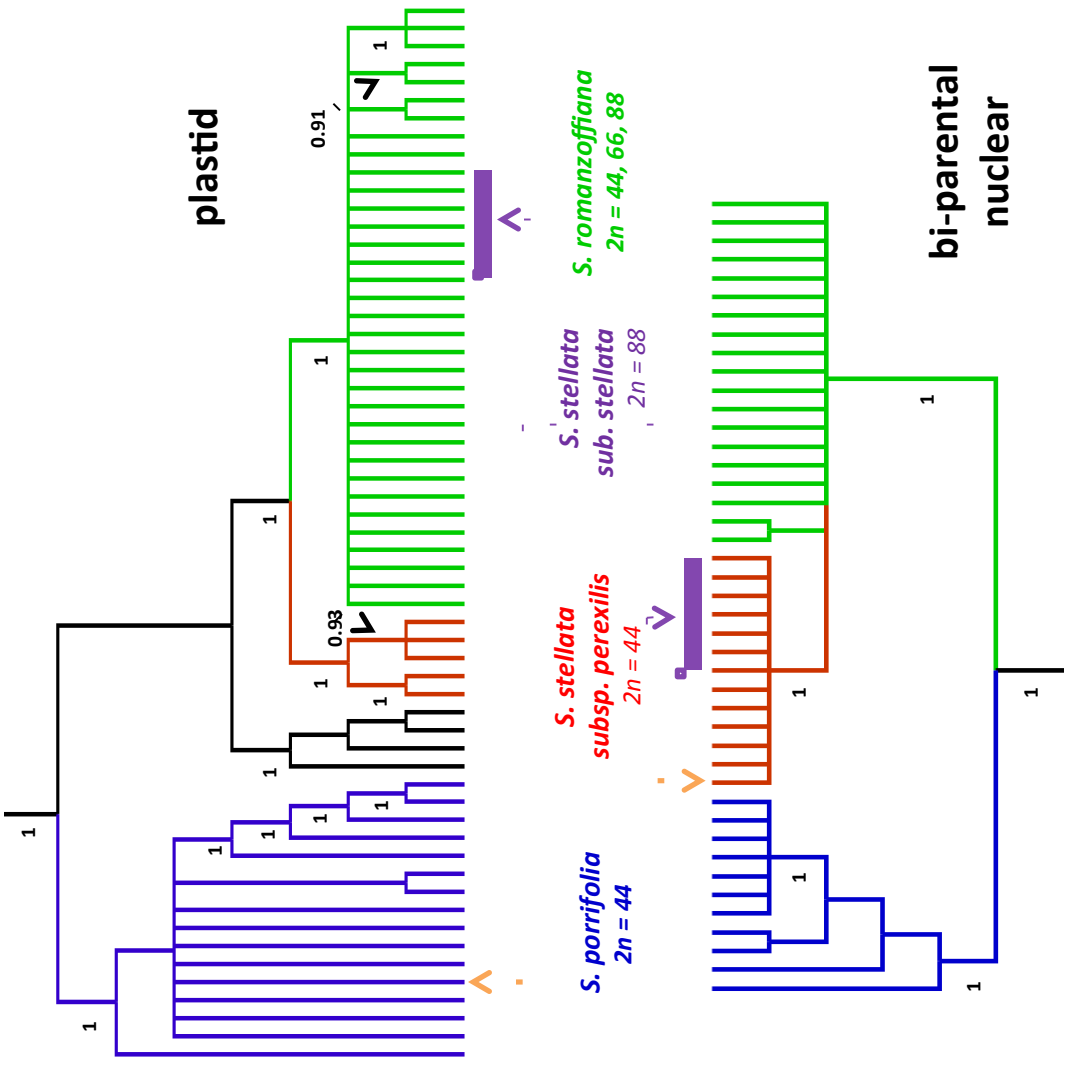
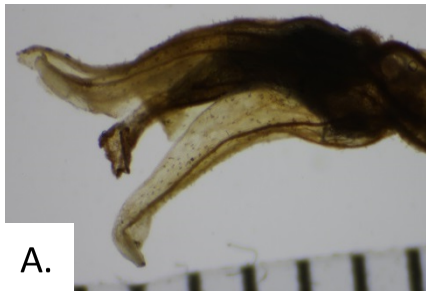
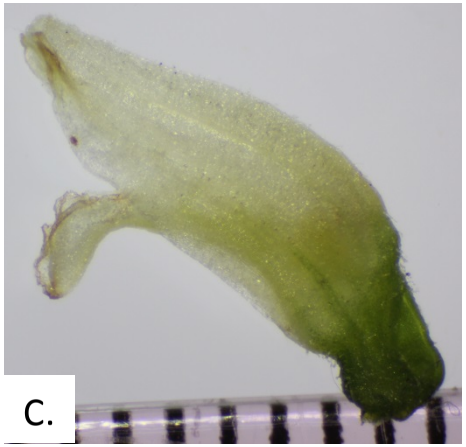
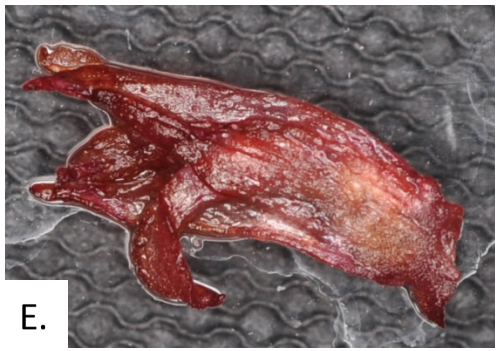

plastid
bi-parental
nuclear
S. porrifolia
2n = 44
S. stellata
subsp. perexilis
2n = 44
S. stellata
sub. stellata
2n = 88
S. romanzoffiana
2n = 44, 66, 88
1
0.93
0.91

Fig. 6. [legend for following page] Rehydrated flowers of herbarium specimens, with corresponding clade placement for nrITS and plastid markers matK, trnL-F, and trnS-M. All images are to scale. **B, D, F,** are the flattened labellum after dissection. **A-B.** *S*. *perexilis*, Stewart 2690. Sample ss6. **C-D.** *S*. *romanzoffiana*. Representative of phylogenetic samples. **C.** fresh collected from Cedarburg Bog, Ozaukee CO., WI. **D.** Labellum dissected and stained with neutral red, **E-F.** *S*. *porrifolia*. Representative of phylogenetic samples. Stained with neutral red. **F.** Representative of the labellum morphological variation of *S*. *porrifolia*.















ITS: *S. perexilis* *S. romanzoffiana* *S. porrifolia*

plastid: *S. perexilis* *S. romanzoffiana* *S. porrifolia*

Fig. 7. [legend for following page] Rehydrated flowers of herbarium samples, with corresponding clade placement for nrITS and plastid markers matK, trnL-F, and trnS-M. All images are to scale. B, D, F, are the flattened labellum after dissection. **A-B.** *S.* ×*stellata*, Horner 2414. Sample 7a. **C-D.** *S. perexilis* × *S. porrifolia*, Sheviak 6995. Sample ss1. **E-F.** *S.* ×*stellata*, Sheviak 7011. Sample ss2. Black and white photo of plant before pressing, originally from Sheviak (2012).

^

.

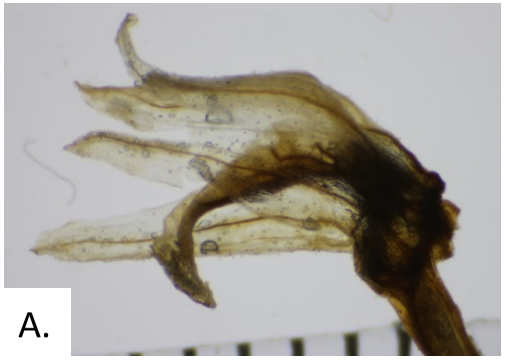
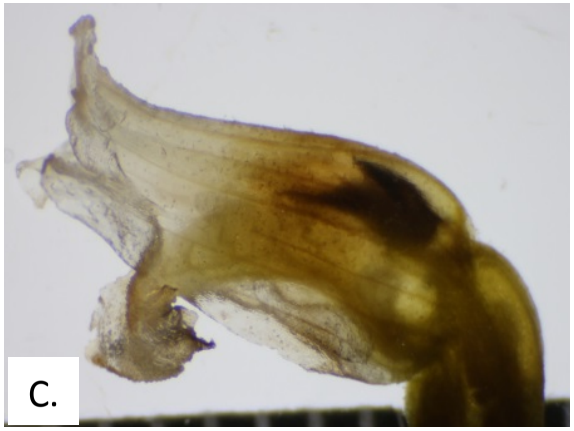
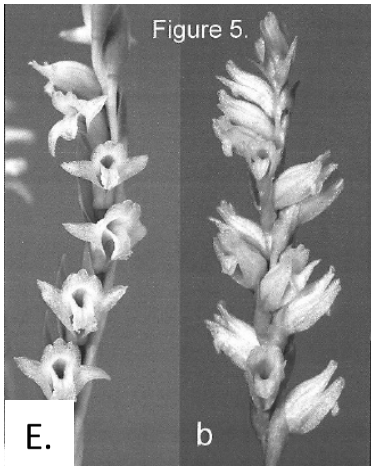
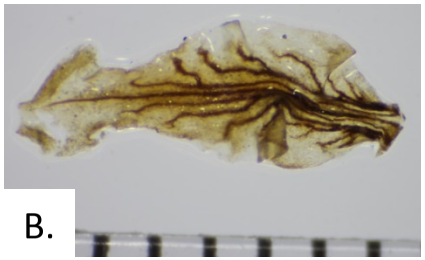
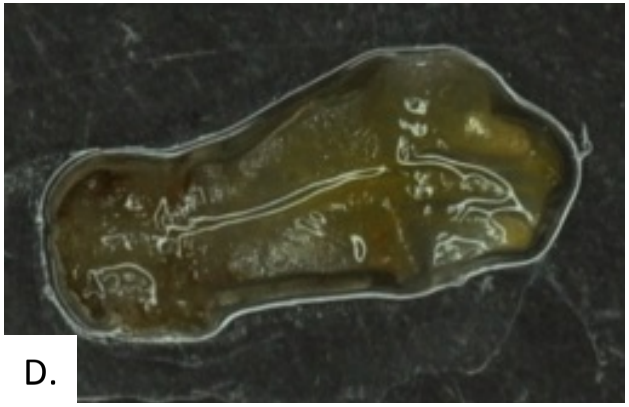
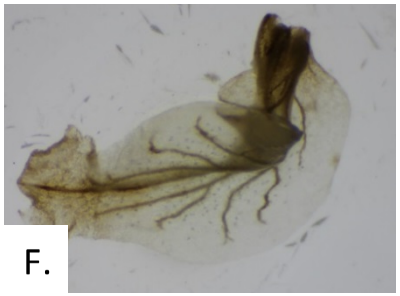
ss7	ss1	ss2
 A.	 C.	 E. b
 B.	 D.	 F.
ITS: *S. perexilis*	*S. perexilis*	*S. perexilis*
plastid: *S. romanzoffiana*	*S. porrifolia*	*S. romanzoffiana*

Fig. 8. [legend for following page] Rehydrated flowers of herbarium samples, with corresponding clade placement for nrITS and plastid markers matK, trnL-F, and trnS-M. All images are to scale. B, D, F, are the flattened labellum after dissection. *Ahart 12,166* (RSA). *Spiranthes* ×*stellata*. **A-B.** Sample 8a. **C-D.** Sample 8b. **E-F.** Sample 8c.

>

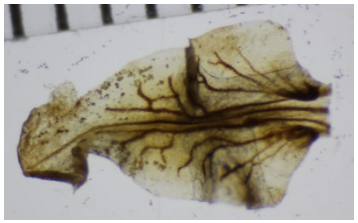
^

8a **8b** **8c**













ITS: *S. romanzoffiana / S. perexilis* *S. perexilis* *S. perexilis*

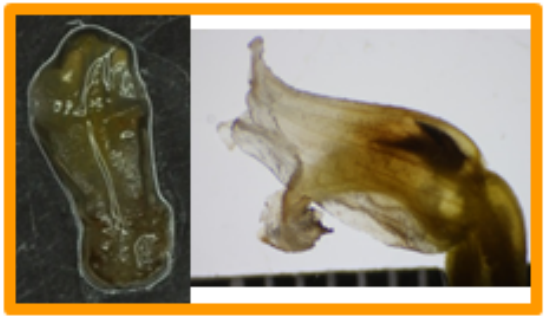
plastid: *S. romanzoffiana* *S. romanzoffiana* *S. romanzoffiana*

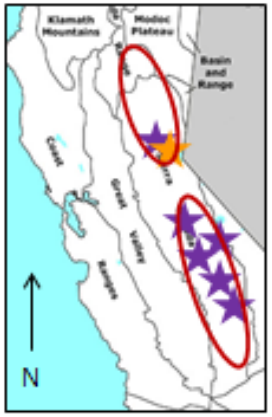
Fig. 9. [legend for following page] Comparison of rehydrated and live flowers of herbarium samples. All images are to scale. The map depicts the region of central California. The red ovals indicate the distribution of *S. perexilis* within CA. The purple stars indicate the locations of *S.* x*stellata* based on annotated herbarium specimens and molecular phylogenetic sampling. The orange star indicates the location of the one know example of *S. perexilis* x *S. porrifolia*. *Spiranthes porrifolia* and *S. romanzoffiana* are both found throughout California. **A.** *Spiranthes perexilis*. **B.** *Spiranthes* x*stellata*. **C.** *Spiranthes romanzoffiana.* **D.** *Spiranthes porrifolia.* **E.** *Spiranthes perexilis* x *Spiranthes porrifolia*.

S. perexilis



S. perexilis
× S. porrifolia





S. ×stellata



S. porrifolia



S. romanzoffiana

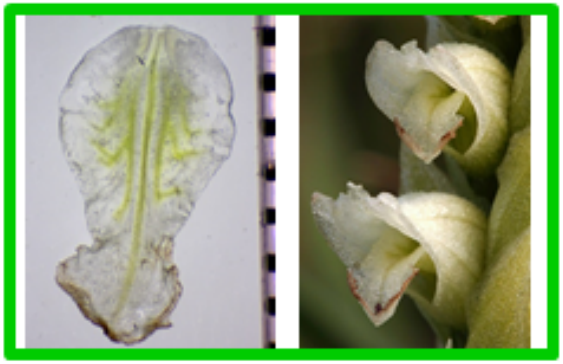


Fig. 10. [legend for following page] Lectotype sheet of *Spiranthes stellata* subsp. *stellata* as designated by Sheviak (2012; *Colwell et al. 07-279*) (= *S*. ×*stellata*). The central, large-flowered-tightly coiled individual is the lectotype. The two smaller-flowered, gracile individuals to either side are individuals of *S*. *stellata* subsp. *perexilis* (= S. *perexilis*).


IMAGED
HOLOTYPE!
Brown, P.M., L.A. Dueck, and K.M. Cameron.
2008. North American Native Orchid Journal 14(1):3-21.
Flora of Yosemite National Park
Rare Plants of Unusual Habitats Survey
ORCHIDACEAE
Spiranthes stellata P.M. Brown, L.A. Dueck & K.M. Cameron
Det. Paul Martin Brown
Mariposa County, California. East end of Merrimac Meadow (Badger Pass Ski Resort), just south of Glacier Point Road. UTM Zone/Easting/Northing 11 North/265304/4171454. Datum NAD27. Elevation 7300 feet.
In fen at the margin of a large meadow. Among dense short-stature vegetation in part shade of Pinus contorta and Abies magnifica forest. Associated species include Carex vesicaria, Eleocharis decumbens, Mimulus primuloides, Pedicularis attollens, Oxypolis occidentalis and Narthecium yosemitense.
Allison Colwell 07-279, Paul Martin Brown, Stan Folsom, Brad Kelley, Robert Lacy. 7/26/2007
Catalog # YOSE 217119 Accession # YOSE 4932
Herbarium of the Yosemite Museum
HERBARIUM OF THE
UC
UNIVERSITY OF CALIFORNIA
copyright reserved

Fig. 11. [legend for following page] Herbarium specimen from which samples 8a, 8b, and 8c were judiciously sampled. *Ahart 12,166* (RSA). *Spiranthes* ×*stellata*.

>


0 1 2 3 4 5 6 7 8 9 10
cm
copyright reserved
WIS
Rancho Santa Ana Botanic Garden
Claremont, CA USA (RSA-POM)
RSA0011916
RANCHO SANTA ANA
715844
BOTANIC GARDEN
8a
8b
8c
Plants of California
Nevada County
Spiranthes porrifolia Lindl.
Orchidaceae
Latitude 39° 19' 02.7" Longitude 120° 38' 24.2"
T17N, R12E, southwest 1/4 Section 21.
Normal size plants, on moist soil, along a small wash, open meadow, under the power lines, about 100 yards west of the paved road, southwest of Lake Spaulding, about ½ mile north of Highway 20. Common. Flowers white. Yellow Pine Forest. Elevation 5160 (GPS) feet.
Lowell Ahart 12,166. 23 July 2005.
Herbarium of California State University, Chico
Specimen data entered into
RSA-POM database

Fig. 12. [legend for following page] Herbarium specimen from which sample ss3 was judiciously taken. These small flowered, gracile, open spiraled plants represent *S*. *stellata* subsp. *perexilis* (= *S*. *perexilis*).

>


NEW YORK STATE MUSEUM
WIS
copyright reserved
0 1 2 3 4 5 6 7 8 9 10
cm
ANNOTATION LABEL
Spiranthes romanzoffiana Cham.
C.J. Shevlak
New York State Museum
2000
Stellate
(cf perexilis)
SS 3
Duplicate of collection at Crater Lake National Park herbarium
KLAMATH COUNTY, OREGON
Spiranthes romanzoffiana Cham.
White flowers, wet meadow, with Eleocharis quinqueflora, headwaters of North Fork of National Creek, elev. 5250 feet, T29S R5E S20 SE¼, west slope of Cascade Mts., Crater Lake National Park
Coll. P. F. Zika 15260 7 August 1994

Fig. 13. [legend for following page] Herbarium specimen from which sample ss6 was judiciously taken. These small flowered, gracile, open spiraled plants represent *S*. *stellata* subsp. *perexilis* (= *S*. *perexilis*).


0 1 2 3 4 5 6 7 8 9 10
cm
copyright reserved
WIS
Rancho Santa Ana Botanic Garden
Claremont, CA USA (RSA-POM)
RSA0020649
RANCHO SANTA ANA
808501
BOTANIC GARDEN
SS6
Stellata
ssp porrifolia
Herbarium of Rancho Santa Ana Botanic Garden
Plants of Tulare Co., California, USA
Orchidaceae
Spiranthes porrifolia Lindl.
Sierra Nevada; Southern Sierra Nevada region: Nobe Young Meadow.
Joan Stewart 2690
25 Jul 2007
Entered into RSA Database (61449RSA)
Specimen data entered into RSA-POM database

CHAPTER 5

PHYLOGENETICS, SPECIES BOUNDARIES, AND CHARACTER EVOLUTION IN *SPIRANTHES* (ORCHIDACEAE)

Matthew C. Pace, Kenneth M. Cameron

***Abstract*—**The goal of a rational and practical system of classification that represents the course of evolution is hampered by a number of factors, including hybridization, incomplete lineage sorting, extinction, extensive inter-specific morphological variation, cryptic speciation, methods that are unable to resolve frequent low levels of phylogenetic divergence among recently derived putative species, and convergent evolution. *Spiranthes* is a genus of orchids which have long presented numerous challenges to systematic identification and evolutionary understanding. Here we present the most expansive taxon and molecular marker sampling of this complicated genus with complimenting ancestral state reconstructions for numerous morphological characters. Although most species were recovered as being reciprocally monophyletic and fully supported (pp =1), relationships between these clades remains unresolved in some cases. Additionally, the molecular phylogenetic relationships of Mesoamerican *S. graminea* and *S. nebulorum* are presented for the first time. Our ancestral state reconstructions show that all characters evaluated have evolved multiple times within *Spiranthes*. This includes changes in labellum color and surface texture, which have changed in the absence of complimentary shifts in pollinators.

***Key words*—**Ancestral state reconstruction; incomplete lineage sorting; reciprocal monophyly; species complex; *Spiranthes sinensis* species complex

One of the fundamental tasks of systematic biology is to describe biodiversity in a natural manner that best reflects evolutionary history. Yet the goal of a rational system of classification that represents the course of evolution –also practically informing conservation management, medicinal discoveries, and economic utility– is hampered by a number of factors, including hybridization and introgression, incomplete lineage sorting, extinction, extensive inter-specific morphological variation, cryptic speciation, methods that are unable to resolve frequent low levels of phylogenetic divergence among recently derived putative species, homoplasy and convergent evolution, difficulties in carrying out pollination studies, and the lack of a universally accepted species concept. The Orchidaceae, with over 27,000 species, offers a nearly unparalleled botanical opportunity to examine the nature of species and the evolution of morphological characters. As molecular phylogenetic reconstructions and complimentary morphological analyses expand across the Orchidaceae, new opportunities arise for clarifying long standing problems in species limits, species identification, and morphological evolution.

The genus *Spiranthes* Rich. (Orchidoideae: Cranichieae: Spiranthinae) (fig. 1) has a long history of taxonomic confusion due to a poor grasp of species boundaries by systematists. Unusually for Orchidaceae *Spiranthes* is most species-rich in the temperate zone, specifically North America. Systematic confusion in *Spiranthes* has resulted from extreme intra-specific variability, confusion over type specimens, shared pollinators and putatively frequent hybridization, and small flowers with few unique and/or discreet characters. Questions regarding species status within *Spiranthes* are an open source of debate, and this can have important consequences for conservation (e.g., *S. parksii* Correll; Walters 2005, Dueck and Cameron 2007, Dueck and Cameron 2008a, Jacobsen et al. 2009, Dueck et al. 2014; Chapter 1, Chapter 2). The extensive morphological and descriptive work of Sheviak and Catling has clarified some

difficulties regarding species status (Sheviak 1973, 1982, 1984, 1989, 1990; Catling 1978, Sheviak and Catling 1980; Catling and Catling 1988), but their work has also raised other questions (Catling 1980; Sheviak 1991, 2012, 2013). More recently, the publications of Brown (e.g., 1999a, 1999b, 2000, 2006) have led to the description of many ambiguous taxa often based on mixed types (e.g., Chapter 1), possibly leading to taxonomic inflation in this genus. Within *Spiranthes*, three studies (Dueck and Cameron 2008b; Chapter 2, Chapter 3) have presented molecular phylogenetic evidence to support species status at the time of description. Furthermore, Chapter 4 presented preliminary evidence regarding several episodes of hybridization among the *Spiranthes* of the North American Western Cordillera, whereas Chapter 3 examined hybridization within the *Spiranthes cernua* species complex. In both cases, our molecular phylogenetic research supports some elements of the hypotheses put forward by Sheviak (1982; 1991; 2013) and Sheviak and Brown (2002) –namely that hybridization is an evolutionary force within these groups, and that some species are of hybrid origin– however hybridization may be less pervasive across the genus than anticipated, and may be strongly correlated with specific geographic regions. Worldwide, there are 45 currently accepted taxa of *Spiranthes* (including subspecies and varieties, named hybrids –as indicated by a "×"– and allopolyloid species of known hybrid origin (e.g., *S. diluvialis* Sheviak)) (Govaerts et al. 2014). Some currently recognized species are of questionable taxonomic status; this is particularly true in Asia, Australia, and eastern North America. The more conservative number of *Spiranthes* species may be closer to 35.

Over the last decade there have been a growing number of papers using molecular phylogenetic tools to disentangle species relationships within this perplexing genus, cumulatively including 29 species of *Spiranthes* (Dueck and Cameron 2007, 2008a, 2008b;

Dueck et al. 2014; Chapter 1, Chapter 2, Chapter 3, Chapter 4). Among the more important findings of this combined body of literature include 1) the recovery of two groupings corresponding to differences in cytology (n = 15 vs. n = 22); 2) the non-monophyly of *S. cernua* s.l.; 3) phylogenetic support for the status of most established species in addition to the description of *S. igniorchis* M.C. Pace and *S. arcisepalum* M.C. Pace, and clarifications regarding the status of *S. triloba* (Small) Schum. emend. M.C. Pace and *S. perexilis* (Sheviak) M.C. Pace; 4) the monophyly of sampled Eurasian species as a clade and their derivation from – as opposed to sister to– the North American species; 5) the position of eastern North American *S. lucida* (H.H. Eaton) Ames within an otherwise Western North American clade; 6) and the lack of support for recognizing *S. parksii*.

Although the most expansive molecular phylogenetic study of *Spiranthes* to date, by Dueck et al. (2014), provides important context for future research, a few limitations of that study must be acknowledged. Taxon sampling for Dueck et al. (2014) was incomplete, with important species from outside North America or of questionable species status not included (e.g., *S. nebulorum*, *S. nivea*). Furthermore, many species are represented in Dueck et al. (2014) by five or fewer accessions, often from geographically close or sympatric populations, and frequently exhibiting 'classic' morphology of the putative taxon it was selected to represent. This is significant because of the great degree of morphological variation and frequent geographic disjunctions displayed by many hypothesized species of *Spiranthes*. Expanded sampling is crucial to understand the morphological and distributional limits of species through a phylogenetic framework (de Queiroz & Gauthier 1992, Pettengill & Neel 2011). Limited geographic phylogenetic sampling was one of the factors leading to confusion regarding *S. perexilis* and *S.* ×*stellata* P.M. Br., Dueck, and K.M. Cameron (Chapter 4 for details).

Additionally, Dueck and Cameron (2007) and Dueck et al. (2014) relied on a distantly related Spiranthinae –*Sacoila* Raf. (Salazar & Jost 2012)– as their only outgroup taxon. Incorrect, incomplete, and distant outgroup sampling is known to possibly result in the reconstruction of erroneous topologies (e.g., Wheeler 1990, Piller & Bart 2009), and does not test the monophyly of the genus. Dueck et al. (2014) did recover strong statistic support for most branching events, but several clades were recovered with moderate to low support (posterior probabilities values < 9.0; particularly along the tree 'backbone') or with no resolution between hypothesized taxa. Finally, these earlier studies were based on a limited number of molecular markers and included no low copy nuclear gene data at all. Thus, there is room for further refinement in reconstructing the phylogenetic history of *Spiranthes* by including more ingroup and outgroup taxa, an increased number of species accessions, additional genetic markers, and by employing alternative molecular phylogenetic methods.

METHODS

Taxon sampling. To capture the full diversity of *Spiranthes*, the first author utilized and greatly expanded upon an established DNA bank of *Spiranthes* at UW – Madison (incorporating the work of Dueck et al. (2014)), and simultaneously conducted fieldwork throughout the U.S., particularly in the North American Coastal Plain, Florida, the Ozarks and Ouachitas, the Great Lakes Basin, New Mexico, and the greater Ohio River Valley. Samples were also collected by citizen scientists of the Native Orchid Conference, and the Virginia and Kentucky Natural Heritage Programs. Herbarium samples (Fig. 2) were judiciously sampled to include disjunct populations, possible hybrids, and geographic areas that were unable to be visited in person.

For samples collected in the field or from greenhouse plants, 3–4 unopened flower buds (or in their absence, ca. 2 cm^2 of leaf material) were placed into empty tea bags, labeled with a unique identifier, and dried with silica gel desiccant. Samples from herbarium specimens followed similar tissue and quantity procedures, but were placed directly into uniquely labeled Eppendorf SafeLock tubes for later extraction of Total gDNA.

Through the above mentioned partnerships and collection strategies, all North America species, many disjunct populations (e.g., *S. magnicamporum*: 7 out of 10 known), and five of the six geographically important non-North American species not sampled by Dueck et al. (2014) were included in this study. Additionally, outgroup genera in the clade sister to *Sprianthes* were incorporated through aliquots of total gDNA generously donated by Dr. G. Salazar (UNAM). After removing several individuals collected from the same population but included in Dueck et al. (2014), overall sampling was increased from 185 to 297 individuals. Vouchered herbarium, spirit, and live plant collections were collected and prepared for depositing at BH, FLAS, NY, US, and WIS.

Loci sampling. Dueck and Cameron (2007) and Dueck et al. (2014) mostly relied on plastid data in addition to nrITS. As hybridization has been suggested to be a potential driver of diversity in *Spiranthes* (Sheviak 1982, 1984, 1991, 2013, Sun 1996, Szalanski et al. 2001, Sheviak and Brown 2002), low-copy nuclear markers are critical to reconstructing the phylogeny of many clades and clarifying the role of putative hybridization within the genus. In this study, we, too, used nrITS, but also included two low-copy nuclear markers: *ACO* and *Xdh* (Fig. 2). Furthermore, to add more resolution to the resulting cladogram, we also expanded plastid marker sampling (focused on loci which might afford increased levels of phylogenetic resolution), for a total of eight loci (Fig. 2).

Molecular techniques and analyses. Silica gel dried material was placed into Eppendorf SafeLock tubes with 2 tungsten coated beads for extraction. Tissues were ground in a TissueLyser (QIAGEN, Germantown, MD) at 30 Hz for ca.1 minute. Genomic DNA was extracted using either the DNEasy Plant Mini Kit (QIAGEN, Germantown, MD), IBI Plant Isolate Kit (IBI Scientific, Peosta, IA), or Maxwell® 16 LEV Plant DNA Kit (Promega, Madison, WI). All herbarium samples were extracted with the IBI Plant Isolate Kit, which employs a CTAB-based approach; it was found that this method consistently yielded high quality and quantity gDNA from specimens as much as 15 years-old. All accessions were amplified for the plastid gene regions *mat*K, *ndh*J, *trn*L intron, *trn*S-fM, and *ycf*1 3', nuclear ribosomal ITS (internal transcribed spacers 1 and 2 and the 5.8S subunit; nrITS), and the low-copy nuclear regions *ACO* and *Xdh* (fig. 2). PCR amplification protocols are as follows: plastid (except *ycf*1) and nrITS: Dueck et al. (2014), *Xdh*: Górniak et al. (2010), *ACO*: Guo et al. (2012), and *ycf*1: Neubig et al. (2009). PCR products were purified using ExoSap-It (Cleveland, OH), and cycle sequencing products were cleaned using Agencourt CleanSeq (Beverly, MA) magnetic beads. Direct sequencing of cleaned cycle sequencing products was performed at the University of Wisconsin – Madison Biotechnology Center. Resulting chromatograms were edited and aligned using software modules available in Geneious 7.1 including MUSCLE. Ambiguities in plastid datasets were coded with standard IUPAC-IUB Symbols for Nucleotide Nomenclature (Cornish-Bowden 1985). We interpreted nuclear sequences with ambiguities as being indicative of hybridization. As a proxy for cloning, ambiguities in nuclear ribosomal and low-copy nuclear sequences were dealt with in the following manner: 1) sequences were duplicated as two distinct contings; 2) based on the ambiguities present across the now doubled sequences, putative parents were identified with complimentary non-ambiguous bases at ambiguous sites; 3) based on the

topology of the combined plastid phylogenetic reconstruction, one of these putative parent species was identified as the likely maternal species, the other species with complimentary bases was designated the tentative paternal parental species; 4) ambiguous positions were coded so that one sequence matched the nucleotide of the likely maternal parent, whereas the other matched the nucleotide present in the likely paternal parent; 5) the names of the doubled sequences were changed so that they represented different operation taxonomic units based on their inferred parentage. If ambiguities were present in the apparent hybrid individual which did not correspond to nucleotides found in the putative parental species, non-ambiguous nucleotides were assigned randomly. If nucleotide ambiguities were found in an apparent hybrid, such that only one tentative parent could be identified, the ambiguous sequence was doubled as above, however one copy –designated 'A'– was coded to match the tentative parent, and the other sequence was coded as its compliment, and did not match the nucleotides found in any other taxon –designated 'B'. This coding approach was used in lieu of cloning in an attempt to retrieve both parental copies of potential hybrids. If samples failed to amplify after repeated attempts for a given locus they were coded as missing data.

Our data matrices were analyzed as distinct partitions: 1) *ACO*; 2) *Xdh*; 3) nrITS; 4) individual plastid loci; 5) combined platsid; 6) full combined total evidence data including all samples; 7) minimal-missing-combined total evidence data in which only one representative accession per taxon was included (the exemplar samples were chosen from the most completely sampled individuals per taxon). In the total evidence combined nuclear + plastid dataset, only the 'A' copy of previously ambiguous nuclear sequences were included. Analyses were performed under Bayesian Inference (MrBayes on XSEDE (3.1.2)) implemented through CIPRES Portal V. 3.3 (Miller et al. 2010). Based on Dueck et al. (2014), the GTR+G (general-time-reversible with

a gamma distribution) model was implemented for all datasets and partitions. Analyses were run for 10,000,000 generations, with a sample frequency of 100,000, nruns = 2, nchains = 6, temp = 0.2, and a burnin of 500,000. Phylogenetic inference of the 50% majority-rule consensus tree was constructed using the "sumt" option based on the remaining trees. The topology of this tree was visualized as trees in FigTree (Rambaut 2014).

Ancestral State Reconstructions (ASRs)—Using the reduced, minimal missing-data partition, we reconstructed the likely ancestral conditions of eight characters for 39 ingroup and six outgroup taxa. The input tree was constructed using BEAST (Drummond et al. 2012) to ensure a lack of polytomies (the presence of which would have inhibited ASRs). Discreet characters analyzed are 1) flower color; 2) central labellum color; 3) labellum texture; 4) lateral sepal position; 5) breeding system; 6) leaf shape; 7) leaf presence at time of anthesis; and 7) root morphology (fig. 11-18). The phenotypic conditions of each character for each taxon are based on field work and reports in the literature. Ancestral state reconstructions were constructed in RStudio (2012) using the ACE (Ancestral Character Estimation using maximum likelihood estimation) function available in the APE package.

Morphometrics—In an attempt to understand morphological variation present within and between select taxa, we performed principle components analyses. Data analysis was performed and PCA visualized in RStudio (2012) using measurements of labella generated from scaled line drawings available in the literature (Sheviak 1989). An additional flower from *DeDecker 5091* (RSA) was rehydrated using a dilute ammonia solution and dissected for measurement. In total 32 individual labella were measured: 12 *S. infernalis* (including individuals from the holotype sheet) and 20 *S. porrifolia*. Measurements included labellum length, width at widest point, width

at isthmus/narrowest point, width of dilated tip, length of dilated tip, and callosity height and width.

RESULTS

***Phylogenetics*—**Overall, sample amplification was not equal between loci. Samples were only included in phylogenetic reconstructions if they amplified for at least one plastid and one nuclear locus (typically *trn*SM and nrITS). Number of amplified samples for each locus are as follows (out of 297 total samples): *ACO* (151), nrITS (273), *mat*K (282), *ndh*J (170), *trn*L-F (275), *trn*S-M (280), *Xdh* (149), *ycf*1 3'(229) (fig. 3). As such, our phylogenetic inferences are based on much missing data; however, our overall topology largely mirrored Dueck et al. (2014), and we feel confident in our resulting topology.

Our individual phylogenetic reconstructions recover similar topologies. Where incongruences do exist they are usually associated with polytomies, short branch lengths (and possible incomplete lineage sorting), clades for which posterior probability support values are low, and/or areas of the tree where hybridization between closely related species is implicated. Within our full combined total evidence data phylogenetic hypothesis (fig. 5), generally, most (25) established species were fully supported as reciprocally monophyletic, except in the following cases: 1) *S. infernalis* is consistently recovered as embedded within *S. porrifolia*; 2) resolution between *S. praecox* and *S. sylvatica* is lacking, with the latter broadly embedded within the former; 3) *S. sinensis* s.l. may be non-monophyletic; 4) *S. lancea* may warrant recognition as distinct from *S. sinensis* s.l.; 5) *S. eatonii* is embedded within *S. lacera*.

Topological reconstruction of the relationships between *S. tuberosa*, the Old World clade, and the *S. praecox*/*S. sylvatica*/*S. graminea*/*S. nebulorum* clade was broadly incongruent

between datasets. Because *S. tuberosa* failed to amplify for *Xdh*, and our matrices are unequal in sample size, we acknowledge comparisons among them must be done with caution. Comparing the full combined total evidence data (fig. 5) vs. the minimal-missing-combined data (fig. 6) reconstructions finds two competing hypotheses with essential identical posterior probability support: a Western Complex + *S. lucida* clade sister to a grade of ((*S. praecox* (*S. graminea, S. nebulorum*)), *S. tuberosa*, Old World taxa, all other North American taxa) vs. a Western Complex + *S. lucida* clade sister to a grade of ((*S. praecox* (*S. graminea, S. nebulorum*)), (*S. tuberosa*, Old World taxa), all other North American taxa).

***Character Evolution*—**Based on the characters we examined, we did not find any clade or complex-specific unique morphological synapomorphies. Instead, many characters appear to have evolved multiple times from a common ancestral state (with a few cases of reversals). However, a few character states, while not exclusive to any particular clade, are displayed by all member species of a specific clade (e.g., lateral sepals held below the flower profile in the *S. lacera* species complex, fig. 11). Symplesiomorphic conditions include an allogamous breeding system, white flowers with a centrally white-colored oblong labellum, tuberous roots, and lanceolate leaves present at the time of flowering. Convergent characters include a centrally yellow or green colored centrally-constricted labellum, linear leaves, and cylindrical roots. Apomixis, autogamy, and/or a mixed breeding system has evolved twice in *Spiranthes*, corresponding to the two most systematically challenging species complexes: the *S. cernua* and *S. sinensis* species complexes. Flower color and labellum color and texture have both changed multiple times within the genus in the absence of a major shift in pollinators.

DISCUSSION

Overall Phylogenetic Relationships. In general, our phylogenetic hypothesis matches that of Dueck et al. (2014) in most instances. The two major differences are 1) relationships of the '*S. lacera* species complex'; and 2) the topological position of *S. tuberosa*, the *S. praecox*/*S. sylvatica*/*S. graminea*/*S. nebulorum* clade, and the Old World Clade (fig. 5 III & 5 IV). In both cases, incomplete lineage sorting among very short back-bone branch lengths likely contributes to the topological uncertainty we recovered here. As discuss in Chapter 6, these branching events likely took place over a short period of geologic time; in the case of the *S. lacera* species complex/grade, the MRCAs of six extant species may have arisen in as little as 6 mybp. The grouping of *S. brevilabris, S. floridana, S. lacera, S. laciniata, S. torta,* and *S. vernalis* into a natural, monophyletic group –as was recovered in Dueck et al. (2014)– seems intuitive based on overall similarities in gross morphology, shared habitats, and specific morphological features such as lateral sepal position (fig. 11). Indeed, our minimal missing data reconstruction (fig. 6), does recover a shared MRCA for these six species, however it is weakly supported (pp = 0.73). The exact topological structure of this portion of the *Spiranthes* phylogeny thus remains unresolved. We hypothesize that a burst of speciation/cladogenesis events may have taken place leading to the evolution of *S. brevilabris, S. floridana, S. lacera, S. laciniata, S. torta,* and *S. vernalis*, and as such further molecular phylogenetic research is needed to clarify deep relationships within this group.

The work we present here is the first to place Mesoamerican *S. graminea* (fig. 7) and *S. nebulorum* (fig. 8) into a molecular phylogenetic context. The surprising relationship between these Mesoamerican species and *S. praecox*/*S. sylvatica* is discussed more fully in Chapter 6.

Previously, *S. graminea* and *S. nebulorum* had been thought to be most closely related to Western North American *S. romanzoffiana*, *S. porrifolia,* and *S. perexilis* (Sheviak 1989, 2012).

Species Limits and Taxonomy

***Spiranthes infernalis* may be a subspecies of *S. porrifolia*.** Taxonomy is a fundamental component and tool of conservation (Mace 2004). As such it has the power to highlight the conservation needs of taxa (e.g., Roca 2001), as well as possibly draw attention away from taxa if the organism is determined to be a synonym, subspecies, or variety of another species (May 1990; e.g., Hind et al 2014, Pedron et al 2014, Powell et al 2014). Particular care must be taken when deciding to elevate, synonymize, or make taxonomic combinations if the taxon in question is endemic or otherwise extremely rare. Concurrently, it is becoming increasingly clear that molecular data is essential to determining the evolutionary relationships and conservation status of taxa, placing them in a proper history-bound evolutionary context, particularly if morphological variability and distribution disjunctions are commonplace. It is within this framework that we discuss the species status of the endemic and globally rare *S. infernalis*.

Spiranthes infernalis is a U.S. Fish and Wildlife Service/Endangered Species Act "species of concern", and is among the rarest orchids in North America, with just 15 known occurrences within an 11.7 km radius, entirely restricted to marshy springs within the Ash Meadows National Wildlife Refuge of southern Nevada (Nevada Natural Heritage Program 2001). Sheviak (1989) described *S. infernalis* based on its geographic isolation, dense inflorescence of small flowers (4–6 mm long), reduced apical labellum apex, and floral color. This last characteristic –with an apparent emerald green hue to the basal portions of the perianth and pale orange coloration to the central labellum– was deemed its "most striking feature" (Sheviak 1989). These observations were based on *ex situ* cultivated plants collected from the

type location. Sheviak has routinely stressed the utility –even superiority– of examining cultivated over wild-collected *Spiranthes* to aide species delimitation (Sheviak 1976, 1982, 1989).

Our molecular phylogenetic data fully (fig. 5 V) support the position of *S. infernlis* within the wider mostly unresolved clade of *S. porrifolia*, indicating at a most basic level that *S. infernlis* and *S. porrifolia* share a most recent common ancestor. None of our reconstructions recover a strongly supported fully resolved sister-relationship between these taxa (as is the case in other closely related species, e.g., *S. perexilis* and *S. romanzoffiana*), although *S. infernalis* is rarely recovered as a subclade within the wider unresolved polytomy of *S. porrifolia*. When examined in concert with our morphological analysis of the labellum, which yields an overlap between the two taxa (fig. 9), and the frequency of distribution disjunctions within the wider genus (e.g., *S. magnicamporum*), the distinctiveness of *S. infernalis* vs. *S. porrifolia* is brought into question. Furthermore, the unequivocal morphological and geographic differential of *S. infernalis* vs. *S. porrifolia* is weakened by the presence of an herbarium specimen of *S. porrifolia* from Fish Slough, CA, in Owens Valley (*DeDecker 5091,* RSA; collected after the description of *S. infernalis*). Although it was collected ca. 220 km NNW of Ash Meadows, this specimen very closely approximates the morphology of *S. infernalis*: the plant is small and gracile in stature, the leaves are linear, and the lower flowers are 3.5–5 mm in length. In our morphometric analysis (fig. 9), *DeDecker 5091* is placed far outside of typical *S. porrifolia*, instead being placed on the *S. infernalis* end of a morphological spectrum. Similarly, the habitat of alkali meadow surrounded by a hot, arid desert flat east of the California Sierra Nevada and west of the Intermountain Region is nearly identical to the conditions present at Ash Meadows. Thus, in keeping with an integrative history-bound phylogenetic species concept, we feel the evolutionary

veracity of *S. infernlis* may be best reflected as a subspecies incorporating both the Ash Meadows and Fish Slough (DeDecker 5091) populations, however we withhold from making a definitive taxonomic judgement at this time.

Sheviak (1989) correctly hypothesized a close relationship between S. *porrifolia* subsp. *porrifolia* and *S. infernalis*, writing "*S. infernalis* evolved from *S. porrifolia*". Both taxa display ochroleucous flowers, free sepal and petal apices, a narrowly oblanceolate to very slightly subpandurate apically papillate labellum that presents a yellowish-center and prominent basal calli, tuberous roots, persistent linear to lanceolate leaves, somatic chromosome counts of 2n = 44 (*S. porrifolia* also includes 2n = 66; Sheviak 2013), and a habitat preference for stream banks and wet riparian meadows. Similarly, *S. porrifolia* and *S. infernalis* share nearly all of the same nucleotide mutations –possible molecular synapomorphies– in all examined loci. *Spiranthes infernalis* can be separated by its smaller, boxier pseudo-cupuliform and slightly tubular flowers, smaller linear leaves, and occurrence east of the California Sierra Mountains, vs. *S. porrifolia* which displays larger tubular flowers, larger linear-lanceolate to lanceolate leaves, and a wider distribution throughout the Cascade, Coast, Klamath, San Bernardino, and Sierra Nevada mountain ranges of CA, NV, OR, and WA. Despite the questions we raise regarding the possible taxonomic rank of *S. infernalis*, we maintain it still warrants protection at all levels. Within Nevada, *S. porrifolia* is restricted to the eastern ring of Sierra Nevada surrounding Lake Tahoe, and *S. infernalis* is found in a few streams within Ash Meadows National Wildlife Refuge. The marshy, spring fed habitats of both species are likely to be severely impacted from the effects of climate change in the Western Cordillera system, particularly decreased snow pack and corresponding droughts.

An unresolved relationship between *Spiranthes sylvatica* and *S. praecox*. Of the loci we sampled, only nrITS provides any strongly supported resolution between *S. praecox* and '*S. sylvatica*' (fig. Supp 1c). Curiously, samples of plants that otherwise match the holotype of '*S. sylvatica*' (ssy2, ssy3; densely coiled inflorescence, larger flowers) fall into the nrITS *S. praecox* clade. Contrastingly, *Xdh* and the individual plastid data reconstructions lack resolution between these taxa, recovering them as broadly embedded within a shared polytomy (fig. Supp 2–8). Morphologically, the flowers are very similar (close morphological examination of these species are ongoing). Moreover, the description provided by Brown (2001) for *S. sylvatica* contains at least one major erroneous measurement: the corolla length of "10–16.5 mm long" does not match the length of even the longest flower on the holotype or any other sheet; a maximum length of 16.5 mm would make the flowers of '*S. sylvatica*' the largest in the genus by ca. 3–4 mm. Moreover, the habitat distinction stressed in its description –"damp to seasonally flooded roadside ditches, open savannahs and pine flatwoods" for *S. praecox* vs. "woodland" and "semi-shaded to shaded habitats also along roadsides and successional old fields as well as within live oak hammocks" for '*S. sylvatica*'– represents an ecological continuum, particularly as fire suppression throughout the prairies and wire-grass ecosystems of the southeastern United States have led to encroachment by woody taxa in many previously sparsely treed habitats.

Following our integrative and history-bound approach, it becomes apparent that '*S. sylvatica*' is evolutionarily contained within *S. praecox*, and may have likely evolved from the latter several times. The evolutionary history of these plants suggests they may be best treated as a subspecies. Based on the slight correlation between larger-flowered and more tightly coiled plants growing in more densely treed habitats, we hypothesize *S. sylvatica* may represent an ecotype of *S. praecox*, however the evolutionary advantage or association between the

morphology of *S. sylvatica* and its environment is unknown. The relationship and boundaries between subspecies and ecotype are challenging to demarcate, and the two concepts are clearly interconnected (Clausen et al. 1940; Stebbins 1950).

A first step toward resolution of the *Spiranthes sinensis* species complex. *Spiranthes sinensis* –in its tradition sense or s.l. (fig. 10)– is among the most morphologically variable species in the genus, and is perhaps one of the most widely distributed orchids on Earth, spanning most of southern and eastern Asia, from Iran to Japan, southern Kamchatka to Tasmania, and scattered across the islands of the southern oceans from La Reunion to western Polynesia. The taxonomy of the complex is vast, with many toponyms (e.g., *Spiranthes papuana* Schltr., *Spiranthes neocaledonica* Schltr., *Spiranthes novae-zelandiae* Hook.f.); *S. sinensis* is something of a 'splitters' dream. The morphological variation and geographic expansiveness of *S. sineneis* s.l. has been subjected to some molecular research, though usually at a local scale (Tsukaya 1994, 2005; Sun 1996, 1997).

Recently new names within the wider complex have been described, resulting from continued exploration and morphological examination, including *Spiranthes alticola* D.L. Jones, *Spiranthes nivea* T.P. Lin & W.M. Lin, and *S. sunii* Boufford & Wen H. Zhang. However these taxa are often endemic or nearly so to one location, as is the case with *S. nivea* and *S. sunii* (*S. alticola* is more widespread, but restricted to swamps of southeastern Australia) and the species status of some names are in doubt: e.g., Chen et al. (2010) propose *S. sunii* may be a synonym of *S. hongkongensis* S.Y. Hu & Barretto (cf. *S. spiralis* × *S. sinensis*). The results we discuss here are the first to put the names/taxa *Spiranthes australis* (R. Br.) Lindl., *Spiranthes lancea* (Thunb. ex Sw.) Backer, Bakh. & Steenis, *S. nivea*, and *S. sunii* in a molecular context, and to place them within the broader perspective of *S. sinensis* from an expansive geographic area.

Although our results must be considered preliminary due to overall limited individual sampling and issues we discuss below, some broad conclusions may be made:

Incongruence and lack of resolution. In general, our datasets are incongruent, with incongruences present between not just plastid vs. non-plastid datasets, but between plastid vs. nrITS vs. *ACO* vs. *Xdh* (fig. Supp 2-8). Furthermore, comparisons are challenging because of the low resolution afforded by some loci (e.g., nrITS, *Xdh*). Alternatively, this low phylogenetic resolution could be interpreted as being informative, indicating that the accessions share very recent common ancestors, and may be considered the same species notwithstanding *a priori* taxonomic bias, wide geographic separation and morphological variability in floral color and shape, and/or overall plant size.

Possible non-monophylly of *S. sinensis*. In reconstructions with higher degrees of resolution (combined nuclear + plastid, combined plastid, *ACO*), the recovered clades indicate *S. sinensis* is non-monophyletic. However these clades do not correspond to previous taxonomies, an obvious geographic explanation such as the Wallace Line, or readily apparent morphological character (e.g., white vs. pink flowers).

Spiranthes lancea may warrant recognition. *Spiranthes lancea* –as indicated by the combined nuclear + plastid, combined plastid, and nrITS datasets– may be distinct from *S. sinensis*, under which it is currently synonymized. Furthermore, *S. lancea* shares a most recent common ancestor with the included accession of *S. sinensis* from Malaysia, indicating this putative taxon may be distributed throughout southeastern Asia and the Malay Peninsula. Morphological examination of these plants and the characters they display are ongoing.

The position of *S. nivea* and its possible affinity with *S. hongkongensis*. *Spiranthes nivea* also appears to be distinct from *S. sinensis*, but its position within the clades of the complex is

incongruent between different datasets. The combined plastid reconstruction indicates *S. nivea* is sister to wide-ranging subclades of *S. sinensis* from East Asia and Australia –including *S. australis* and *S. sunii*. In contrast, *Xdh* and *ACO* place *S. nivea* within broad groupings of sometimes poorly resolved *S. sinensis*. However nrITS recovers *S. nivea* as sister to *S. lancea*, which in turn are fully supported as being distinct from *S. sinensis*.

Observations of *S. nivea* in the greenhouse indicate it is autogamic. The ovaries of plants grown in the UW–Madison Botany Greenhouses without insects all swelled, often just at full anthesis, and all produced fruit and seed –the viability of this seed was not explored. Most seeds lacked embryos, and those that did present embryos were monoembryonic, suggesting autogamy. Polyembryonic seeds arising through facultative adventitious embryonic apomixis have been documented in the *S. cernua* species complex (Leavitt 1900, 1901; Schnarf 1929; Swamy 1948; Catling, 1982; Sheviak 1982; Schmidt and Antifinger 1992). It is possible these seeds arose through parthenogenesis, but this has not been documented elsewhere in the genus. Morphological examination finds that the rostellum of *S. nivea* is poorly developed, and it lacks a viscidium (supporting the original description), thus hindering or making impossible the removal of pollinia under natural conditions. Although the pollinaria do not appear to come into direct contact with the main surface area of the stigmatic surface, they do abut the upper area of the stigma; thus supporting the occurrence of autogamy. The role of apomixis vs. autogamy in *S. nivea* –indeed the within the whole *S. sinensis* species complex– is deserving of continued inquiry. Comparisons of the role of apomixis vs. autogamy in this wider group would complement similar work focusing on the *S. cernua* species complex (Sheviak 1982; Catling 1982).

The likelihood of autogamy, lack of a viscidium, and a poorly developed rostellum, when combined with similarities in labellum, callosity, and gross floral morphology and color, indicate an affinity and possible relationship –synonymy? – between *S. nivea* and *S. hongkongensis*. Upon close examination the labellum and column of *S. nivea* vs. *S. hongkongensis* are nearly indistinguishable. Similarly, Hong Kong and Taiwan are located in the same general area and experience the same regional humid subtropical climate. The only apparent differences between the two putative taxa are a larger floral bract in *S. nivea,* and papillate labellum and pubescent sepals in *S. hongkongensis* (vs. glabrous in *S. nivea*). These may be valid morphological differences, however the presence of labellum papillae is known to vary in other species (e.g., *S. magnicamporum* (Chapter 1)).

Spiranthes sunii. The position of *S. sunii* remains clouded. The included sample, a leaf fragment from the isotype sheet graciously donated by Boufford, failed to amplify for most included loci; subsequently our reconstructions of its topological placement are necessarily based on limited molecular information. As with *S. nivea*, we were unable to directly test the hypothesis that *S. sunii* and *S. hongkongensis* are synonyms because material of the later species was unavailable for sampling. However, if *S. sinensis* s.l. in part –minus *S. lancea* and *S. sinensis* from Malaysia– is accepted, then *S. sunii* is a synonym of *S. sinensis* due to its recovery with this expansive clade based on our combined plastid dataset. Conversely, if *S. sinensis* s.l. is instead interpreted as broadly non-monophylletic, consisting of at least 3 clades –i.e. tentative taxa– then *S. sunii* may be either considered a distinct species or part of one of the disparate *S. sinensis* clades.

Recommendations for continued research within the *S. sinensis* species complex. Because of the issues and uncertainties we discuss above, we do not recommend any formal taxonomic

changes for the *S. sinensis* species complex at this time. Continued, far more geographically, morphologically, and taxonomically expansive and comprehensive sampling is clearly needed, particularly from Australia, India, Indonesia, New Zealand, Southeast Asia, and the greater Himalayan and Hengduan mountain systems and Sichuan Basin. Similarly, Next-Generation Sequencing methods represent a promising avenue for resolving this challenging complex. Additionally, research into the possibility of peloria, apomixis, autogamy, and pollination biology are clearly necessary.

Character Evolution. One of the main challenges in *Spiranthes* systematics is the similarity of most species: they are mostly uniform to a large degree in habitat preference, floral color, and leaf shape. A few species have just one distinct unique character (e.g., pointed trichomes in *S. vernalis*), but many species do not appear to have any such exclusive features, and are best distinguished based on unique suites of characters. Our morphometric analyses confirm this, showing that most of the characters we included have evolved multiple times across the genus.

Within *Spiranthes*, it appears that from an ancestral suite of characters – centrally white-colored oblong labellum, tuberous roots, lanceolate leaves present at the time of flowering, and an allogamous breeding system– other characters have repeatedly evolved (such as a yellow labellum). An example of a reversal –evolving a yellow labellum from a white labellum, and then back to white– can be seen in the clades of *Spiranthes* from Eastern North America: the *S. lacera* species complex/grade and *S. cernua* species complexes (fig. 12). A yellow labellum evolved in the shared common ancestor of *S. odorata* and the *S. lacera* complex grade + the *S. cernua* species complex s.l. This feature is retained in most of the species within the *S. lacera* species complex/grade, and *S. longilabris* and *S. igniorchis*. A reversal to a white labellum

occurred in the split between the *S. acrisepalum* + *S. cernua* s.s. + *S. ochroleuca* clade and the *S. magnicamporum* + *S. ovalis* + *S. triloba* clade, with a two distinct secondary re-evolutions of a yellow labellum in the sister-species pair of *S. magnicamporum* and *S. triloba*, and *S. ochroleuca* and *S. casei*.

Shifts in labellum color (fig. 12), flower color (fig. 13), and labellum texture (fig. 14) appear to be de-coupled from shifts in pollinator or perceivable floral fragrance. The vast majority of *Spiranthes* are pollinated by multiple species of *Bombus*, with the same *Bombus* species pollinating yellow, green or yellow-green, and white labella. *Spiranthes magnicamporum, S. triloba*, and *S. ochroleuca* (to a lesser extent) are fragrant with a yellow labellum, whereas *S. longilabris, S. vernalis,* and *S. brevilabris* are not fragrant and also have a yellow labellum.

One feature which provides some utility in delimiting clades of species is lateral sepal position (fig. 11). From an ancestral condition of the sepals being held in-line with the profile of the flower (a condition retained by *S. lucida* and *S. infernalis* of the Western Cordillera clade, the *S. praecox*/*S. sylvatica*/ *S. graminea*/*S. nebulorum* clade, and many species of the Old World species), three main types have evolved: 1) semi-oblique (present in the Western Cordillera clade, and *S. igniorchis* of the *S. cernua* species complex); 2) below the profile of the flower (present primarily in the *S. lacera* species complex/grade, *S. tuberosa,* and *S. sinensis*; and 3) ascending (a condition that predominates within the *S. cernua* species complex, in addition to some members of the Western Cordillera complex.

CONCLUSIONS.

Although we present the most complete taxon sampling of *Spiranthes* both in terms of number of individual samples and species, and progress has been made in understanding evolutionary and morphological relationships within the genus, many questions still remain. The characteristics that define *Spiranthes* –within species phenotypic plasticity, shared pollinators and habitats, small flowers with few discreet characters– make it both an interesting and challenging genus to study. It is our hope that the present contribution can serve as a foundation for continued molecular phylogenetic and morphological research within clades of species. Although individual plants of *Spiranthes* are easily overlooked in the field, these small but intriguing plants provide important insights into the evolution of the North American and global orchid flora.

Balogh, P. 1982. Generic redefinition in subtribe Spiranthinae (Orchidaceae). American Journal of Botany, 69: 1119–1132.

Brown, P.M. 1999a. Recent taxonomic and distributional notes from Florida 1. North American Native Orchid Journal, 5(1): 7–15.

Brown, P.M. 1999b. Recent taxonomic and distributional notes from Florida 4. North American Native Orchid Journal, 5(4): 358–362.

Brown, P.M. 2000. Recent taxonomic and distributional notes from Florida 5. North American Native Orchid Journal, 6(1): 64–66.

Brown, P.M. 2001. Recent taxonomic and distributional notes from Florida 11: *Spiranthes sylvatica* P.M. Brown, a new species of ladies'-tresses from the southeastern United States. North American Native Orchid Journal, 7(3): 193–205.

Brown, P.M. 2006. North American Native Orchid Journal, 12: 61.

CalPhotos. 2012. *Spiranthes infernalis*. Regents of the University of California, Berkeley. Accessed on 28 January 2015. Available online at: http://calphotos.berkeley.edu/.

Catling, P.M. 1978. Taxonomic notes on *Spiranthes casei* Catling & Cruise and *Spiranthes* × *intermedia* Ames. Rhodora, 80: 377–389.

Catling, P.M. 1980. Taxonomy of autumn-flowering *Spiranthes* species of southern Nova Scotia. Canadian Journal of Botany,

Catling, P.M. 1982. Breeding systems of northeastern North American *Spiranthes* (Orchidaceae). Canadian Journal of Botany, 60: 3017–3039.

Catling, P.M., and V.R. Catling. 1988. *Spiranthes nebulorum* (Orchidaceae), a new species from southern Mexico and Guatemala. Rhodora, 90(862): 139–145.

Chen, X., S.W. Gale, and P.J. Cribb. 2010. *Spiranthes*. *In* Flora of China; eFloras (2008). Published on the Internet http://www.efloras.org [accessed 15 January 2015]. Missouri Botanical Garden, St. Louis, MO and Harvard University Herbaria, Cambridge, MA

Clausen, J., D.D. Keck, and W.M. Hiesey. 1940. Experimental studies on the nature of species. I. Effects of varied environments on western North American plants. Carnegie Inst. Wash. Publ. 520pp.

Clausen, J., D.D. Keck, and W.M. Hiesey. 1948. Experimental studies on the nature of species. III. Environmental responses of climatic races of *Achillea*. Carnegie Inst. Washington Pub. No. 581. 129 pp.

Cornish-Bowden, A. 1985. Nomenclature for incompletely specified bases in nucleic acid sequences: recommendations 1984. Nucleic Acids Research, 13(9): 3021–3030.

Drummond, A.J., Suchard, M.A., Xie, D. & Rambaut, A. 2012. Bayesian Phylogenetics with BEAUti and the BEAST 1.7. Molecular Biology and Evolution, 29, 1969–1973.

Dueck, L.A., and K.M. Cameron. 2007. Sequencing re-defines *Spiranthes* relationships, with implications for rare and endangered taxa. Lankesteriana, 7(1–2): 190–195.

Dueck, L.A., and K.M. Cameron. 2008a. Molecular evidence on the species status and phylogenetic relationships of *Spiranthes parksii*, an endangered orchid from Texas. Conservation Genetics, 9: 1617–1631.

Dueck, L.A., and K.M. Cameron. 2008b. Molecular evidence for new species – *Spiranthes stellata*. North American Orchid Journal, 14(1): 12–21.

Dueck, L.A., D. Aygoren, and K.M. Cameron. 2014. A molecular framework for understanding the phylogeny of *Spiranthes* (Orchidaceae), a cosmopolitan genus with a North American center of diversity. American Journal of Botany, 101(9): 1551–1571.

Górniak, M., O. Paun, and M.W. Chase. 2010. Phylogenetic relationships within Orchidaceae based on a low-copy nuclear coding gene, *Xdh*: Congruence with organellar and nuclear ribosomal DNA results. Molecular Phylogenetics and Evolution, 56: 784–795.

Govaerts, R., P. Bernet, K. Kratochvil, G. Gerlach, G. Carr, P. Alrich, A.M.Pridgeon, J. Pfahl, M.A. Campacci, D. Holland Baptista, H. Tigges, J. Shaw, P. Cribb, A. George, K. Kreuz, J. Wood. 2014. World Checklist of Orchidaceae. Facilitated by the Royal Botanic Gardens, Kew. Published on the Internet; http://apps.kew.org/wcsp/ Retrieved 2014-12-12.

Guo, Y-Y, Y-B Luo, Z-J Liu, and X-Q Wang. 2012. Evolution and Biogeography of the Slipper Orchids: Eocene Vicariance of the Conduplicate Genera in the Old and New World Tropics. PLoS ONE 7(6): e38788. doi:10.1371/journal.pone.0038788

Katharine, R.H., P.W. Gabrielson, S.C. Lindstrom, and P.T. Martone. 2014. Misleading morphologies and the importance of sequencing type specimens for resolving coralline taxonomy (Corallinales, Rhodophyta): *Pachyarthron cretaceum* is *Corallina officinalis*. Journal of Phycology, 50(4): 760–764.

Jacobsen, S., W. Brown, B. McGee, J. McIntyre, and C. Best. 2009. 5-Year Review: Navasota Ladies'-Tresses/*Spiranthes parksii* Correll. U.S. Fish and Wildlife Service Austin Ecological Services Field Office Austin, Texas.

Leavitt, R.G. 1900. Polyembryony in *Spiranthes cernua*. Rhodora, 2: 227–228.

Leavitt, R.G. 1901. Notes on the embryology of some New England orchids. Rhodora, 3: 61–63.

Lindley, J. 1830-1840. The genera and species of orchidaceous plants. Ridgways, London, U.K.

Mace, G.M. 2004. The role of taxonomy in species conservation. Philosophical Transactions of the Royal Society London B, 360: 289–295.

May, R.M. 1990. Taxonomy as destiny. Nature, 347: 129–130.

Miller, M.A., Pfeiffer, W., Schwartz, T., 2010. Creating the CIPRES science gateway for inference of large phylogenetic trees. Proceedings of the Gateway Computing Environments Workshop (GCE), New Orleans, LA, pp 1–8.

Nevada Natural Heritage Program. 2001. Rare Plant Fact Sheet: *Spiranthes infernalis*. [http://heritage.nv.gov/sites/default/files/atlas/spirainfer.pdf]. Accessed 27 Jan 2015.

Neubig, K.M., W.M. Whitten, B.S. Carlsward, M.A. Blanco, L. Endara, N.H. Williams, and M. Moore. 2009. Phylogenetic utility of *ycf*1 in orchids: a plastid gene more variable than *mat*K. Plant Systematic Evolution, 277: 75–84.

Pedron, M., C.R. Buzatto, A.J. Ramalho, B.M. Carvalho, J.A. Radins, R.B. Singer, and J.A Batista. 2014. Molecular phylogenetics and taxonomic revision of *Habenaria* section Pentadactylae (Orchidaceae, Orchidinae). Botanical Journal of the Linnean Society, 175(1): 47–73.

Piller, K.R., and Bart Jr., H.L. 2009. Incomplete sampling, outgroups, and phylogenetic inaccuracy: A case study of the Greenside Darter complex (Percidae: *Etheostoma blennioides*). Molecular Phylogenetics and Evolution, 53: 340–344.

Potter, D., and J.V. Freudenstein. 2005. Character-Based Phylogenetic Linnaean Classification: Taxa Should Be Both Ranked and Monophyletic. Taxon, 54(4): 1033–1035.

Powell, R.F., J.S. Boatwright, and A.R. Magee. 2014. A taxonomic revision of the *Cotula coronopifolia* group (Asteraceae) and implications for the conservation statuses of the species. South African Journal of Botany, 93: 105–117.

RStudio. 2014. RStudio: Integrated development environment for R (Open Source Edition; Version v0.98.932) [Computer software]. Boston, MA. Retrieved September 25, 2014.

Rambaut. A. 2014. FigTree v1.4.2, released 2014-07-09. http://tree.bio.ed.ac.uk/software/figtree/

Rapp, R.A. and J.F. Wendel. 2005. Epigenetics and plant evolution. New Phytologist, 168: 81–91.

Roca, A.L., N. Georgiadis, J. Pecon-Slattery, and S.J. O'Brien. 2001. Genetic evidence for two species of elephant in Africa. Science, 293(5534): 1473–1477.

Salazar, G.A., M.W. Chase, M.A. Soto, and M. Ingrouille. 2003. Phylogenetics of Cranichideae with emphasis on Spiranthinae (Orchidaceae, Orchidoideae): evidence from plastid and nuclear DNA sequences. American Journal of Botany, 90: 777–795.

Salazar, G.A., L.I. Cabrera, S. Madriñán, and M.W. Chase. 2009. Phylogenetic relationships of Cranichidinae and Prescottiinae (Orchidaceae, Cranichideae) inferred from plastid and nuclear DNA sequences. Annals of Botany, 104: 403–416.

Salazar, G.A., and L. Jost. 2012. *Quechua*, New Monotypic Genus of Andean Spiranthinae (Orchidaceae). Systematic Botany, 37(1): 78–86.

Schmidt, J.M., and A.E. Antifinger. 1992. The level of agamospermy in a Nebraska population of *Spiranthes cernua* (Orchidaceae). American Journal of Botany, 79(5): 501–507.

Schnarf, K. 1929. *Embryologie der Angiospermen*. Borntrager, Berlin.

Sheviak, C.J. 1973. A new *Spiranthes* from the grasslands of central North America. Botanical Museum Leaflets 23(7): 287–297.

Sheviak, C.J. 1982. Biosystematic study of the *Spiranthes cernua* complex. New York State Museum Bull. 448: 1–73.

Sheviak, C.J. 1984. *Spiranthes diluvialis* (Orchidaceae): a new species from the western United States. Brittonia 36(1): 8–14.

Sheviak, C.J. 1989. A new *Spiranthes* (Orchidaceae) from Ash Meadows, Nevada. Rhodora, 91:225–234.

Sheviak, C.J. 1990. A new *Spiranthes* (Orchidaceae) from the cienegas of southernmost Arizona. Rhodora, 92(872): 213–231.

Sheviak, C.J. 1991. Morphological variation in the compliospecies *Spiranthes cernua* (L.) Rich.: ecologically-limited effects of gene flow. Lindleyana, 6(4): 228–234.

Sheviak, C.J. 2012. A new and rare *Spiranthes* from the Sierra Nevada. The Native Orchid Conference Journal, 9(4): 1–6.

Sheviak, C.J. 2013. A cytogeographic survey of *Spiranthes romanzoffiana* Cham. and its taxonomic implications. The Native Orchid Conference Journal, 10 (2): 1–22.

Sheviak, C.J., and P.M. Brown. 2002. *Spiranthes*. In *Flora of North America*, Volume 26. Oxford University Press, USA.

Sheviak, C.J., and P.M. Catling. 1980. The identity and status of *Spiranthes ochroleuca* (Rydberg) Rydberg. Rhodora, 82(832): 525–562.

Stebbins, L.S. 1950. Variation and Evolution in Plants. Columbia University Press, New York, 643 pp.

Sun, M. 1996. Effects of population size, mating system, and evolutionary origin on genetic diversity in *Spiranthes sinensis* and *S. hongkongensis*. Conservation Biology, 10 (3): 785–795.

Sun, M. 1997. Genetic diversity in three colonizing orchids with contrasting mating systems. American Journal of Botany, 84(2): 224–232.

Swamy, B.G.L. 1948. Agamospermy in *Spiranthes cernua*. Lloydia, 11: 149–162.

Tsukaya, H. 1994. *Spiranthes sinensis* var. *amoena* in Japan contains two seasonally differentiated groups. Journal of Plant Research, 107(1086): 187–190.

Tsukaya, H. 2005. Molecular variation of *Spiranthes sinensis* (Orchidaceae) in Japan, with special reference to systematic treatment of seasonally differentiated groups and a dwarf form, f. gracilis, from Yakushima Island. Journal of Plant Research, 118: 13–18.

Walters, C. 2005. Genetic relationships among *Spiranthes parksii* and congeneric species. Master's Thesis. Texas A&M University

Wheeler, W.C. 1990. Nucleic acid sequence phylogeney and random outgroups. Cladistics, 6: 363–367.

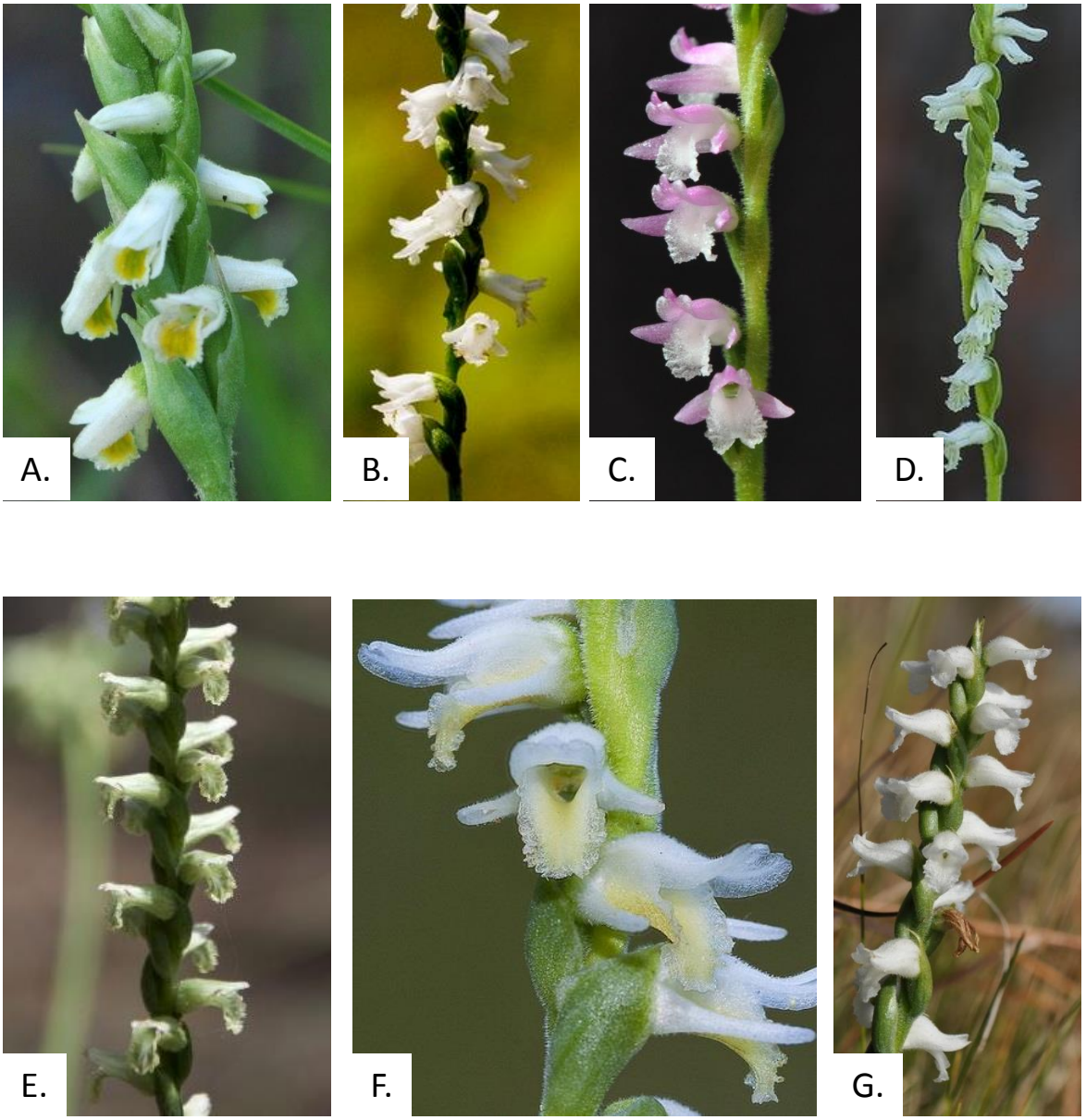


Fig1. Examples of morphological variation of *Spiranthes*.
A. *Spiranthes lucida*. **B.** *Spiranthes tuberosa*. **C.** *Spiranthes sinensis*.
D. *Spiranthes praecox*. **E.** *Spiranthes sylvatica*. **F.** *Spiranthes vernalis*.
G. *Spiranthes cernua*.

Fig. 2. [Following nine pages] Sample, amplification, and voucher information for individual samples used in molecular phylogenetic analyses. Sample ID, taxon, collection site, collection date, collector, loci/spacer/gene region, and voucher information at top. An “X” indicates a positive reaction.



ID #	Species/Variety	Collection Site	Collector	Coll.Date	ACO	ITS	matK	ndhj	trnLF	trnSM	Xdh	ycf1 3'	Herbarium specimen	Photo Voucher
	S. aestivalis													
21058	*S. aestivalis*	#21058 - Kew micropropagation	RBG-Kew	(rec'd	x	x	x	x	x	x	x	x	K-21058	
21059	*S. aestivalis*	#21059 - Switzerland	RBG-Kew	4/10/06	x	x	x	x	x	x	x	x	K-21059	
	S. australis													
Sau1	*S. australis*		G. Salazar		x	x	x	x	x	x	x	x	Clemants S-346	
	S. brevilabris													
1a	*S. brevilabris*	Levy, FL	PMBrown	5/7/03			x	x	x	x				
1e	*S. brevilabris*	Walker Co., TX	Eric Keith	4/6/07		x	x	x	x	x		x	82213	yes
1g	*S. brevilabris*	Levy Co. (Goethe St.For.), FL	Scott Stewart	4/1/06	x	x	x	x	x	x	x	x		
	arcisepalum. Northeast/Appalachian													
4c	*S. cernua*	Giles Co., VA	Stan Bentley	9/6/03			x	x	EU384709	x		x		
4e	*S. cernua*	Armstrong Co., PA (ne-Pit)	Scott Shriver	9/20/03		x	x	x	EU384711	x		x		
4f	*S. cernua* (1 of 2 bags)	Somerset Co., PA (se-Pit)	Scott Shriver	9/20/03		x	x	x	x	x		x		
4g	*S. cernua*	Westmoreland Co., PA (e-Pit)	Scott Shriver	9/20/03		x	x	x	x	x		x		
4s	*S. cernua* (moldy)	Arookstook Co., ME	PMB	9/13/06			x	x	x	x		x		
4u	*S. cernua* (lg lf)	Seneca Co., OH	David McCann	9/25/06	x	x	x	x	x	x	x	x		yes
4y	*S. cernua* (giant)	Oneida Co., NY (Oriskany)	Charles Ufford	10/3/06	x	x	x	x	x	x	x	x	82220	yes
4z	*S. cernua* (giant)	Singer Bog, OH	Tim M. Jones	10/1/06	x	x	x	x	x	x	x	x	82216-7*	yes
NY1	*S. cernua*	Delaware Co., NY	M.C. Pace	_2015		x	x	x	x		x		Pace s.n.	yes
sc30a		Seneca Co., OH	M.C. Pace	_2015		x	x	x	x		x		Pace s.n.	yes

	ochroleuca/casei													
2a	*S. casei* var. *casei*	Montmorency Co., MI	Fred Case	~9/04	x	x	x	x	x	x	x	x	80510	
2b	*S. casei* var. *casei* (atyp?)	Newaygo Co., MI	Fred Case	9/3/04	x	x	x	x	x	x		x		
2d	*S. casei* var. *casei*	Marinette Co. (Pond 518), WI	Kip Knudson	9/15/06	x	x	x	x	x	x	x	x		yes
2e	*S. casei* var. *casei*	Hamilton Co., NY (Speculator)	Charles Ufford	9/16/06	x	x	x	x	x	x	x	x		
4h	*S. ochroleuca*	Randolph Co., WV	Scott Shriver	10/19/03			x	x	EU384712	x		x		
4n	*S. ochroleuca*	Essex Co., NY (Muller Pond)	Ken Cameron	9/4/06	x	x	x	x	x	x	x	x	82209	
4p	*S. ochroleuca*	Washington Co., ME	Lucy Dueck	8/26/06		x	x	x	x	x	x	x		yes
4wx	*S. ochroleuca*	Lucas Co., OH	Lucy Dueck	10/4/06	x	x	x	x	x	x		x	82206, 82208	yes
16a	*S. ochroleuca*	Oconee Co., SC	Jim Fowler	10/25/02		x	x	x	x	x		x	80494	yes
16b	*S. ochroleuca*	Craig Co., VA	Stan Bentley	9/13/03	x	x	x	x	x	x		x		
16c	*S. ochroleuca*	NH	PMBrown	9/14/03	x	x	x	x	x	x			80498	yes
16e	*S. ochroleuca*	Allegany Co., MD	Scott Shriver	10/4/03		x	x	x	x	x		x		
16f	*S. ochroleuca*	Candia, NH	Jean Stefanik	9/13/04	x	x	x	x	x	x		x	80505-6	
16g	*S. ochroleuca*	Saginaw Co., MI	Fred Case	9/8/04	x	x	x	x	x	x		x		
16h	*S. ochroleuca*	Oneida Co., NY (Camden)	Charles Ufford	10/3/06	x	x	x	x	x	x		x	82219	yes
ci1a	*S. casei*	Ontario, Canada	Nancy Van Alstine	9/1/14	x	x	x							
	S. ×kapnosperia (cernua x ochroleuca)													
16i	*S. ochroleuca* (was **4d**)	Oconee Co., SC (Blue Hole Falls)	Lucy Dueck	11/1/03		x	x	x	EU384710	x		x		yes
sc20a	*S.cernua*	Macon Co., NC	Wen	9/27/07	x	x		x	x	x		x	Wen 10003 (US)	
sh2	*S. ochroleuca*	Transylvania Co., NC	Jim Fowler	10/8/13	x	x	x	x	x	x		x		
	cernua/parksii /Coastal Plain/Piedmont													
4b	*S. cernua* (**BCDEFGH**)	Greenville Co., SC	Jim Fowler	10/25/02		x	x		EU384708	x		x	80495 (7)	yes
4j	*S. cernua* (scw21)	Brazos Co., TX	Cathy Walters (DNA)	10/20/03		x	x		EU384713	x			TAMU	
4k	*S. cernua* (scp61)	Brazos Co., TX	Cathy Walters (DNA)	10/20/03		x	x		EU384714	x			TAMU	
4L	*S. cernua* (green)	Henderson Co., TX	Lucy Dueck	11/6/04	x	x	x	x	EU384715	x			80491	yes
4m	*S. cernua* (deep south race)	Liberty Co., FL (via UFL)	Scott Stewart	7/3/06	x	x	x	x	x	x	x	x		yes
4cc	*S. cernua* **(JF-B1)**	Marlboro Co., SC. (Bennettsvl.)	Jim Fowler	10/21/06	x	x	x	x	x	x		x		yes
4dd	*S. cernua* **(JF-B3)**	Brunswick Co., NC (BoilSprLk)	Jim Fowler	10/29/06	x	x	x	x	x	x		x		
4ee	*S. cernua* - Chadd's Ford **(17k)**	cultivar - White Flwr. Farm, CN	Lucy Dueck/KC	8/19/06	x	x	x	x	x	x	x	x		
4ff	*S. cernua* - Chadd's Ford **(17L)**	cultivar - Sunshine Grdns., WV	Jeff Patton	10/5/06	x	x	x		x	x	x	x		
sc1b	*S. cernua*	New Castle Co., DE	M.C. Pace	9/29/13	x	x		x	x	x		x		
sc6d	*S. cernua*	Cape May, NJ	M.C. Pace	10/19/13	x	x	x	x	x	x	x	x	yes	yes
sc8d	*S. cernua*	MD	M.C. Pace	10/23/13	x	x	x	x	x	x	x	x		
sc9a	*S. cernua*	Little Creek Reservoir, James City, VA	M.C. Pace	10/25/13	x	x		x	x	x		x		
sc15b	*S. cernua*	Wayne Co, GA	M.C. Pace	11/6/13	x	x	x	x	x	x		x		
sc22	*S. cernua*	Brasstown Seeps, Towns Co., GA	Jim Fowler			x	x			x				yes
sc25	*S. cernua*	NC	Jim Fowler			x	x							yes
sc28	*S. cernua*	Aberdeen Proving Grounds, Monks Island, Harford Co., MD		10/13/13	x	x			x	x		x		
AR29	*S. cernua*		M.C. Pace		x	x	x						Pace s.n.	yes
TX1	*S. parksii*		M.C. Pace		x	x	x	x	x			x		yes
20a	*S. parksii* - **a** (sp5)	Brazos Co., TX	Cathy Walters (DNA)	10/8/03		x	x	x	x	x		x	TAMU	yes
20b	*S. parksii* - **b** (sp137)	Brazos Co., TX	Cathy Walters (DNA)	10/22/03		x	x	x	x	x		x	TAMU	
20c	*S. parksii* - **c** (sp175)	Brazos Co., TX	Cathy Walters (DNA)	11/22/03		x	x		x	x			TAMU	
sp70	*S. parksii*	Brazos Co., TX	Cathy Walters (DNA)	10-11/03		x	x		x	x			TAMU	
sp146	*S. parksii*	Brazos Co., TX	Cathy Walters (DNA)	10-11/03		x	x		x	x			TAMU	
sp167	*S. parksii*	Brazos Co., TX	Cathy Walters (DNA)	10-11/03		x	x		x	x			TAMU	
sp267	*S. parksii*	Brazos Co., TX	Cathy Walters (DNA)	10-11/03		x	x		x	x			TAMU	

	S. cernua x S. magnicamporum Upper Plains and Great Lakes Basin													
4a	*S. cernua* (S. cernua x S. magni)	NE	R.Bischof/NGPC (DNA)	≤2001		x	gone?		EU384707	x				
4t	*S. cernua* (S. cernua x S. magni)	Marinette Co. (Spur Lake), WI	Kip Knudson	9/15/06	x	x	x	x	x	x	x	x		
4v	*S. cernua* (S. cernua x S. magni)	Seneca Co., OH	David McCann	9/25/06	x	x	x	x	x	x	x	x		yes
4aa	*S. cernu* (S. cernua x S. magni)	Kalamazoo Co., MI	Nick Stoynoff/MSU herb.	8/26/82		x	x		x	x			MSC283303	
4bb	*S. cernua* (S. cernua x S. magni)	Huron Co., MI	Julian Donahue/MSU herb.	9/20/63		x	x		x	x			MSC190339	
15a1	*S. magnicamporum* (SM1) [S. cernua x magni]	IA	R.Bischof/NGPC (DNA)	≤2001		x	x (14)		x	x			NEB	
sm1a	*S. cernua x S. magni*	Arboretum, Dane Co., WI	M.C.Pace	_2013		x	x	x	x	x	x	x	Pace s.n.	yes
sm1c	*S. cernua x S. magni*	Arboretum, Dane Co., WI	M.C.Pace	_2013	x	x	x	x	x	x	x	x	Pace s.n.	yes
sc31a		Portage Co., OH	M.C.Pace	_2015		x	x	x	x		x		Pace s.n.	yes
sc32a		Clark Co., OH	M.C.Pace	_2015		x	x	x	x		x		Pace s.n.	yes
sc33a	*S. cernua*	Ontario, Canada	M.C.Pace	_2015	x	x	x	x	x		x		Pace s.n.	yes
sc35a	*S. cernua*	Ontario, Canada	M.C.Pace	_2015		x	x	x	x				Pace s.n.	yes
MI1		MI		_2015		x		x	x			x		yes
sm21r		Chiwaukee Prairie, Kenosha Co., WI	M.C.Pace	_2015	x		x	x	x		x	x	Pace s.n.	yes
sm23b		Illinois Beach State Park, Lake Co., IL	M.C.Pace	_2015	x		x	x	x			x	Pace s.n.	yes
sm27d	*deistogomous*	Marquette Park, Lake Co., IN	M.C.Pace	_2015			x					x	Pace s.n.	yes
soch10a		Orleans Co., VT	M.C. Pace	_2015	x	x	x	x	x				Pace s.n.	yes
	S. delitescens													
5a	*S. delitescens*	Santa Cruz Co., AZ	Aaron Hicks	5/2005	x	x	x	x	x	x	x	x	in hort.	
5b	*S. delitescens*	Santa Cruz Co., AZ	Aaron Hicks	5/2005	x	x	x	x	x	x	x	x	in hort.	
5c	*S. delitescens*	Santa Cruz Co., AZ	Aaron Hicks	5/2005	x	x	x	x	x	x	x	x	in hort.	
	S. diluvialis													
6a1	*S. diluvialis* (**SD1**)	NE	R.Bischof/NGPC (DNA)	<2001		x	x	x	x	x		x	NEB	
sd1	*S. diluvialis*	Fort Hall Indian Reservation, Bannock Co., I	C.J. Sheviak	8/10/2009	x	x	x	x	x	x		x	C.J. Sheviak 7087 (NYS)	
sd2	*S. diluvialis*	Chelan Co., WA	Kathryn A. Beck	8/3/2000		x	x	x	x	x		x	K.A Beck 200025 (NYS)	
	S. eatonii													
7a	*S. eatonii*	Clay Co., FL	PMBrown	5/8/03		x	x	x	x	x		x		
7b	*S. eatonii*	Clay Co., FL	PMBrown	4/2003		x	x		x	x	x	x		
	S. floridana													
8a	*S. floridana*	Bradford Co., FL	PMBrown	5/8/03		dbl	x	x	x	x				
8b	*S. floridana*	Bradford Co., FL	PMBrown	4/2003	x	x	x		x	x				
8c	*S floridana* (**Sbf**)	Franklin Co., FL	John Churchill/MSU herb.	4/27/73		dbl/faint	x (1-4)		x	x			MSC243112	
8f	*S. floridana*	Duval Co. (Jennings St.For.), FL	Scott Stewart	4/2/06	x	x	x	x	x	x		x		

	S. graminea													
SG1	*S. graminea*		G. Salazar		x	x	x	x	x	x	x	x		
	S. infernalis													
9a	*S. infernalis*	Nye Co., NV	Carol Siegel/Jody Fraser	6/19/04	x	x	x		x	x	x	x	80511*	yes
9b	*S. infernalis*	Nye Co.,NV	Carol Siegel/Jody Fraser	6/19/04	x	x	x		x	x	x	x	82229	yes
9c	*S. infernalis*	Nye Co.,NV	Carol Siegel/Jody Fraser	6/19/04	x	x	x		x	x	x	x		yes
	S. lacera													
10c	*S. lacera var. lacera*	Shannon Co., MO	Bill Summers	8/31/04	x	dbl	x	x	x	x	x		80500	
10d	*S. lacera var. lacera*	Oconto Co., WI	Kip Knudson	7/28/06	x	x	x		x	x	x			yes
	S. lacera var. gracilis													
11a	*S. lacera var. gracilis*	Pickens Co., SC	Jim Fowler	7/12/03	x	x	x	x	x	x	x	x	80497	
11b	*S. lacera var. gracilis*	Monroe Co., WV	Stan Bentley	9/1/03	x	x	x		EU384724	x		x		
11c	*S. lacera var. gracilis*	Forsyth Co., NC	David McAdoo	8/8/2004	x	x	x (14)	x	x	x		x	80504	
11d	*S. lacera var. gracilis* (sg2)	Brazos Co., TX	Cathy Walters (DNA)	9/23/03		x	x	x	x	x		x	TAMU	yes
11e	*S. lacera var. gracilis*	Shannon Co., MO	Bill Summers	8/24/04		x	x (1-4)		x	x			80502	
11f	*S. lacera* var. *gracilis* (AR1)	Pope Co., AR	George Johnson	9/22/06	x	x	x		x	x	x		UARK-5731	yes
	S. igniorchis													
12b	*S. laciniata*	Charlton Co., GA	Jim Fowler	6/18/05	x	x	x	x	x	x	x	x	80503	
Slt1a	*S. laciniata*	APARF, Polk Co., FL	Steve Orzell	6/17/14	x	x				x		x	Orzell & Bridges 26,728	
slt1b	*S. laciniata*	APARF, Polk Co., FL	Steve Orzell	6/17/14	x		x		x	x				
slt1c	*S. laciniata*	APARF, Polk Co., FL	Steve Orzell	6/18/14	x				x	x				
slt2a	*S. laciniata* calcareous wet prairie	APARF, Polk Co., FL	Steve Orzell	8/22/14	x	x	x	x		x		x	Orzell 26733	
slt2b	*S. laciniata* calcareous wet prairie	APARF, Polk Co., FL	Steve Orzell	8/22/14	x	x		x	x	x		x	Orzell 26733	
slt2c	*S. laciniata* calcareous wet prairie	APARF, Polk Co., FL	Steve Orzell	8/22/14	x	x		x	x	x		x	Orzell 26733	
slt3a	*S. laciniata* acidic wet prairie	APARF, Polk Co., FL	Steve Orzell	8/25/14	x	x	x	x	x	x		x	Orzell 26735	
slt3b	*S. laciniata* acidic wet prairie	APARF, Polk Co., FL	Steve Orzell	8/25/14	x	x		x		x		x	Orzell 26735	
slt3c	*S. laciniata* acidic wet prairie	APARF, Polk Co., FL	Steve Orzell	8/25/14	x	x		x	x	x		x	Orzell 26735	
slt4a	*S. laciniata* calcareous wet prairie	APARF, Polk Co., FL	Steve Orzell	8/25/14	x	x		x	x	x		x	Orzell 26734	
slt4b	*S. laciniata* calcareous wet prairie	APARF, Polk Co., FL	Steve Orzell	8/25/14	x	x		x	x				Orzell 26734	
26728A	*S. laciniata*	APARF, Polk Co., FL	Steve Orzell		x	x	x	x	x	x		x		
26735B	*S. laciniata* acidic wet prairie	APARF, Polk Co., FL	Steve Orzell		x	x	x	x	x	x		x		
26734B	*S. laciniata* calcareous wet prairie	APARF, Polk Co., FL	Steve Orzell		x		x	x		x		x		
26733A	*S. laciniata* calcareous wet prairie	APARF, Polk Co., FL	Steve Orzell		x			x	x	x		x		
slt5a	*S. laciniata*				x		x	x	x	x		x		

	S. longilabris													
13a	*S. longilabris*	Brunswick Co., NC	Frank Galloway	10/31/03	x	x	x	x	x	x	x	x		
13b	*S. longilabris*	(Little Is.) Brunswick Co., NC	Jim Fowler	10/24/04	x	x	x	x	x	x	x	x		
13c	*S. longilabris*	Levy Co., FL	Scott Stewart	11/20/04	x	x	x	x	x	x	x	x		
13f	*S. longilabris*	Harden Co., TX	Joe Liggio	11/21/04		x	x	x	x	x		x	82222	
sloAvon	*S. longilabris*	Polk Co, FL	Steve Orzell		x		x							
	S. lucida													
14a	*S. lucida*	Giles Co., VA	Stan Bentley	6/8/03		x	x	x	x	x	x			
14b	*S. lucida*	Pittsburgh, PA	Scott Shriver/SBentley	6/9/03			x		x	x				
14d	*S. lucida*	Newaygo Co., MI	Fred Case	6/02->9/04	x	x	x		x	x	x			
SL1a	*S. lucida*	Layfayette Fen, NY	M.C. Pace	21-Jun-12	x	x	x	x	x	x	x	x	Pace s.n.	yes
SL2a	*S. lucida*	Salmon River Falls, NY	M.C. Pace	26-Jun-13	x	x	x		x	x	x	x	M.C. Pace 558	yes
SL5a	*S. lucida*	Black River, Watertown, NY	M.C. Pace	26-Jun-13		x		x			x		Pace s.n.	yes
SL6a	*S. lucida*	Robert Moses State Park, Masena, NY	M.C. Pace	27-Jun-13	x	x	x			x	x	x	Pace s.n.	yes
SL9a	*S. lucida*	Winooski River, Burlington, VT	M.C. Pace	29-Jun-13	x		x	x	x	x	x	x	Pace s.n.	yes
SL13d	*S. lucida*	Ozark National Forest, AR	M.C. Pace	19-May-13	x	x	x	x	x	x	x	x	M.C. Pace 576	yes
SL14b	*S. lucida*	Willow Spring Sec. MTNF, MO	M.C. Pace	20-May-13	x	x	x	x	x	x	x	x	Pace s.n.	yes
SL15b	*S. lucida*	Eleven Point Sec. MTNF, MO	M.C. Pace	21-May-13	x	x	x	x	x	x	x	x	Pace s.n.	yes
SL16a	*S. lucida*	Duck Creek Wildlife Management Area, MO	M.C. Pace	22-May-13	x	x	x	x	x	x	x	x	M.C. Pace 582	yes
SL17a	*S. lucida*	Salem Sec. MTNF, MO	M.C. Pace	23-May-13	x	x	x	x	x	x	x	x	M.C. Pace 584	yes
SL18b	*S. lucida*	Potosi Sec. MTNF, MO	M.C. Pace	24-May-13	x	x	x	x	x	x	x	x	M.C. Pace 589	yes
SL19b	*S. lucida*	Mammoth Creek, MO	M.C. Pace	24-May-13	x	x	x	x	x	x	x	x	M.C. Pace 590	yes
SL20n	*S. lucida*	Menomonee River, WI	M.C. Pace	23-Jun-13	x	x	x	x	x	x	x	x	Pace s.n.	yes
sl21a	*S. lucida*	IN	M.C. Pace		x	x	x	x		x		x	Pace s.n.	yes
sl23a	*S. lucida*	TN	M.C. Pace		x	x	x	x		x		x	Pace s.n.	yes
	S. magnicamporum													
15b	*S. magnicamporum* (sm7)	Blanco Co., TX	Cathy Walters (DNA)	10/17/04		x	x (24)		x	x			TAMU	yes
15c	*S. magnicamporum*	Ozark Co., MO	Bill Summers	10/18/04		x (poor)	x	x	x	x	x		80501	
15e	*S. magnicamporum*	Grimes Co., TX	Joe Liggio	10/31/04	x	x	x	x	x	x			80489	
15f	*S. magnicamporum* - (sm)	Walworth Co., WI	Jeff Hapeman	9/16/06	x	x	x	x	x	x	x	x	82207	
15g	*S. magnicamporum* - (lg)	Walworth Co., WI	Jeff Hapeman	9/16/06	x	x	-	x	x	x	x	x		
sm7h	*S.magnicamporum*	NM	M.C.Pace	9/21/13	x	x	x	x	x	x	x	x		
sm8a	*S.magnicamporum*	Russell Co., VA		10/15/13	x	x	x		x	x	x	x		
sm11a	*S.magnicamporum*	Russell Co., VA		10/17/13	x	x	x	x	x	x	x	x		
sm12a	*S.magnicamporum*	GA	Jim Fowler	11/3/13	x	x	x	x	x	x	x	x		yes
sm13a	*S.magnicamporum*	Lanark Co., Ontario, Canada	Joyce Reddoch	9/25/13	x	x	x	x	x	x	x	x		
sm15a	*S.magnicamporum*	Marthaville, Lambton Co, Ontario, Canada	Oldham	9/7/11	x	x	x	x	x	x	x	x	Oldham 39307 (NYS)	
sm16a	*S.magnicamporum*	Black Belt Prairie, Starkville, MS	McCabe	10/29/91		x	x		x	x			McCabe s.n. (NYS)	
sm17a	*S.magnicamporum*	Cowan, Franklin co., TN	Sheviak	11/8/06	x	x		x	x	x	x	x	Sheviak 7064 (NYS)	
sm18a	*S.magnicamporum*	Honey Creek, Pottawattamie Co., IA	Rahel	9/1/98					x	x		x	Rahel s.n. (NYS)	
sm19a	*S.magnicamporum*	Chandlerville, Cass Co., IL	Sheviak	9/25/81	x	x	x		x	x		x	Sheviak 2142 (NYS)	
sm22c	*S.magnicamporum*	North Beach Park, Racine Co., WI	M.C.Pace		x		x					x	Pace s.n.	yes
sm23e	*S.magnicamporum*	Illinois Beach State Park, Lake Co., IL	M.C.Pace		x		x						Pace s.n.	yes
sm25g	*S.magnicamporum*	Van Vlissingen Prairie, Cook Co., IL	M.C.Pace		x		x					x	Pace s.n.	yes
sm26k	*S.magnicamporum*	Van Vlissingen Prairie, Cook Co., IL	M.C.Pace		x		x					x	Pace s.n.	yes
sm28h	*S.magnicamporum*	Spinn Prairie, White Co., IN	M.C.Pace		x		x					x	Pace s.n.	yes

	S. nebulorum													
SN1	S. nebulorum	Mexico	G. Salazar		x	x	x	x	x	x	x	x		
	S. nivea													
Sni	S. nivea	Taiwan	M.C. Pace		x	x	x	x	x	x	x	x	Pace s.n.	yes
	S. odorata													
17c	*S. odorata* (non-flwr'g)	Rice's Crk Br., Brunswick Co., NC	Lucy Dueck	8/8/04			x	x	x	x				
17d	*S. odorata*	Clark's Bridge, Brunswick Co., NC	Frank Galloway	10/31/03		x	x	x	x	x	x	x		
17e	*S. odorata*	Gilbert's Bridge, Brunswick Co., NC	Frank Galloway	10/31/03		x	x	x	x	x				
17g	*S. odorata*	Levy Co., FL	Scott Stewart	11/20/04		x	x	x	x	x		x		
17i	*S. odorata*	San Jacinto Co., TX	Joe Liggio	10/24/04		x	x	x	x	x	x	x	80488	
17opq	*S. odorata*	Effingham Co., GA	Darlene (L) Vincent	12/2/06	x	x	x	x	x	x	x	x	82211	yes
17z	*S. odorata*	Levy Co. (Goethe St.For.), FL	Scott Stewart	11/20/04		x	x	x	x	x	x	x		
So1d	*S. odorata*	Piney Island, NC	M.C. Pace	10/26/2013	x	x	x	x	x	x	x	x	Pace s.n.	yes
So5h	*S. odorata*	Bridge, Brunswick Co., NC	M.C. Pace	11/4/2013	x	x	x		x	x	x	x	Pace s.n.	yes
So10i	*S. odorata*	Roadside, Taylor Co., FL	M.C. Pace	11/9/2013	x	x	x		x	x	x	x	Pace s.n.	yes
so11	*S. odorata*	Kentucky	Sheviak			x	x		x	x	x	x	Sheviak 2408	
so12	*S. odorata*	Tennessee	Durr	10/1/2001	x	x	x	x	x	x	x	x	Durr s.n.	
so13	*S. odorata*	Back Bay NWR, Princess Anne Co., VA	Statler	11/4/2006	x	x			x	x		x	Statler s.n. (NYS)	
	S. ovalis var. ovalis													
18b	*S. ovalis var. ovalis*	Columbia Co., FL	PMBrown	11/11/06		x	x		x	x		x		
	S. ovalis var. erostellata													
19a	*S. ovalis var. erostellata* - **a**	Giles Co., VA	Stan Bentley	9/28/03		x	x	x	x	x		x		
19b	*S. ovalis var. erostellata* - **b**	Barnwell Co., SC (SRS)	Lucy Dueck	9/30/03		x	x		x	x			80493	yes
19c	*S. ovalis var. erostellata* - **c**	Rutherford Co., NC	Jim Fowler	10/11/03		x	x	x	x	x		x		
19d	*S. ovalis* var. *erostellata* /hybrid?	McCormick Co., SC (F659D)	Lucy Dueck	10/11/06		x	x	x	x	x		x	82210	yes
19e	*S. ovalis* var. *erostellata* - **e**	Columbia Co., FL	PMBrown	11/11/06		x	x		x	x		x		
sov2	*S. ovalis var. erostelalta*	Waukesha Co., WI	M.C. Pace		x		x					x	Pace s.n.	yes
sov3	*S. ovalis var. erostelalta*	Orange Co., NC	Jim Fowler		x		x					x		yes
	S. porrifolia													
21d	*S.porrifolia*	Curry Co. (Oak Flat), OR	Veva Stansell	6/18/05		fungus	x (1-4)		x	x			USFS-OR	yes
21f	*S.porrifolia*	Butte Co.(Lk. Oroville), CA	Lucy Dueck	6/14/06		fungus	x-f2		x	x			82215	yes
21j	*S.porrifolia*	Sierra Co. (Dn'vl., mm17.86), CA	Lucy Dueck	6/15/06		x	x		x	x	x	x	82214	yes
21m	*S.porrifolia*	Sierra Co. (Dn'vl., mm10.37), CA	Lucy Dueck	6/15/06		x	x		x	x	x	x		yes
21n	*S. porrifolia*	Curry Co. (Agness), OR	Mike Parsons	~6/14/06		x	x		x	x	x	x		yes
21o	*S. porrifolia*	Sierra Co., (Downieville), CA	Mike Parsons	~6/18/06		x	x		x	x	x	x		yes
21q	*S. porrifolia*	Marin Co., CA	Brad Kelley	6/11/06		bad-dbl	x (14)		x	x	x	x		yes
21r	*S. porrifolia*	Curry Co., OR (Lobster Crk.)	Don Heller/Veva Stansell	6/26/06		fungus	x		x	x				
21s	*S. porrifolia*	Sierra Co. (Dn'vl., mm17.86), CA	Lucy Dueck	6/15/06		x	x		x	x	x	x	(presrvd)	yes
21t	*S. porrifolia*	Josephine Co., OR	William Gray	7/1/06		dbl	x (14)		x	x		x	82223	
21u	*S. porrifolia*	San Diego Co., CA	Nick Crawford	7/12/06		x	x		x	x	x	x	SD180698	yes
21x	*S. porrifolia*	Douglas Co., OR	Hope Stubbs	Aug-06		fungus	x (14)		x	x		x		yes

	S. praecox													
22a	*S. praecox*	Bradford Co., FL	Paul Martin Brown	5/8/03		x	x	x	x	x	x	x		
22b	*S. praecox*	Charleston Co., SC	Lucy Dueck	5/18/03		x	x		x	x	x	x		
22c	*S. praecox* (spr3)	Brazos Co., TX	Cathy Walters (DNA)	4/30/03		x	x		x	x		x	TAMU	yes
22d	*S. praecox*	Bradford Co., FL	Jim Fowler	4/2003		x	x	x	x	x				
22e	*S. praecox*	Brunswick Co., NC	Lucy Dueck	5/17/03		x	x	x	x	x	x			
22f	*S. praecox*	Charleston Co., SC	Lucy Dueck	5/18/03		x	x	x	x	x	x	x		
22i	*S. praecox*	Alachua Co., FL	Scott Stewart	5/20/05		x	x	x	x	x	x	x		
23a	*S. praecox* form *albolabia*	Levy, FL	Paul Martin Brown	5/7/03		x	x		x	x		x		
	S. roamanzoffiana													
24a	*S. romanzoffiana* (**SR3**)	WY	R.Bischof/NGPC (DNA)	≤2001		x		x	x	x		x		
24b	*S. romanzoffiana*	Presque Isle Co., MI	Fred Case	8/16/04		x	x	x	x	x	x	x		
24d	*S. romanzoffiana*	Fairbanks, ALASKA	Jason Grant/KCameron	7/0/00	x	x	x	x	x	x	x	x		
24e	*S. romanzoffiana*	Bunchgrass Co., WA	R. Bursik/U.ID. Herb.	7/26/87		x	x	x	x	x	x	x	ID-100680	yes
24f	*S. romanzoffiana*	Deschutes Co., OR	R&J Mastrogiuseppe/UID	7/6/92		x	x	x	x	x	x	x	ID-124784	yes
24g	*S. romanzoffiana*	Garfield Co., UT	SWelsh/DAtwd./U.ID.Herb.	8/17/95			x		x	x			ID-131440	yes
24i	*S. romanzoffiana*	Flathead Co., Mont.	M.Mantas/U.ID.Herb.	8/14/91			x		x	x			ID-110994	yes
24j	*S. romanzoffiana*	Ravalli Co., Mont.	R. Bursik/U.ID. Herb.	8/4/88		x	x	x	x	x	x	x	ID-132379	yes
24m	*S. romanzoffiana*	Whiteboro, NY	Charles Ufford	8/26/05		x	x	x	x	x	x	x		
24n	*S. romanzoffiana*	Monterey Co., CA	Brad Kelley	6/30/06		x	x	x	x	x	x	x		yes
24o	*S. romanzoffiana*	Marinette Co., WI (Spur Lake)	Kip Knudson	7/24/06		x	x	x	x	x	x	x		yes
24pq	*S. romanzoffiana*	Marin Co., CA (Pt. Reyes)	Brad Kelley	7/30/06		x	x	x	xx	xx		x		yes
24r	*S. romanzoffiana* - **Sr85**	Scotland-Isle of Colonsay (southern)	R.Gulliver/A.Forrest-RBGE	2001	x	x	x		x	x	x	x	E-AM001143	
24s	*S. romanzoffiana* - **Sr154**	Scotland-Isle of Coll (northern)	E.Grant/A.Forrest-RBGE	2001		x	x	x	x	x	x	x	E-AM001212	
24t	*S. romanzoffiana*	Lane Co., OR (Patterson Mtn.)	Tanya Harvey	9/4/06		x	x	x	x	x	x			yes
24u	*S. romanzoffiana* **(was PxR23)**	Lane Co., OR (Patterson Mtn.)	Tanya Harvey	9/4/06		x	x	x	x	x	x	x		
24w	*S. romanzoffiana*	Unalaska Is. (Aleutians), AK	Robert Lauri	7/24/06	x	x	x		x	x	x	x		
sr2	*S. romanzoffiana*	Manitoba, Canada	C.J. Cheviak	7/15/03		x				x			Sheviak 6947 (NYS)	
sr3	*S. romanzoffiana*	Rio Arribo Co., NM	C.J. Cheviak	8/10/97		x				x			Sheviak 6089 (NYS)	
sr4	*S. romanzoffiana*	Mt. Graham, Grahama Co., AZ	C.J. Cheviak	8/5/84		x				x			Sheviak 2642 (NYS)	
sp1	*S. romanzoffiana (as porriflia)*	Craig Mountain, Nez Perce Co., ID	Karen L. Gray	Aug-98		x	x		x	x		x	K. Gray 1518 (NYS)	
	S. sinensis													
2184	*S. sinensis*	Malaysia	Ken Cameron/NYBG	1995	x	x	x	x	x	x	x	x		
Si17	*S. sinensis*	#14317 - Yunnan, China	RBG-Kew	via	x	x	x		x	x	x	x	K-14317	
Ssi4	*S. sinensis*	Russia	Sheviak	1/17/1994	x	x	x		x	x	x	x	Sheviak 5448 (NYS)	
Ssi5	*S. lancea*	Yunnan, China	Heng			x			x	x		x	Heng 14401 (F)	
Ssi6	*S. sinensis*	Australia	Bruhl	2/7/2001		x			x	x			Bruhl 2035 (MO)	
Ssi7	*S. sinensis*	La Reunion	K.M. Cameron	1/1/2014	x	x	x		x	x	x	x		yes
Ssi8	*S. sinensis*	Hangzhou, Zhejiang, China	K.M. Cameron	6/5/2014	x	x	x			x		x	Cameron s.n.	

	S. triloba													
FL36	*S. triloba*	Florida Panther NWF, FL	M.C. Pace	11-Jan-13	x	x	x		x			x	Pace s.n.	yes
FL130	*S. triloba*	Fakahatchee Strand, FL	M.C. Pace	16-Jan-13	x	x	x	x	x	x			Pace s.n.	yes
FL135	*S. triloba*	Corkscrew Swamp Sanctuary, FL	M.C. Pace	17-Jan-13	x	x	x	x	x	x	x	x	Pace s.n.	yes
FL146	*S. triloba*	Wakulla Co., FL	M.C. Pace	9-Nov-13		x	x			x	x	x	Pace s.n.	yes
Flpolk	*S. triloba*	Polk Co., FL	Steve Orzell			x	x	x		x		x	Orzell 26717	yes
FL164	*S. triloba*	Citis Co., FL	M.C. Pace				x			x		x	Pace s.n.	yes
FL165	*S. triloba*	Jesserson Co., FL	M.C. Pace				x			x		x	Pace s.n.	yes
17h	*S. odorata*	Collier Co. (Naples), FL	Scott Stewart	5/25/05			x-h2		x	x		x		
17m	*S. odorata*	Columbia Co.,FL (Ich.Spr.S.P.)	PMBrown	11/11/06			x (14,1-4)	x	x	x		x		
17n	*S. odorata* (dwarf)	Levy Co., FL	PMBrown	11/11/06	x	x	x	x	x	x	x	x		
Ich2	*S. x ichetuckneensis*	Columbia Co., FL	PMBrown	11/11/06		x	x	x	x	x	x			
si2	*S. triloba*	Ichetucknee Springs, Columbia Co., FL	M.C. Pace	11/13/13	x	x	x	x		x	x	x	Pace s.n.	yes
	S. spiralis													
Sp51	*S. spiralis*	#10451 - U.K.	RBG-Kew	NYBG)	x	x	x	x	x	x		x	K-10451	
	S.perexilis													
31a	*S. stellata* **(PxR1)**	Douglas Co., OR	Hope Stubbs	8/12/06		x	x	x	x	x	x	x		yes
31e	*S. stellata* **(PxR7)**	Lane Co., OR (Happy Lakes)	Tanya Harvey	9/7/06		x	x	x	x	x	x	x		yes
31f	*S. stellata* **(21y = PxR9)**	Tulare Co., CA	Sarah DeGroot (viaRobL)	7/25/06		x	x		x	x		x		yes
sst3	S. stellata (cf. perexilis)	Crater Lake NP, Klamath Co., OR	Peter F. Zika	8/7/94		x	x		x	x		x	Zika 15260 (NYS)	
sst6	S. stellata (open spiral cf perexilis)	Nobe Young Meadow, Tulare Co., CA	J. Stewart	7/25/07		x	x		x	x			Stewart 2690 (RSA)	
	S. xstellata													
sst1	S. stellata (stellata x porrifolia)	El Dorado Co., CA	Sheviak	0 0 2009		x	x		x	x	x	x	Sheviak 7084	
sst2	S. stellata (stellata x rom)	Tom's Place, Inyo Co. CA	Sheviak	8/5/04		x	x		x	x	x	x	Sheviak 7000	
sst4	S. stellata (stellata x rom)	Union Valley Reservoir A, El Dorado Co., CA	C.J Sheviak	6/30/87		x	x		x	x			Sheviak 2925a (NYS)	
sst5	S. stellata (stellata x rom)	Union Valley Reservoir D, El Dorado Co., CA	C.J Sheviak	6/30/87		x	x		x	x			Sheviak 2925d (NYS)	
sst7	S. stellata (stellata x rom)	Granite seeps, Fresno Co., CA	M. Honer	9/1/06		x	x		x	x			Honer 2414 (RSA)	
sst8a	S. stellata (stellata x rom) wide leaves)	Nevada Co., CA	L. Ahart	7/23/05		x	x		x	x			Ahart 12166 (RSA) a	
sst8b	S. stellata (stellata x rom) (linear leaves)	Nevada Co., CA	L. Ahart	7/23/05		x	x		x	x			Ahart 12166 (RSA) b	
sst8c	S. stellata (stellata x rom) (linear leaves)	Nevada Co., CA	L. Ahart	7/23/05		x	x		x	x			Ahart 12166 (RSA) c	
	S. sunii													
Sun	S. sunii	Ganzu, China				x	x		x	x				

	S. sylvatica													
25a	*S. sylvatica*	Levy, FL	PM Brown	5/7/03		dbl	x		x	x		x		
25b	*S. sylvatica*	Barnwell Co., SC (SRS)	Lucy Dueck	5/7/03		x	x		x	x	x	x	80507-8	yes
25c	*S. sylvatica*	Brunswick Co., NC	Lucy Dueck	5/17/03		dbl	x		x	x				
25d	*S. sylvatica*	Alachua Co., FL	Scott Stewart	5/20/05	x	x	x	x	x	x	x	x		
ssy1	*S. sylvatica*	Camp Whispering Pines, Tangipahoa Parish	W.J. Platt	4/22/02		x			x	x		x	Platt s.n. (LSU 00113853)	
ssy2	*S. sylvatica*	Holland Bottoms, Lonoke Co., AR	Nunn	6/3/04		x				x			Nunn 9935 (UARK)	
ssy3	*S. sylvatica*	Pine City Natural Area, Monroe Co., AR	Nunn	6/4/03		x			x	x				
	S. torta													
28a	*S. torta* (lg flwr & stem)	Miami-Dade Co., FL	PMB/RH	6/20/06		x	x	x	x	x	x	x	82221	yes
28b	*S. torta* (sm flwr & stem)	Miami-Dade Co., FL	PMB/RH	6/20/06	x	x	x		x	x	x	x	82221	yes
28c	*S. torta* (flwr only)	Miami-Dade Co., FL	PMB/RH	6/20/06	x	x	x		x	x	x	x	82221	yes
28f	*S. torta*	Puerto Rico	Jim Ackerman	4/1/07	x	x	x (1-4)	x	x	x	x	x	82230	
28g	*S. torta*	Puerto Rico	Jim Ackerman	4/1/07	x	x	x (1-4)		x	x	x	x	82230	
28h	*S. torta*	Puerto Rico	Jim Ackerman	4/1/07	x	x	x (1-4)	x	x	x	x	x	82230	
	S. tuberosa													
26b	*S. tuberosa* (late blm.)	S. Aiken Co., SC	Lucy Dueck	8/19/03	x	x	x	x	x	x		x	82218	yes
26c	*S. tuberosa*	Monroe Co., WV	Stan Bentley	9/6/03	x	x	x	x	x	x		x		
26e	*S. tuberosa*	Marion Co., FL	Scott Stewart	6/1/04		x	x	x	x	x		x		
26f	*S. tuberosa* (early blm.)	N. Aiken Co. (front yard), SC	Lucy Dueck	10/20/04		x	x	x	x	x		x	80499	yes
26g	*S. tuberosa*	Marion Co. (X-Fla Greenway), FL	Scott Stewart	11/21/04		x	x		x	x				
	S. vernalis													
27a	*S. vernalis* (**SV1**)	IA	R.Bischof/NGPC (DNA)	≤2001		x	bad		x	x			NEB	
27b	*S. vernalis*	Levy, FL	Paul Martin Brown	5/7/03		x	x		x	x		x		
27c	*S. vernalis*	Aiken Co., SC (crystal palace)	Lucy Dueck	5/26-29/03		dbl	x (14,1-4)		x	x		x		
27d	*S. vernalis*	Alleghany Co., NC (BRP)	Stan Bentley	8?/15/03		x (poor/dbl)	x		x	x				
27e	*S. vernalis*	Ritchie Co., WV	Scott Shriver	8?/17/03		x	x		x	x	x			
27f	*S. vernalis* (sv29)	Brazos Co., TX	Cathy Walters (DNA)	5/13/03		x	x		x	x		x	TAMU	
27g	*S. vernalis*	Levy, FL	Jim Fowler	4/2003		x	x		x	x		x		
27h	*S. vernalis*	Aiken Co., SC (w.SREL pkg.lot)	Lucy Dueck	5/28/03		x	x		x	x		x	80509	yes
27k	*S. vervalis*	Alachua Co., FL	Scott Stewart	5/20/05		x	x		x	x	x	x		
27m	*S. vernalis*	Bradford Co., FL	Scott Stewart	5/25/05	x	x	x		x	x	x	x		
27n	*S. vernalis*	Jefferson Co., TX	Joe Liggio	5/28/04		x	x		x	x		x	80952	
27o	*S. vernalis*	Jasper Co., TX	Joe Liggio	5/23/04		bad - dbl.	x (14)		x	x	x		80486	
	Outgroup taxa													
30	*Sacoila lanceolata* var. *lanc.*	Levy Co., FL	Scott Stewart	6/1/04	x	x	x		x	x		x		
GP	Goodyera pubescens	Baxters Hollow, WI	M.C. Pace	May-13		x		x		x		x		yes
Dici	Dichromanthus cinnabara		G. Salazar		x	x	x	x	x	x	x	x		
Mepo	Mesadenella polyantha		G. Salazar		x	x	x		x	x	x	x		
Dedi	Deiregyne diaphana		G. Salazar			x	x		x	x	x	x		
Scll	Schiedeella llaveana		G. Salazar			x	x		x		x	x		



Fig. 4. [Following page] Screen shot of nrITS alignment. Samples of *Spiranthes magnicamporum* at the top (where the mutations of G & T are highlighted), whereas *S*. *cernua* s.s. is toward the bottom. Highlighted at the center are samples identified as being hybrid in origin: *Spiranthes* ×*incurvum*.

Originally, direct sequencing of nrITS produced an R at position 503, and a Y at position 521 for the samples of *S*. ×*incurvum*. To resolve this ambiguity, the individual sequences were doubled, and then coded as putative parental copies. In this example, samples with a G at position 503 and a T at position 521 were identified as being derived from S. magnicamporum (highlighted in red), and samples with an A at position 503 and a C at position 521 were inferred to be derived from *S*. *cernua* s.s. (highlighted in black).

Comparing against the plastid data matrix, we inferred the copy derived from *S*. *cernua* to likely be maternal in origin, and the copy derived from *S*. *magicamporum* to likely be paternal in origin.

Fig. 4

S. cernua *S. ×incurvum* *S. magnicamporum*

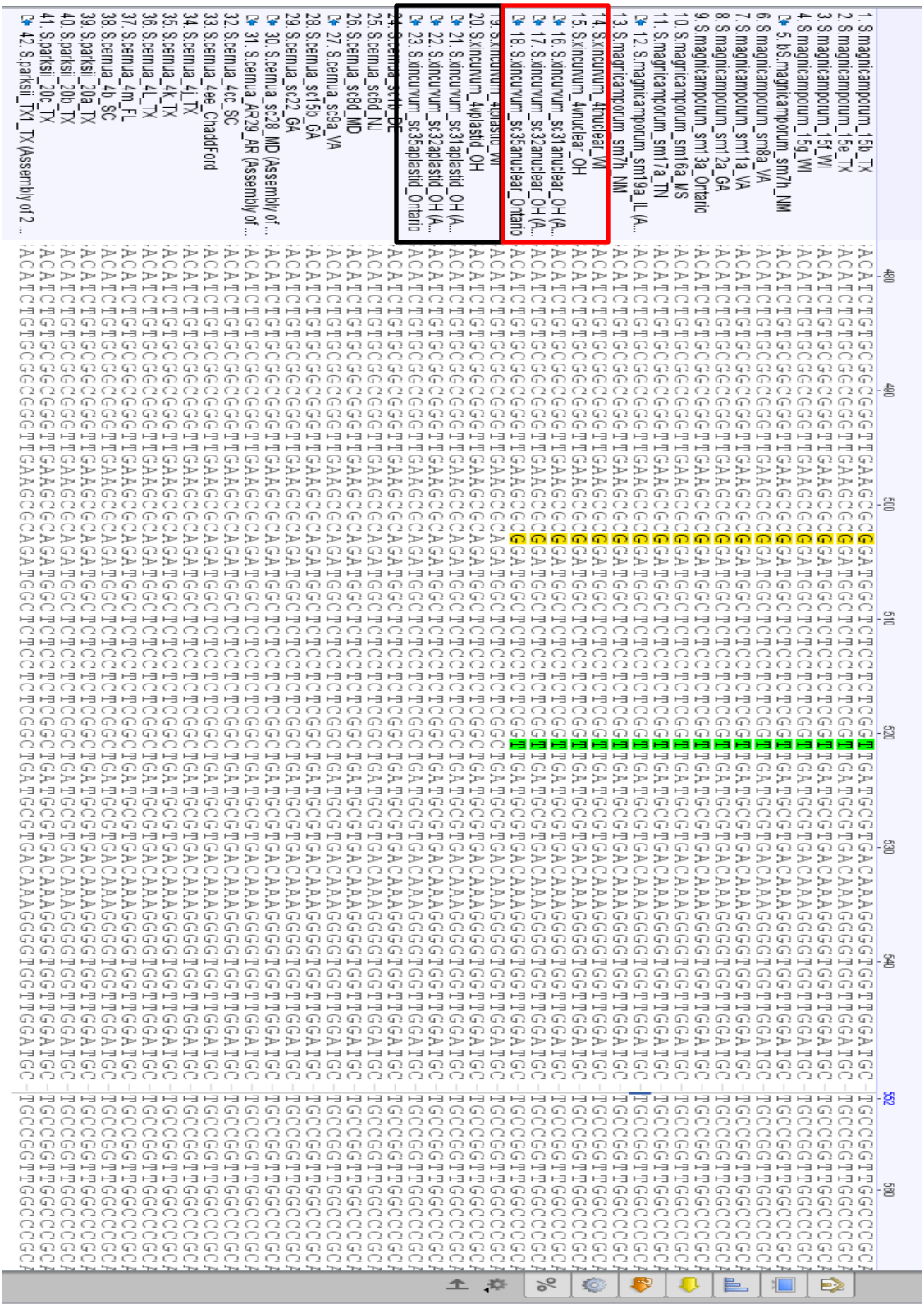


Fig. 5. Bayesian Majority Rule reconstruction of *Spiranthes* from the combined data set (*ACO*, nrITS, *mat*K, *ndh*J, *trn*F-L, *trn*S-M, *ycf*1 3', *Xdh*). 308 total individual samples, including out-group taxa.

Gray-scale bars (number I-V) indicate sections of the tree which are presented in closer detail in the subsequent pages. The black bar with the asterisk indicates out group taxa.

Posterior probabilities of the backbone branches are presented. See sub-sections for posterior probabilities of derived clades.

<u>Clade colors are as follows:</u>
Blue: *Spiranthes cernua* species complex s.l. (fig. 5 I & fig. 5 II)

Green: *Spiranthes lacera* complex grade (fig. 5 III)

Mustard Yellow: *Spiranthes odorata* (fig. 5 III)

Pink: Old World clade (fig. 5 IV)

Black: *Spiranthes tuberosa* (fig. 5 IV)

Orange: Gulf & Mesoamerican clade (fig. 5 IV)

Red: Western North American Cordillera + *Spiranthes lucida* (fig. 5 V)

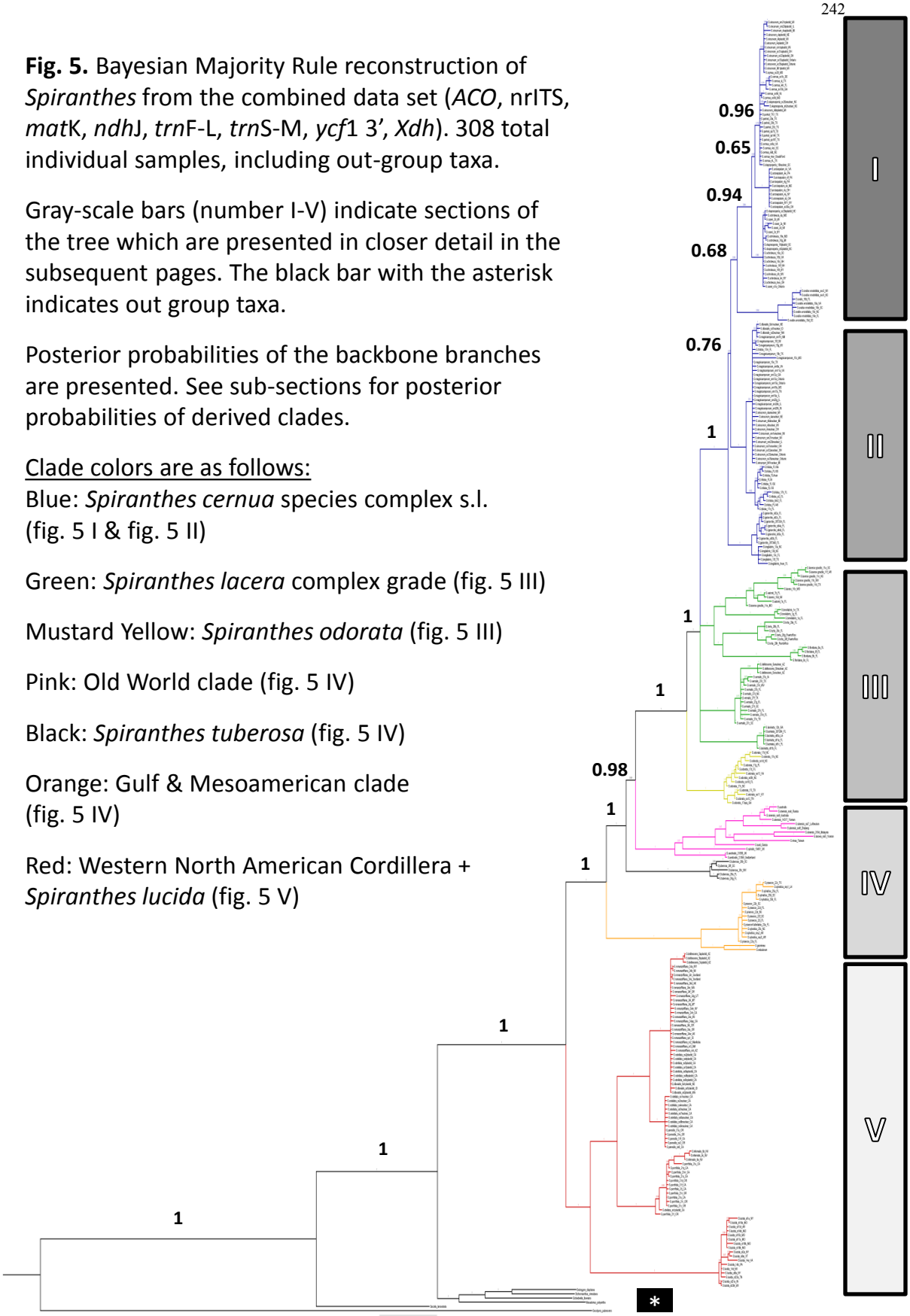
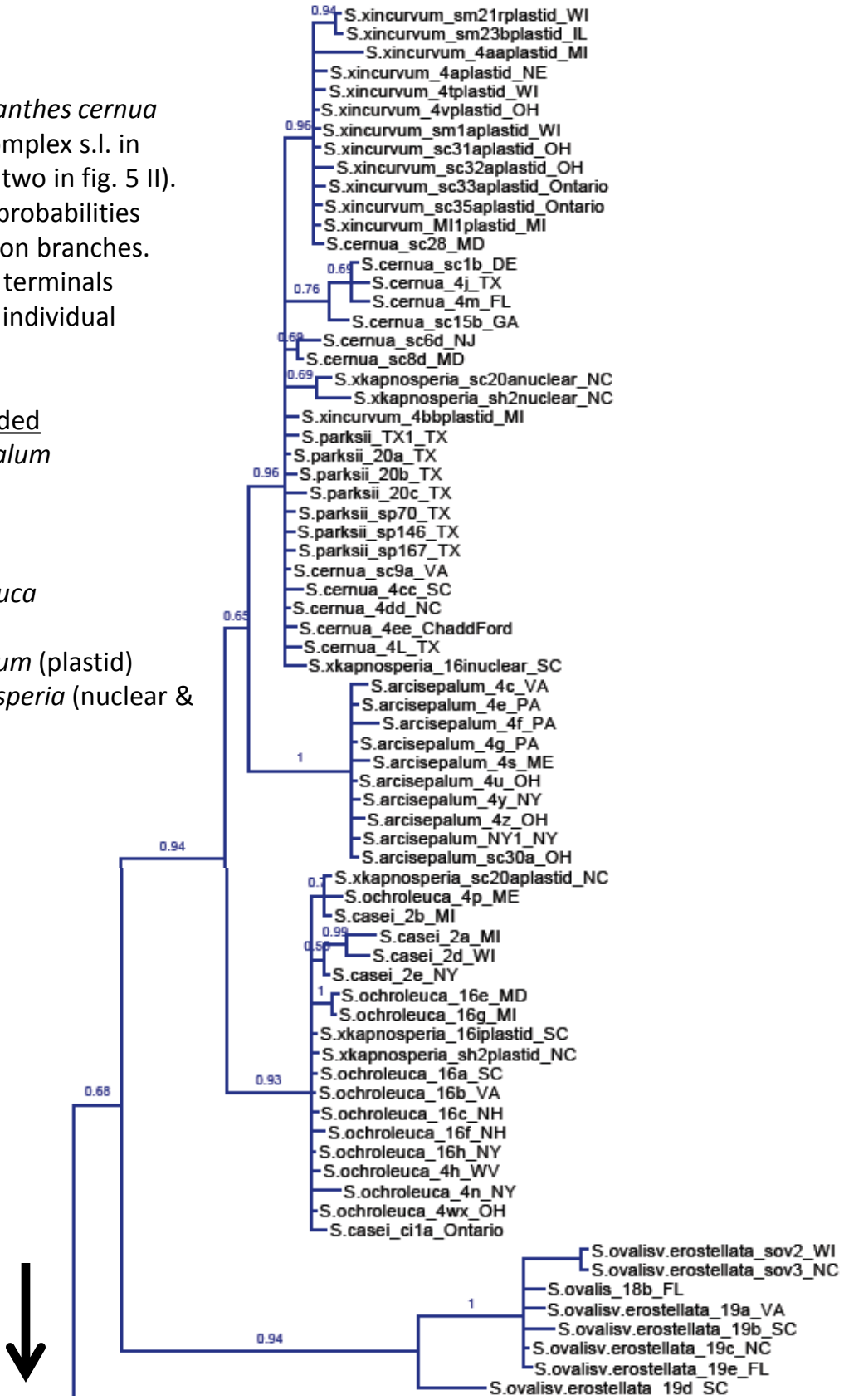


Fig 5 I

Blue: *Spiranthes cernua* species complex s.l. in part (part two in fig. 5 II). Posterior probabilities displayed on branches. Individual terminals represent individual samples.

<u>Taxa included</u>

S. arcisepalum
S. casei
S. cernua
S. parksii
S. ochroleuca
S. ovalis
S. ×*incurvum* (plastid)
S. ×*kapnosperia* (nuclear & plastid)



to fig. 5 II

Fig 5 II

Blue: *Spiranthes cernua* species complex s.l. in part (part one in fig. 5 I). Posterior probabilities displayed on branches. Individual terminals represent individual samples.

<u>Taxa included</u>
S. diluvialis (nuclear)
S. igniorchis
S. longilabris
S. magnicamporum
S. triloba
S. ×incurvum (nuclear)

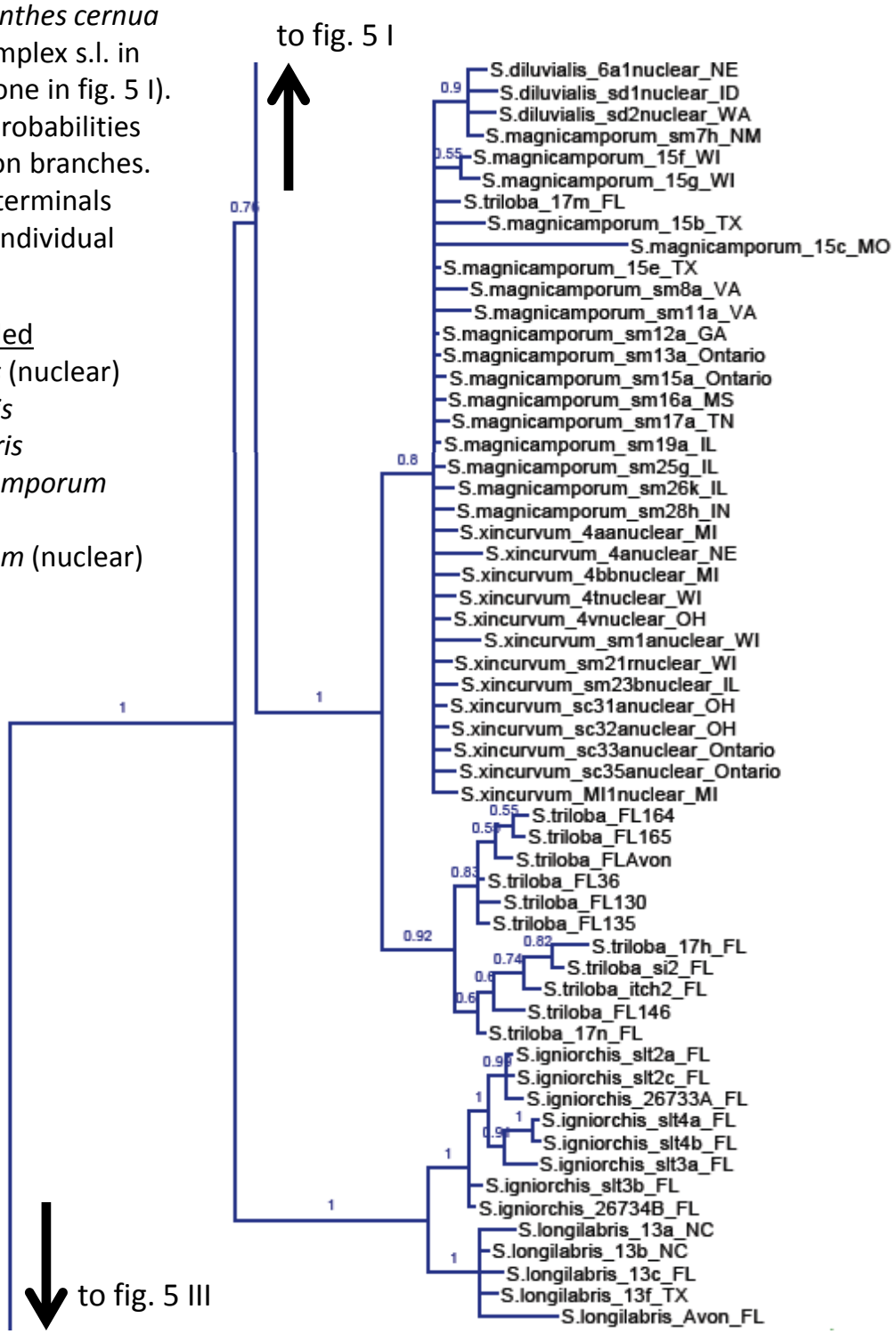
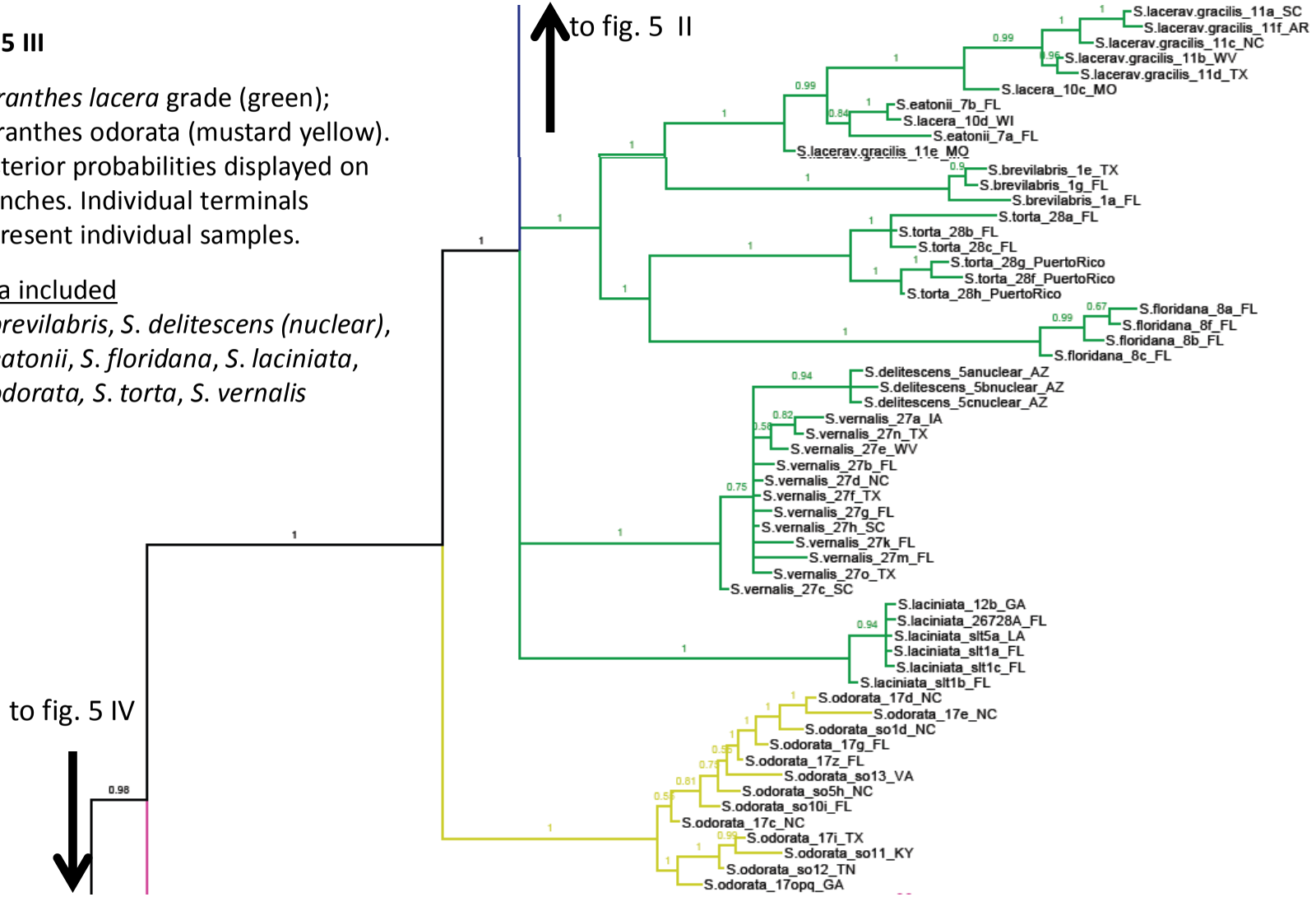


Fig 5 III

Spiranthes lacera grade (green); Spiranthes odorata (mustard yellow). Posterior probabilities displayed on branches. Individual terminals represent individual samples.

Taxa included

S. brevilabris, *S. delitescens (nuclear)*, *S. eatonii*, *S. floridana*, *S. laciniata*, *S. odorata, S. torta*, *S. vernalis*



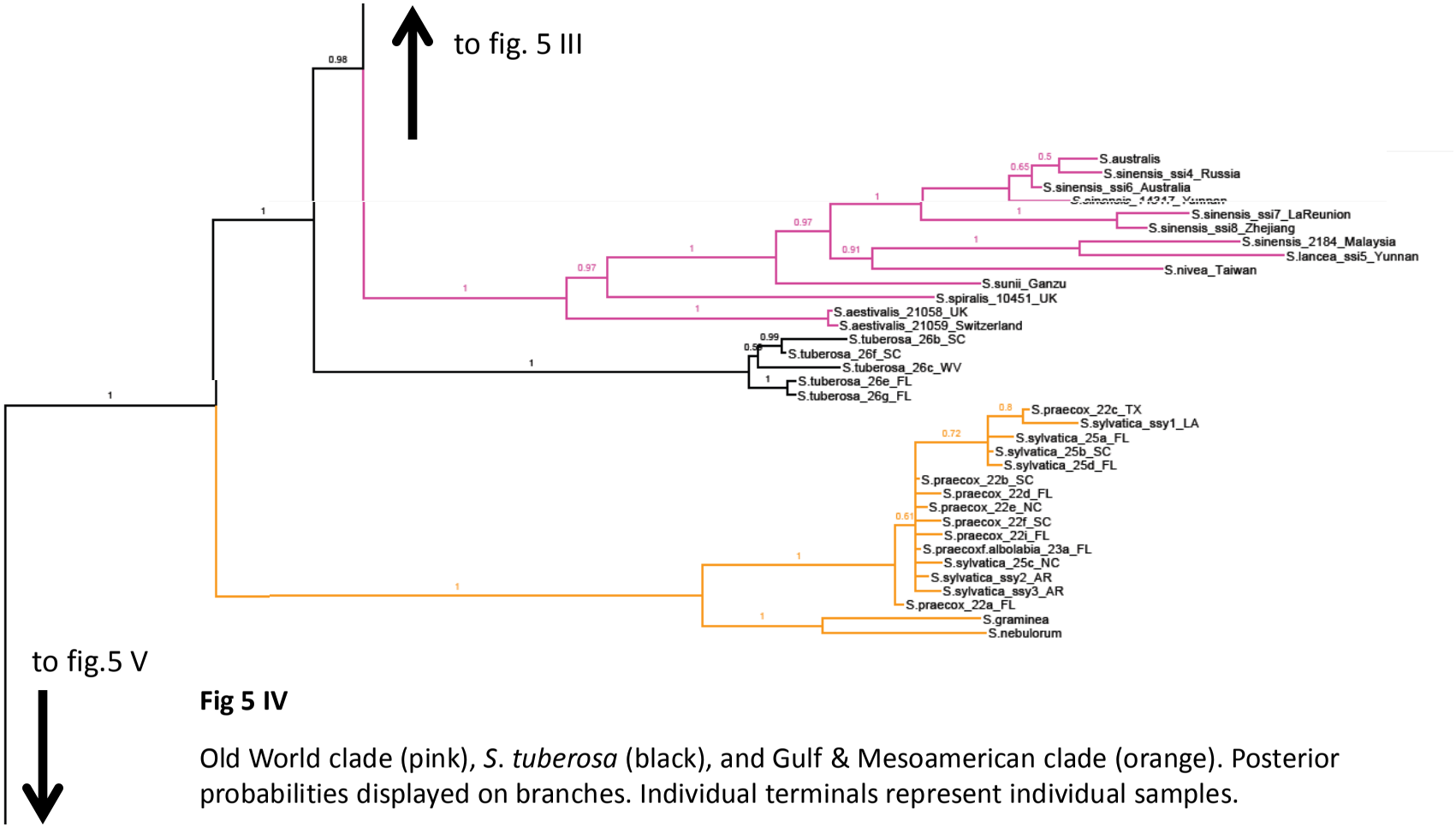


Fig 5 IV

Old World clade (pink), *S. tuberosa* (black), and Gulf & Mesoamerican clade (orange). Posterior probabilities displayed on branches. Individual terminals represent individual samples.

<u>Taxa included</u>

S. aestivalis, *S. australis*, *S. graminea*, *S. lancea, S. nebulorum*, *S. nivea*, *S. praecox*, *S. sinensis*, *S. spiralis*, *S. sunii*, *S. sylvatica*

Fig XX 5 V

Red: Western North American Cordillera + *Spiranthes lucida*. Posterior probabilities displayed on branches. Individual terminals represent individual samples.

<u>Taxa included</u>

S. delitescens (plastid), *S. diluvialis* (plastid), *S. infernalis*, *S. lucida*, *S. perexilis*, *S. porrifolia*, *S. ×stellata* (nuclear & plastid)

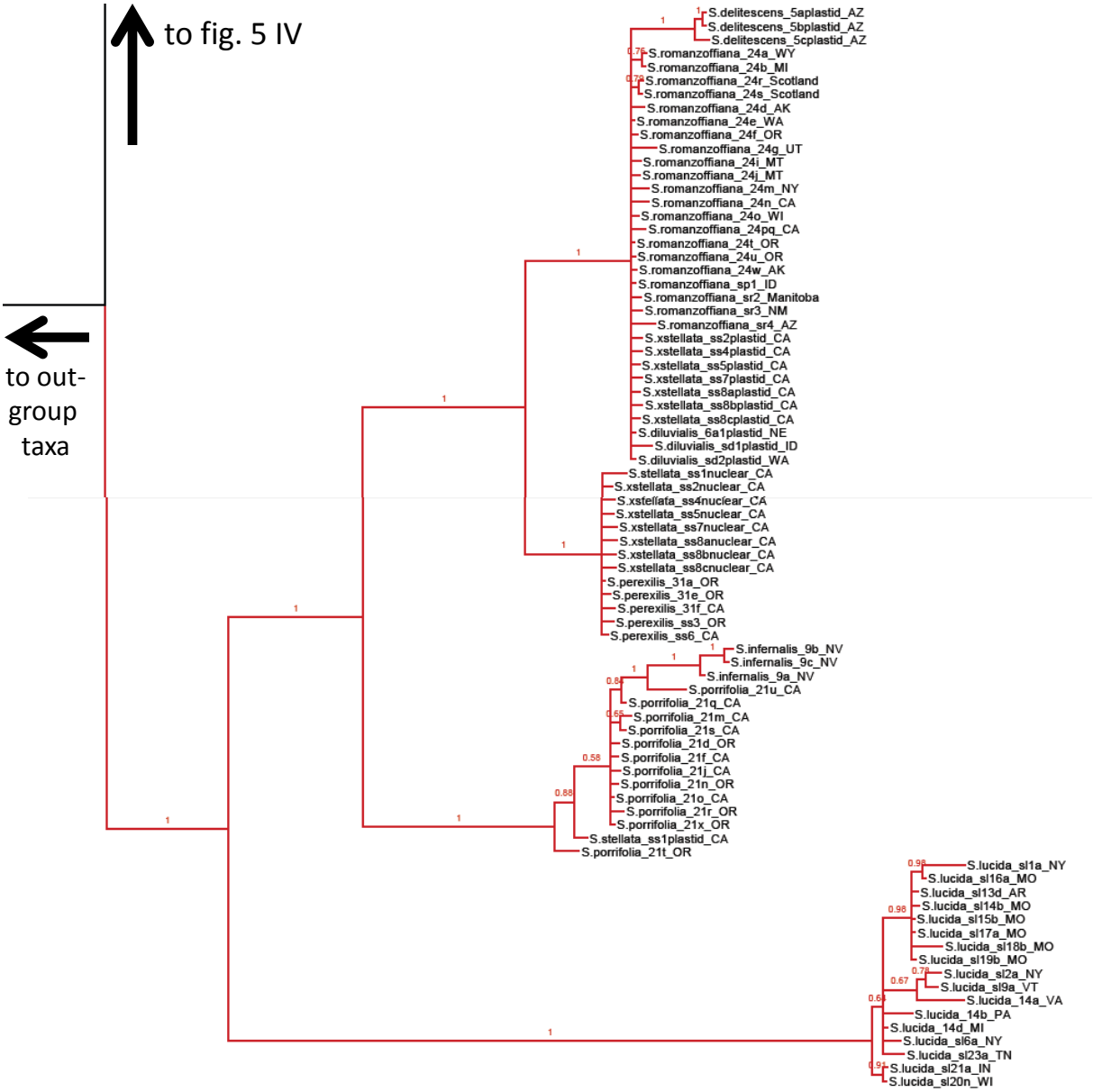


Fig. 6. Minimal missing data BEAST reconstruction (to ensure a lack of polytomies for ancestral state reconstructions) of *Spiranthes* from the combined data set (*ACO*, nrITS, *mat*K, *ndh*J, *trn*F-L, *trn*S-M, *ycf*1 3', *Xdh*). One representative sample per taxon.

Posterior probabilities are presented on branches.

<u>Clade colors are as follows (modified from fig. 5):</u>
Blue: *Spiranthes cernua* species complex s.l.

Green: *Spiranthes lacera* complex (subtended by a branch with pp = 0.73)

Mustard Yellow: *Spiranthes odorata*

Pink: *Spiranthes tuberosa* + Old World Clade (subtended by a branch with pp = 0.96)

Orange: Gulf & Mesoamerican clade

Red: Western North American Cordillera complex + *Spiranthes lucida*

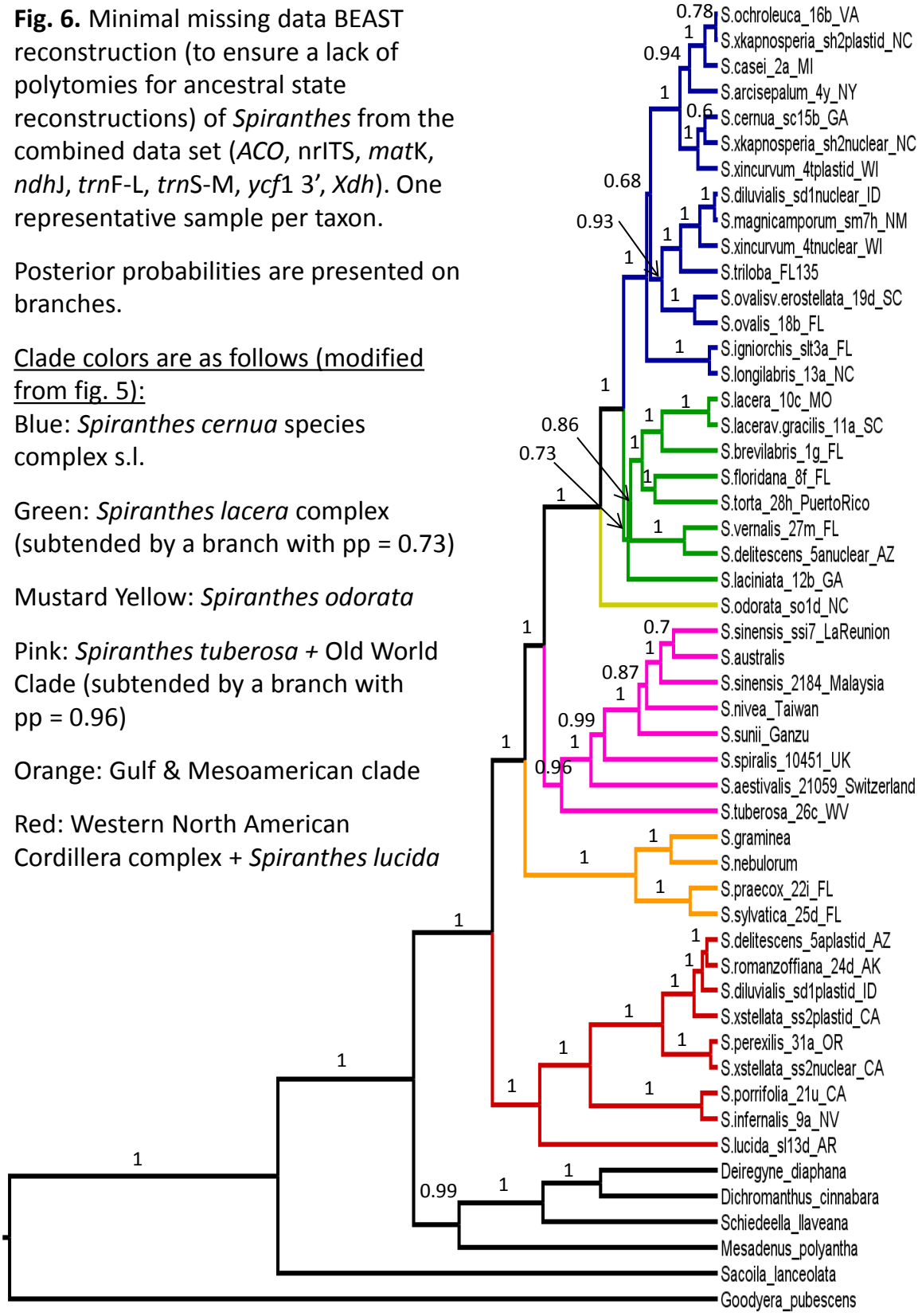




Fig. 7. *Spiranthes graminea*). A. Herbarium specimen, Pueblo, Mexico (*Catling M52*, UMEX). B. Inset: inflorescence detail.



Fig. 8. *Spiranthes nebulorum* paratype (originally misidentified as *S. graminea*). A. Herbarium specimen, Hidalgo, Mexico (*Moore 3323*, UMEX). B. Inset: inflorescence detail.

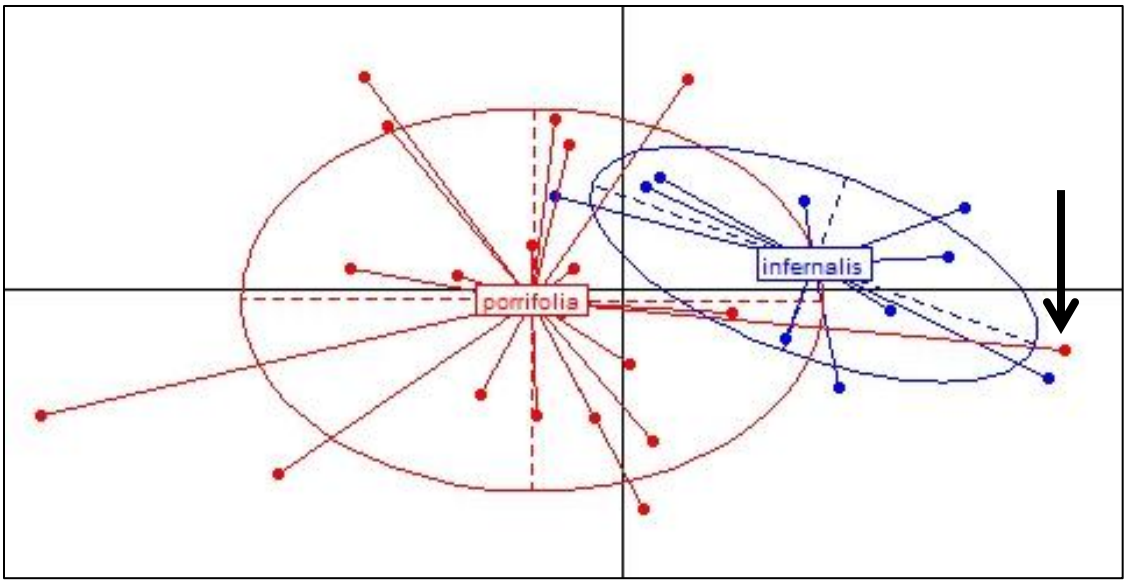


Fig. 9. Visualization of PCA of measured *Spiranthes infernalis* (blue) and *Spiranthes porrifolia* (red) labella. The sample from Fish Slough, CA, (*DeDecker 5091*) is highlighted by the black arrow.

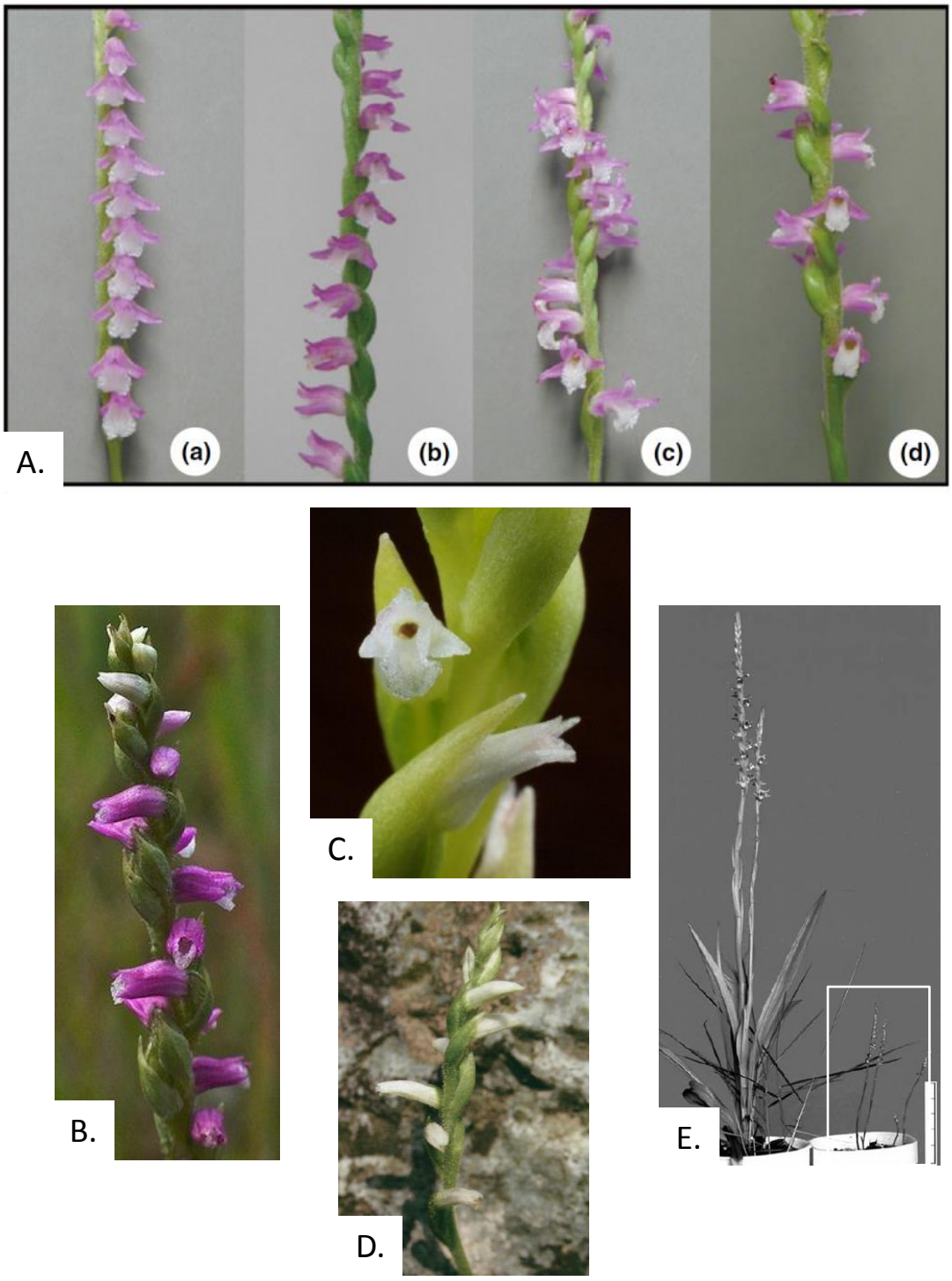


Fig. 10. Morphological variation of *Spiranthes sinensis* and its associated species. **A.** Variation of inflorescence spiraling (Iwata et al. 2012) **B.** *Spiranthes novae-zelandiae* (New Zealand). **C.** *Spiranthes nivea* (greenhouse grown; originally from Taiwan). **D.** *Spiranthes sunii* (Ganzu, China. Photo from original publication). **E.** typical *S. sinensis* and *S. sinensis* f. gracilis (Japan).

Fig. 11. ASR of lateral sepal position.
Yellow: ascending
Green: in-line w/flower profile
White: oblique
Red: semi/sub-oblique
Blue: below flower profile

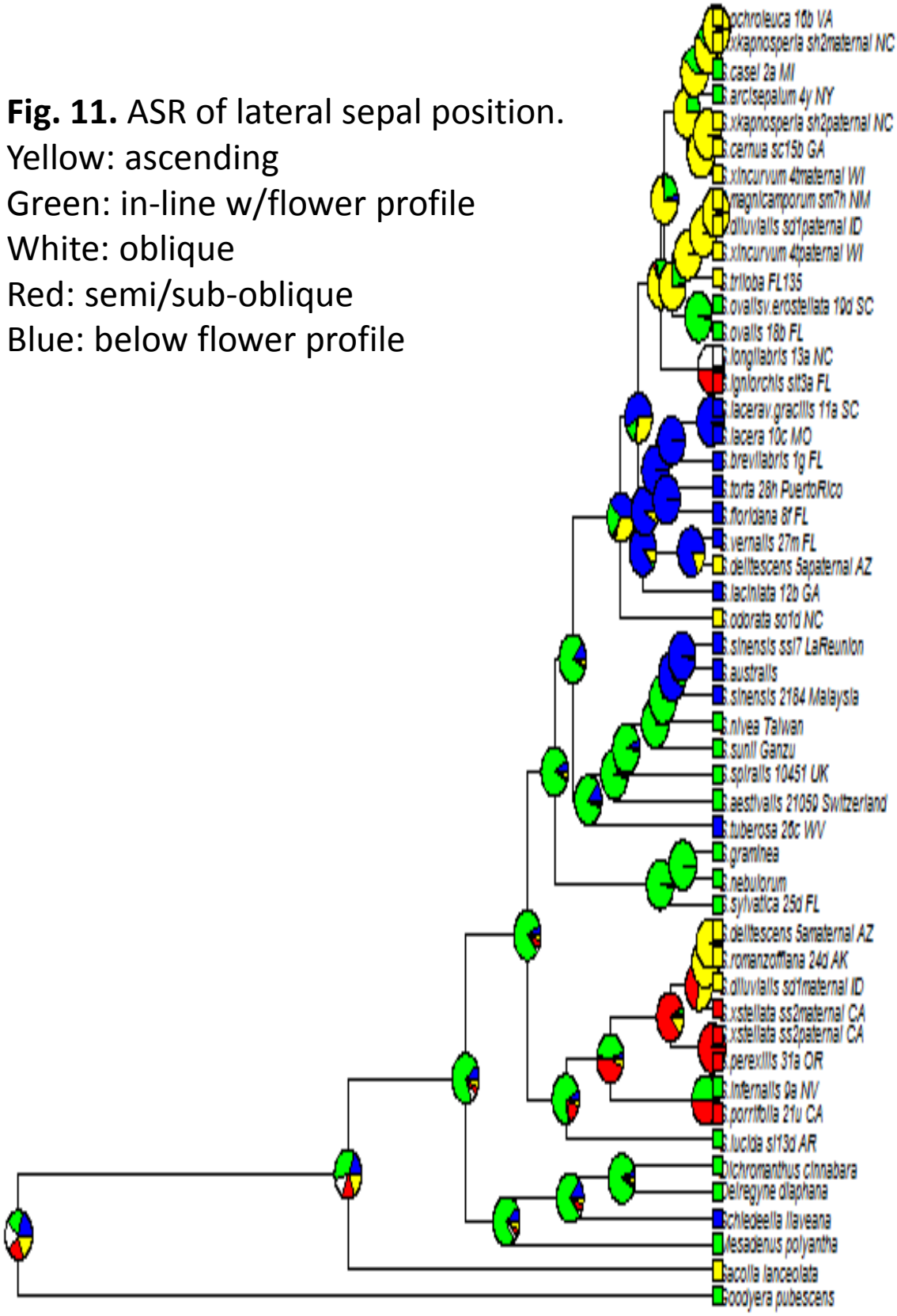


Fig. 12. ASR of labellum color.
White = white
Yellow: yellow
Green: green
Blue: greenish-yellow
Pink: pink
Brown: brown

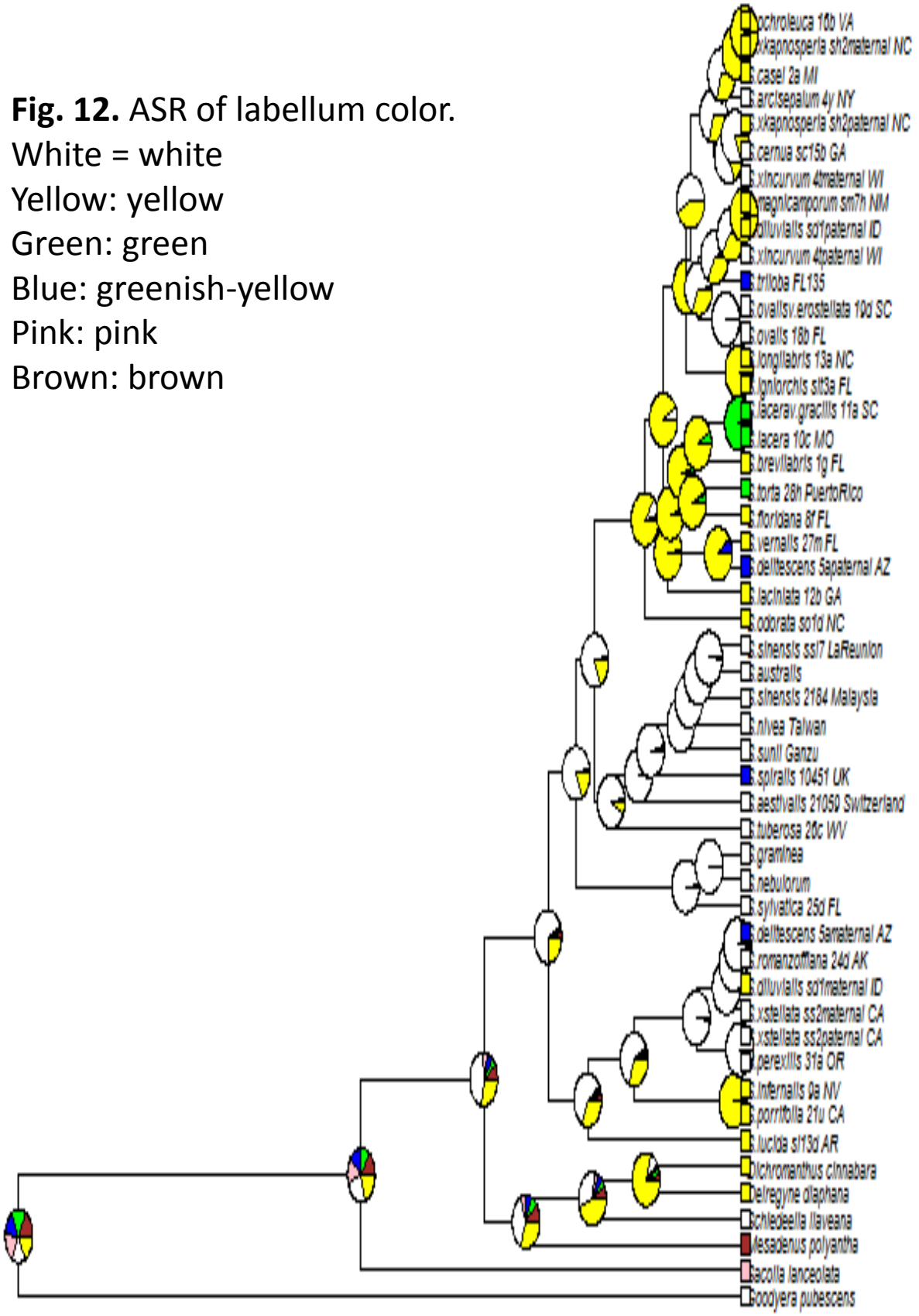


Fig. 13. ASR of flower color.
White = white
Yellow: ochroleucous
Green: ivory
Pink: pink
Red: red
Brown: brown

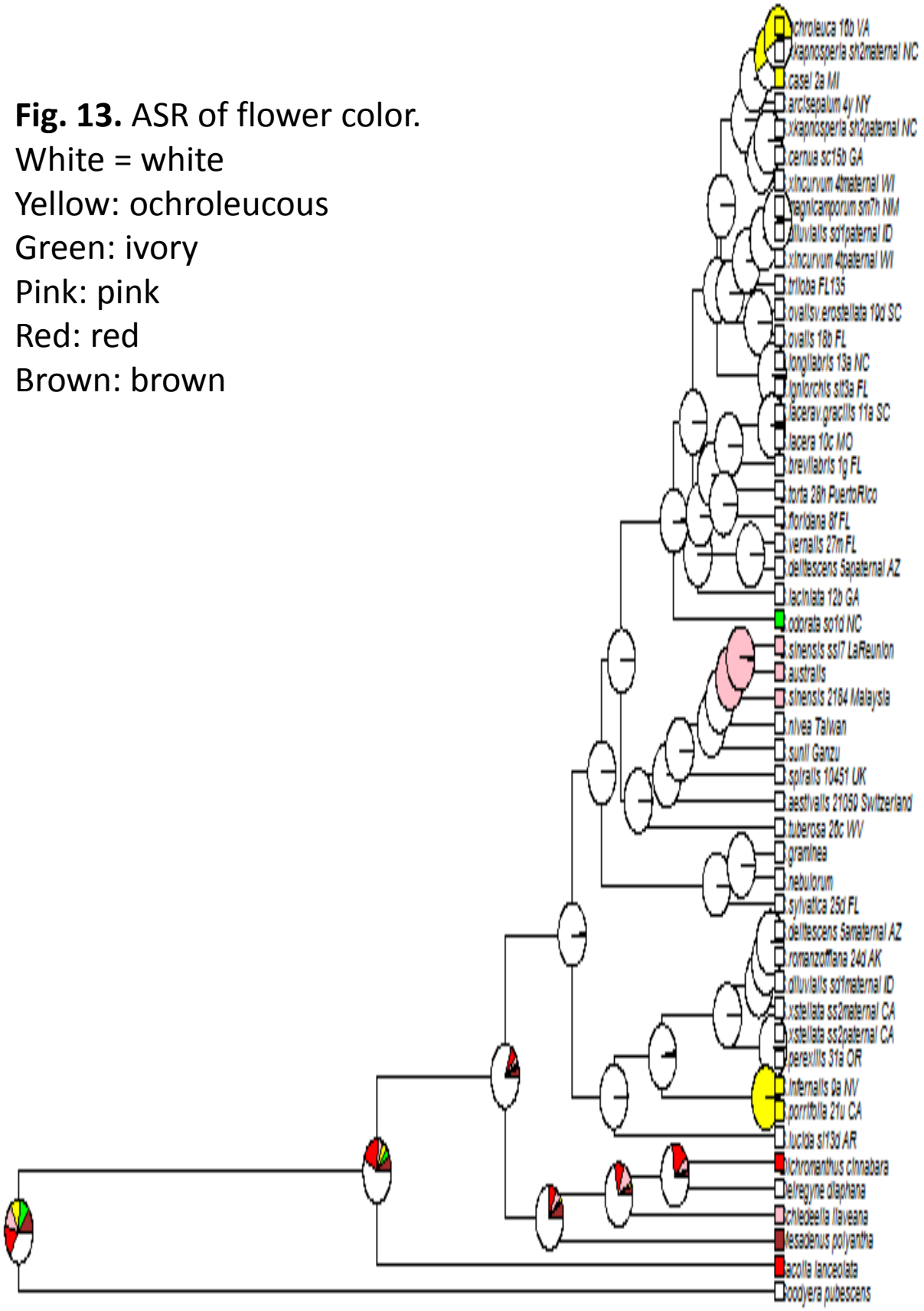


Fig. 14. ASR of labellum surface texture
Yellow: glabrous
Red: papillate
Blue: glabrous OR papillate

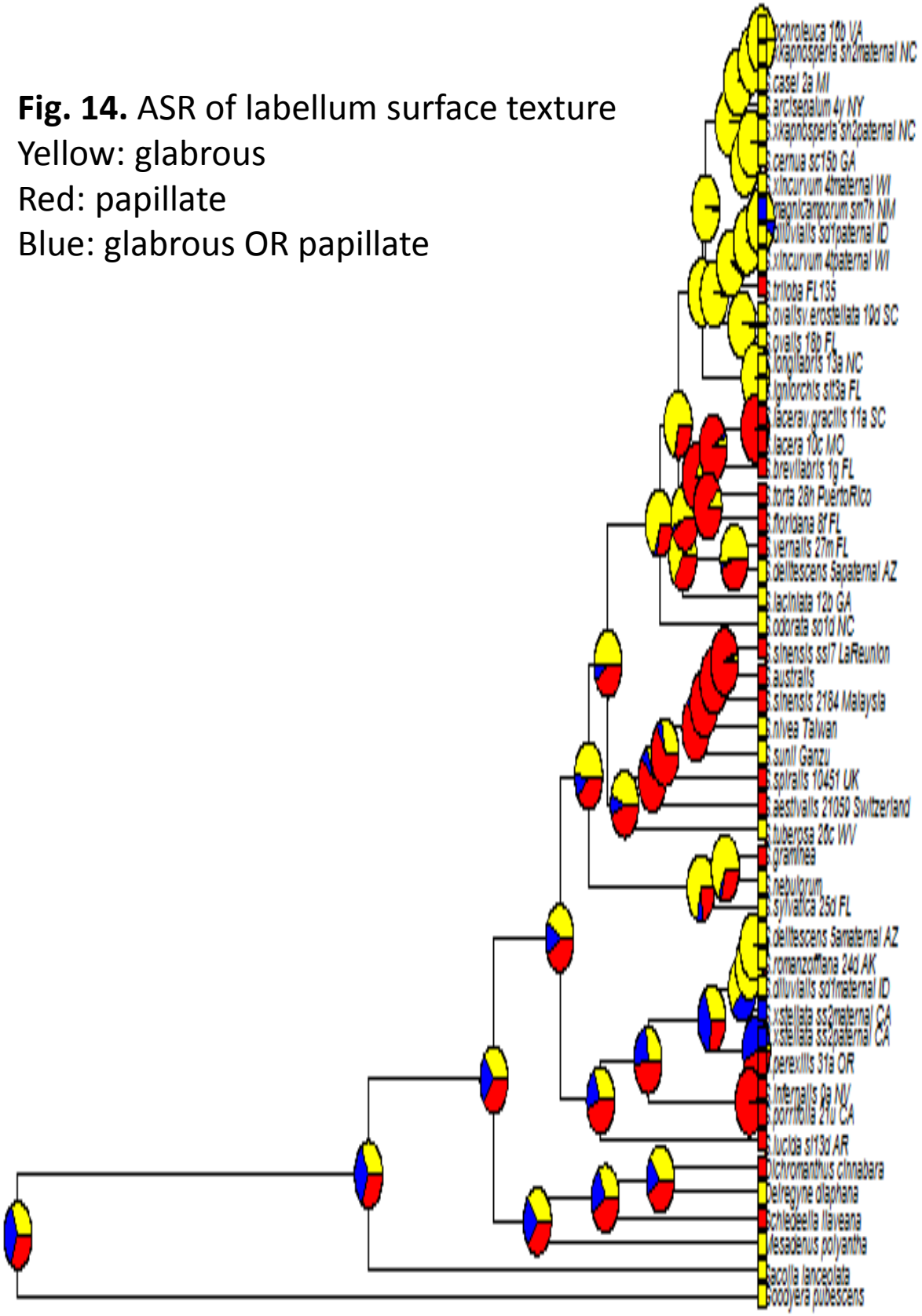


Fig. 15. ASR of breeding system.
Green = allogamous
Blue: autogamous
Yellow: mixed auto/allo/apomictic
Red: apomictic

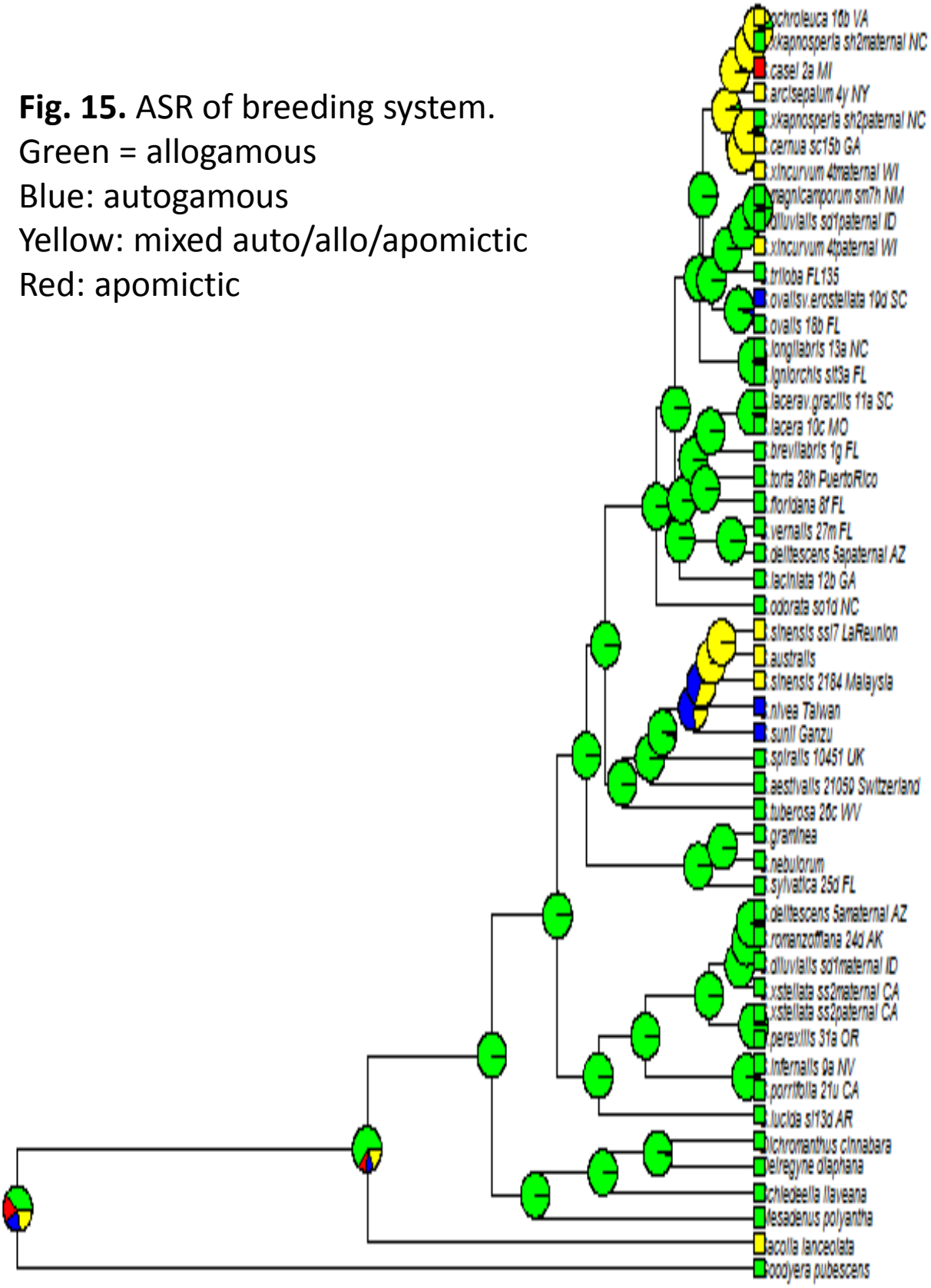


Fig. 16. ASR of leaf presence at time of anthesis.
Green: present
White: typically fugaceous (absent)

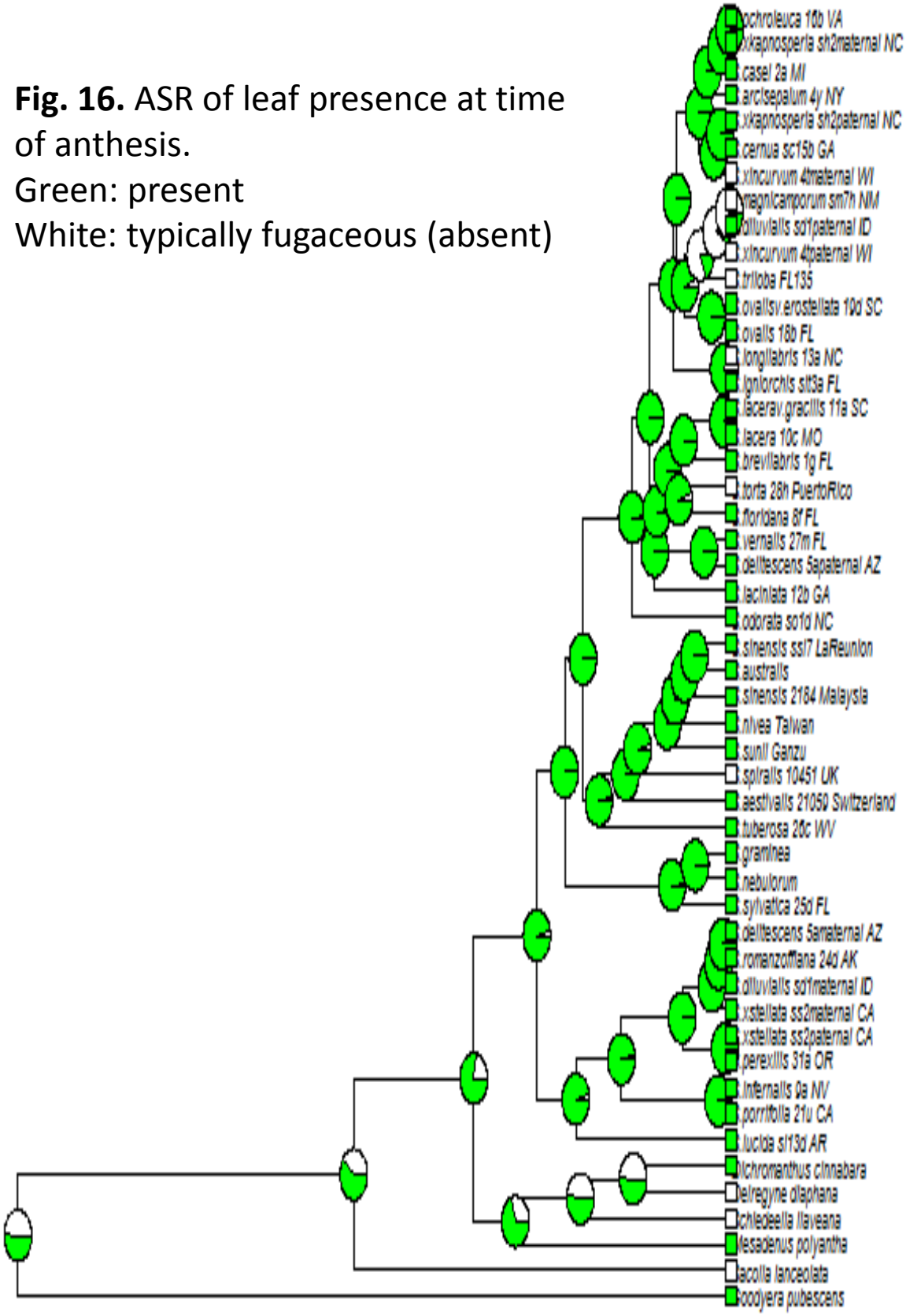


Fig. 17. ASR of leaf shape.
Green: lanceolate
Blue: linear
Yellow: ovate
Red: spatulate

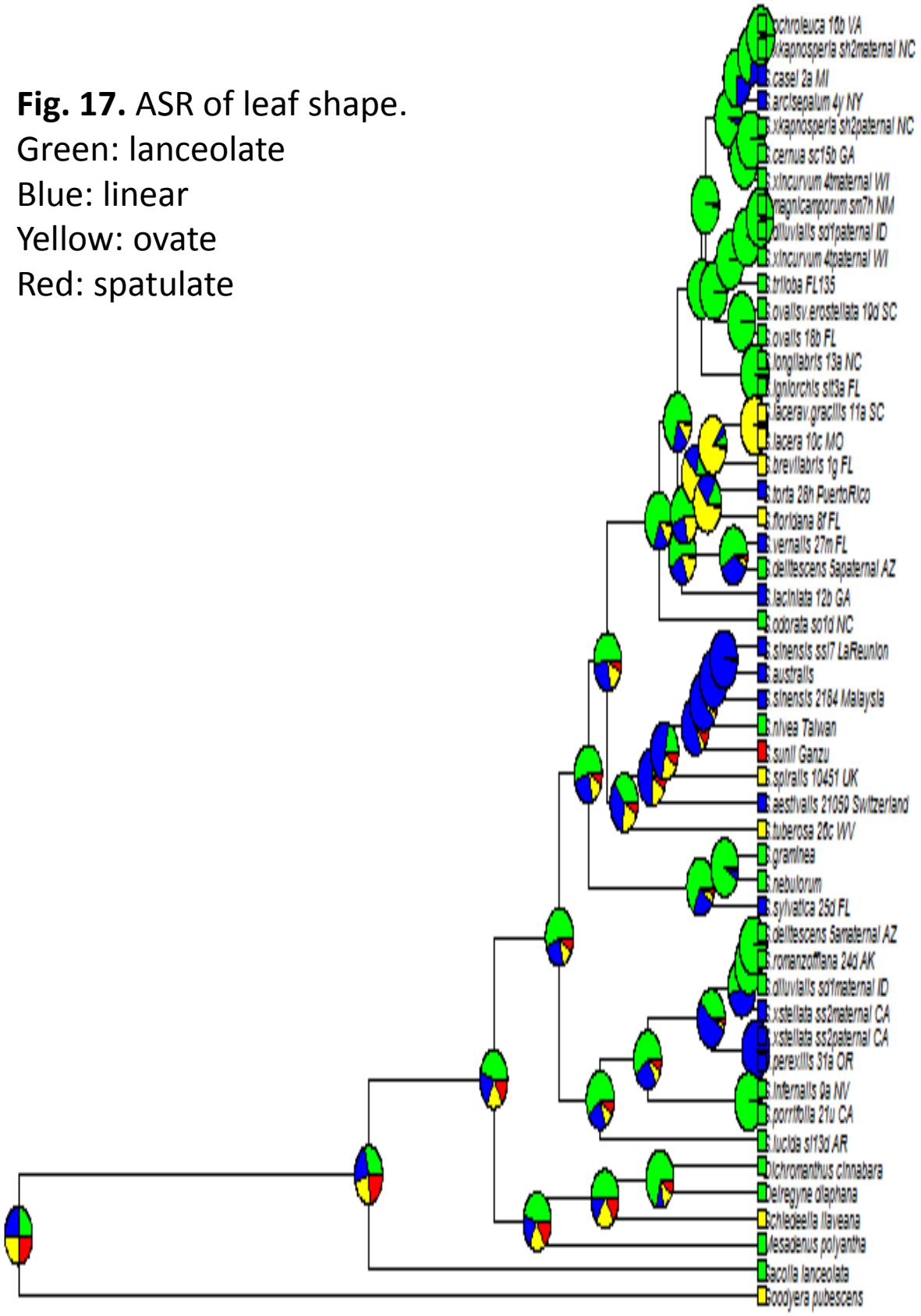


Fig. 18. ASR of root type.
Red: slender
Green: rhizomatous
Blue: cylindrical (sub-tuberous)
White: tuberous

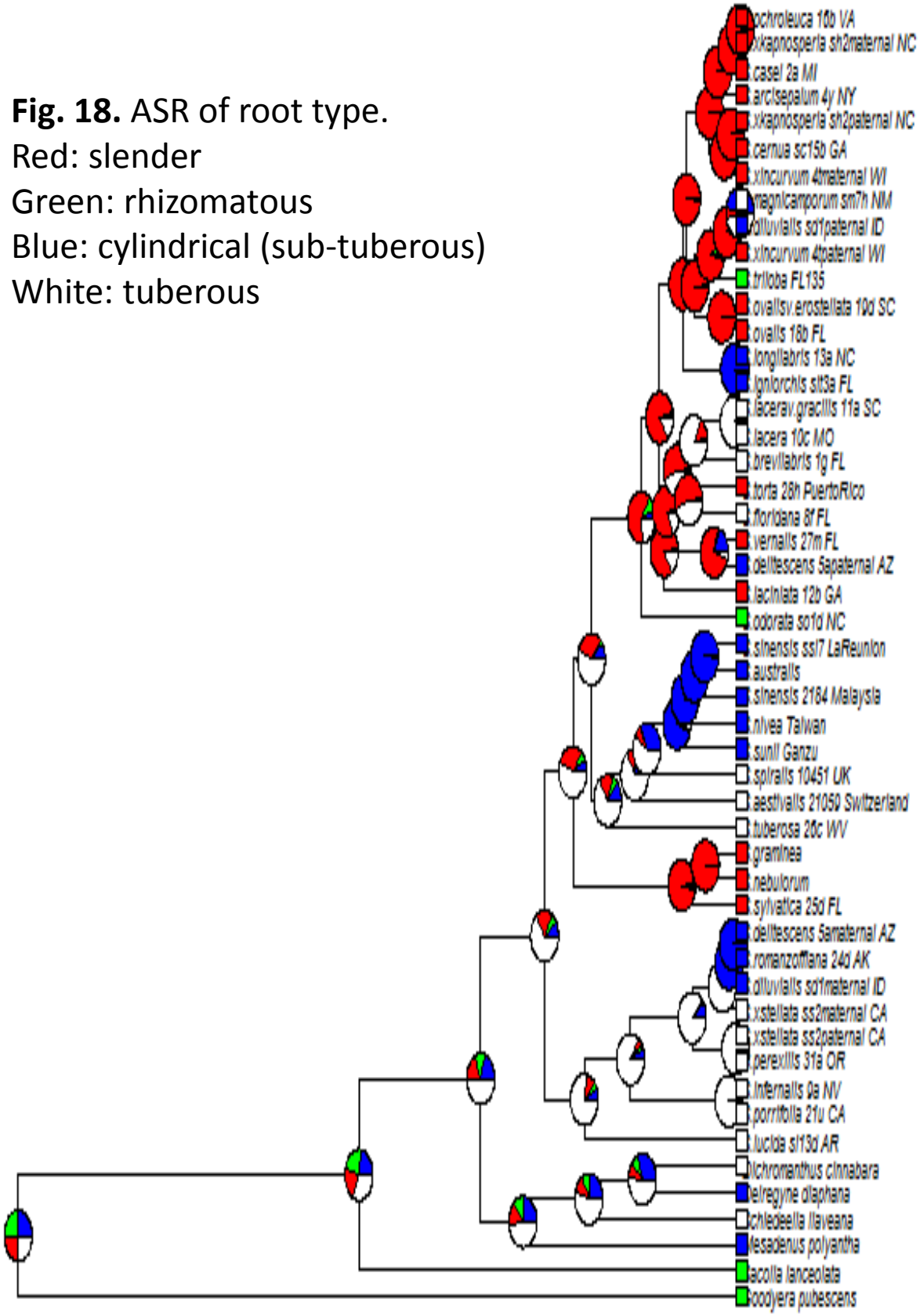


Fig Supp. 1 (A)

Bayesian Majority Rule reconstruction of nrITS in-part. (337 samples, sequence length = 667). Posterior probabilities displayed on branches. Individual terminals represent individual samples.

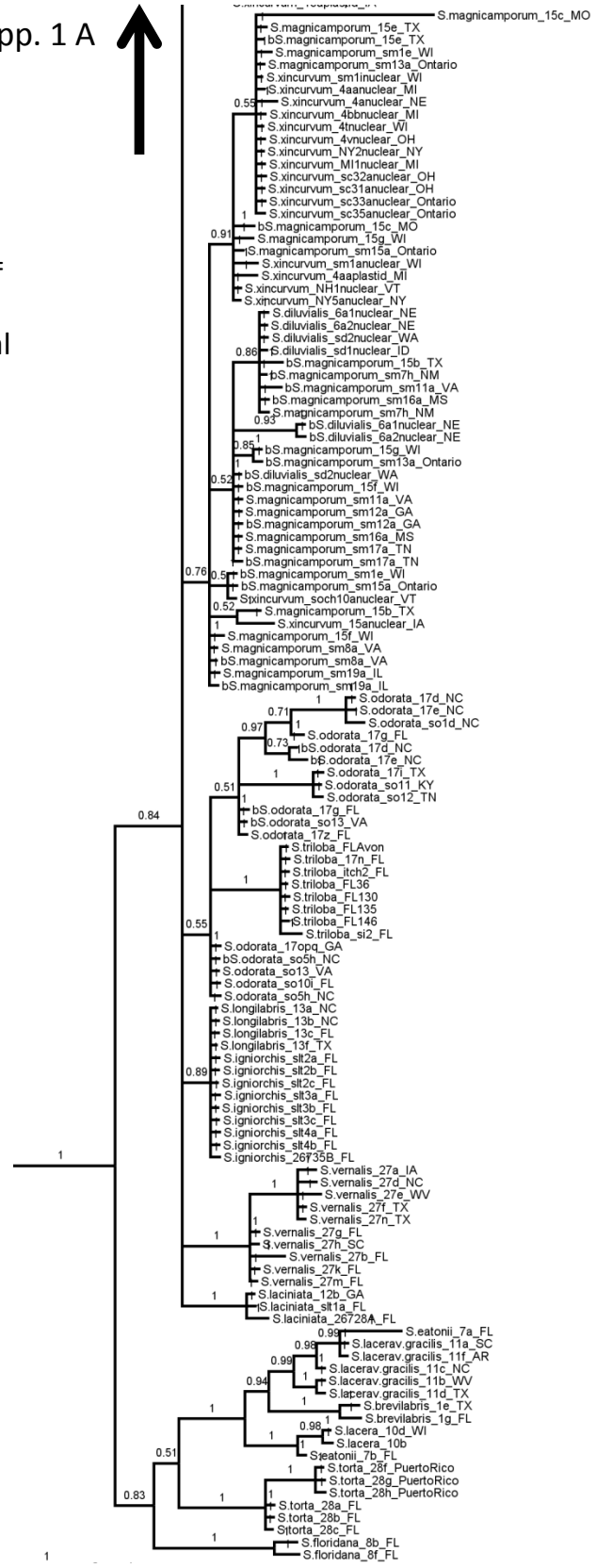


to fig. Supp. 1 B

to fig. Supp. 1 A

Fig Supp. 1 (B)

Bayesian Majority Rule reconstruction of nrITS in-part. (337 samples, sequence length = 667). Posterior probabilities displayed on branches. Individual terminals represent individual samples.



to fig. Supp. 1 C

to fig. Supp. 1 B

Fig Supp. 1 (C)

Bayesian Majority Rule reconstruction of nrITS in-part. (337 samples, sequence length = 667). Posterior probabilities displayed on branches. Individual terminals represent individual samples.

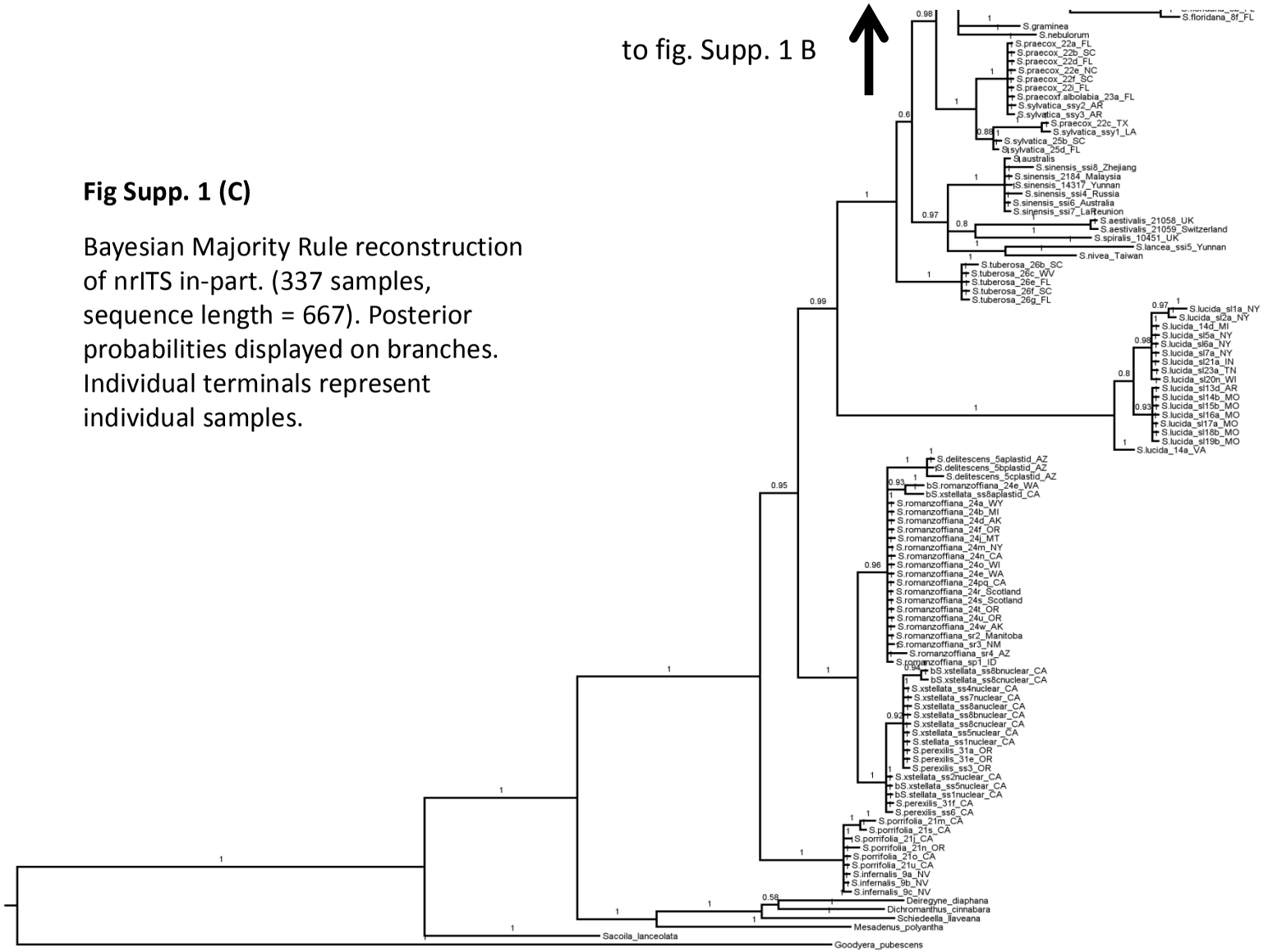


Fig Supp. 2

Bayesian Majority Rule reconstruction of *Xdh* (165 samples, sequence length = 942). Posterior probabilities displayed on branches. Individual terminals represent individual samples.

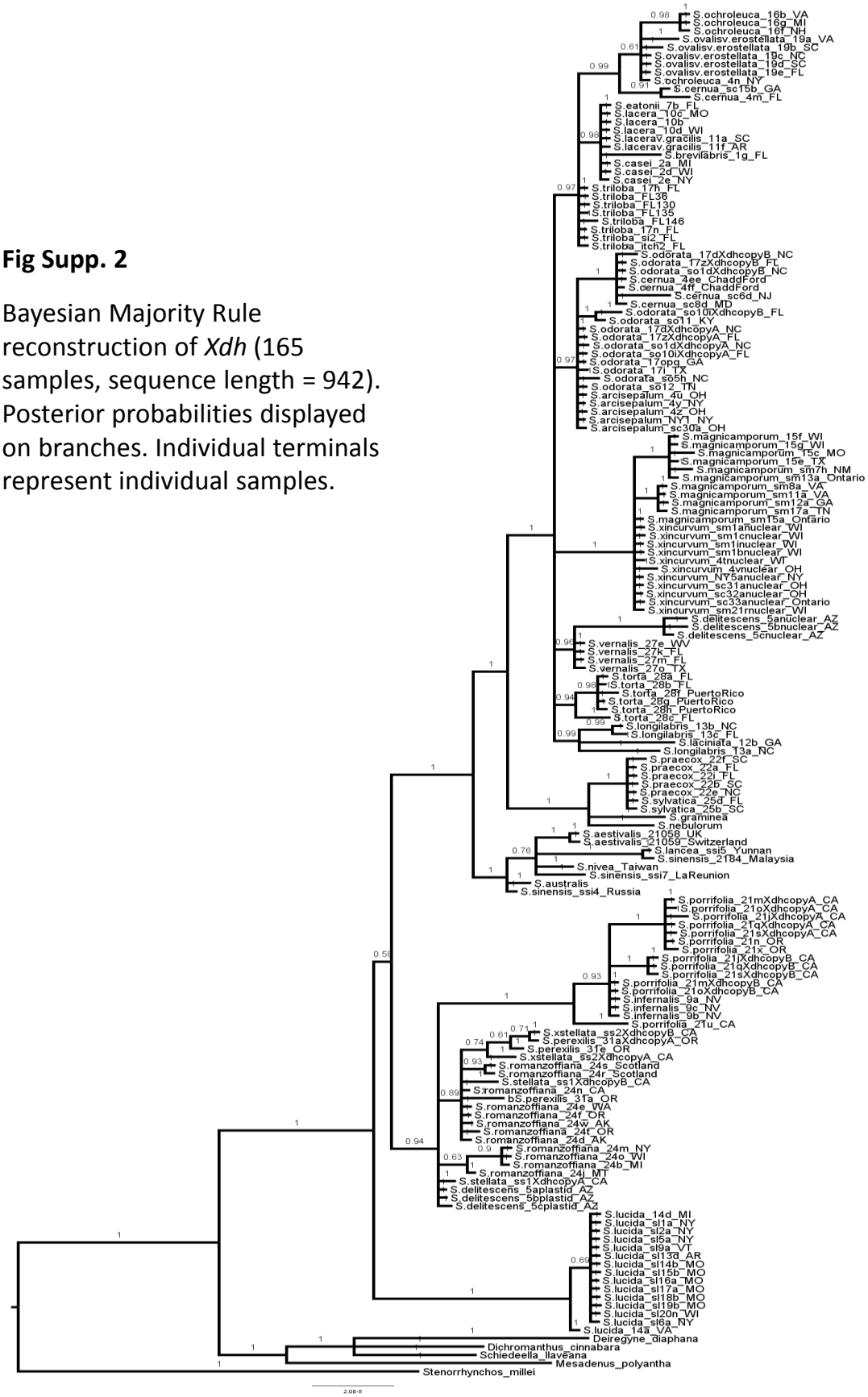
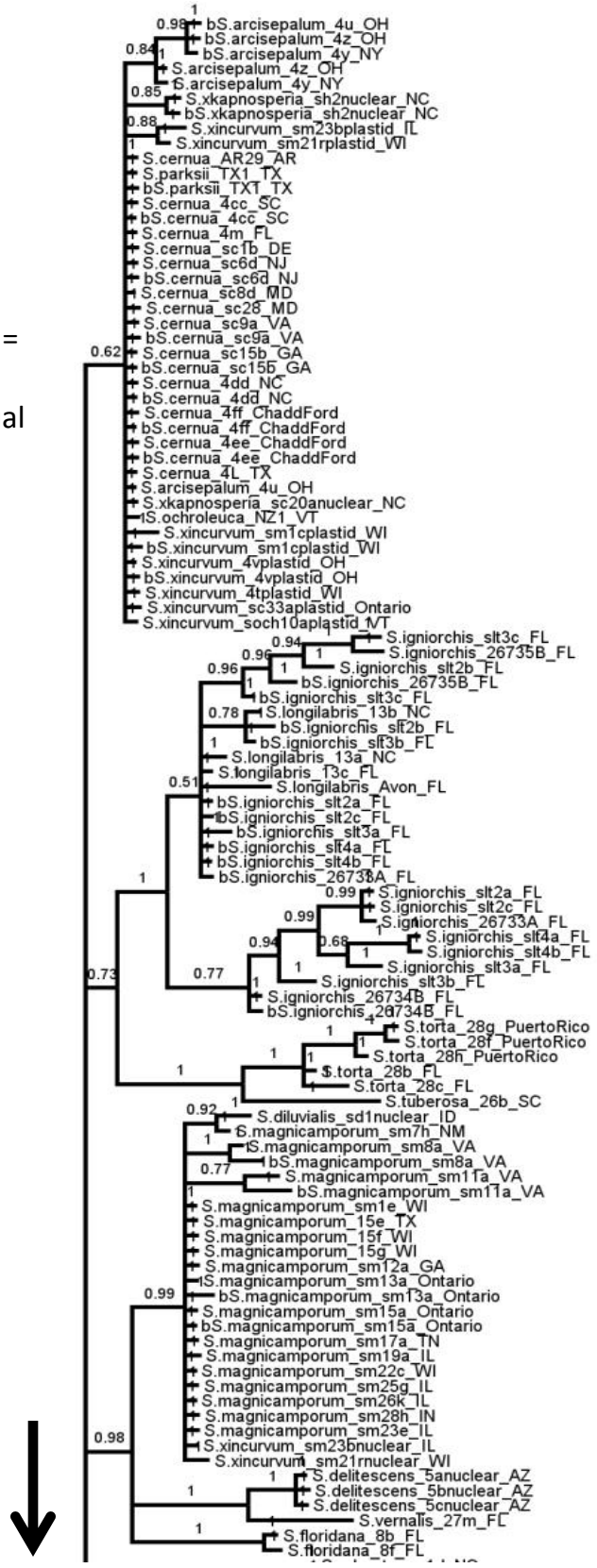


Fig Supp. 3(A)

Bayesian Majority Rule reconstruction of *ACO* in-part. (151 samples, sequence length = 1039). Posterior probabilities displayed on branches. Individual terminals represent individual samples.



to fig. Supp. 3B

Fig Supp. 3 (B)

Bayesian Majority Rule reconstruction of *ACO* in-part. (151 samples, sequence length = 1039). Posterior probabilities displayed on branches. Individual terminals represent individual samples.

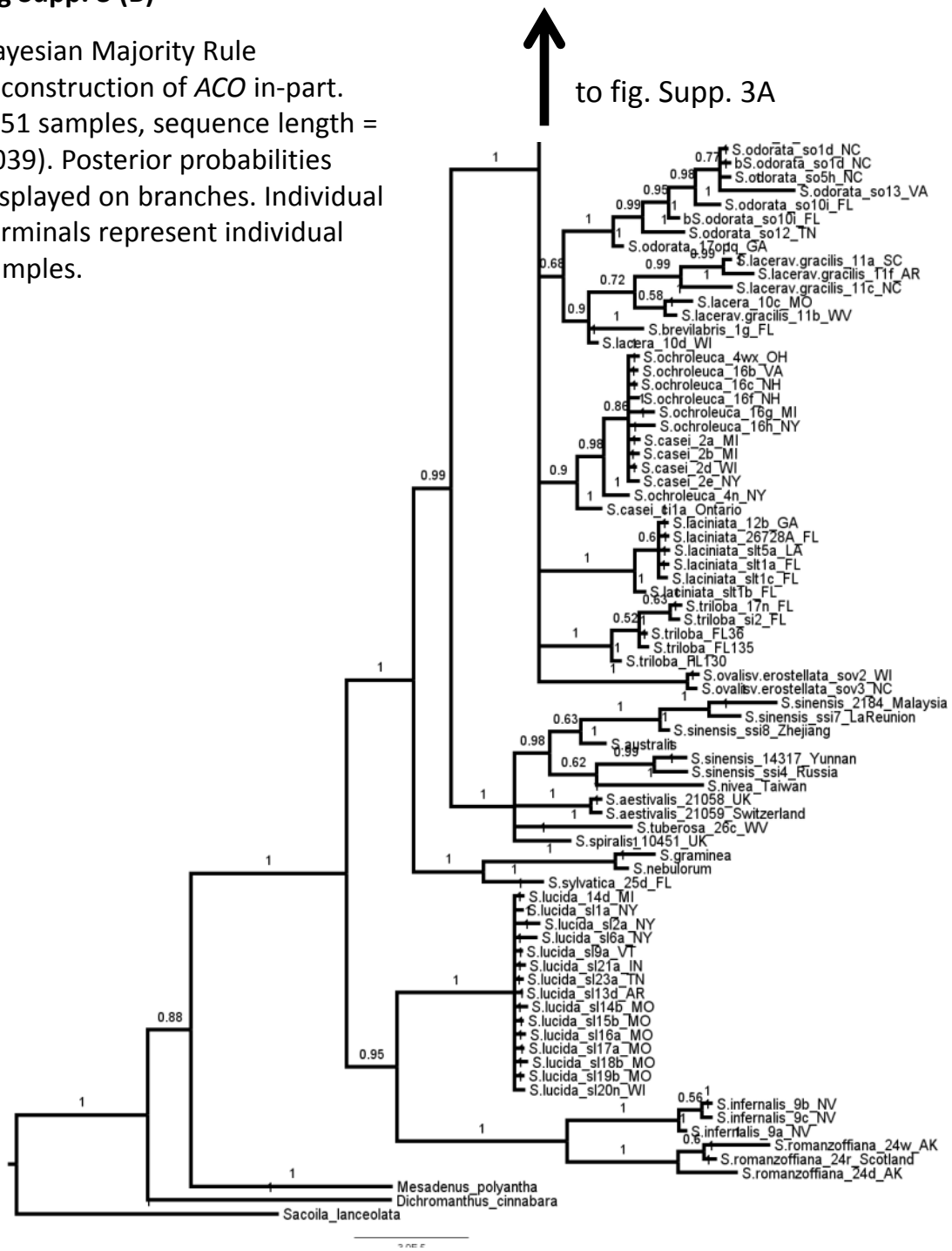
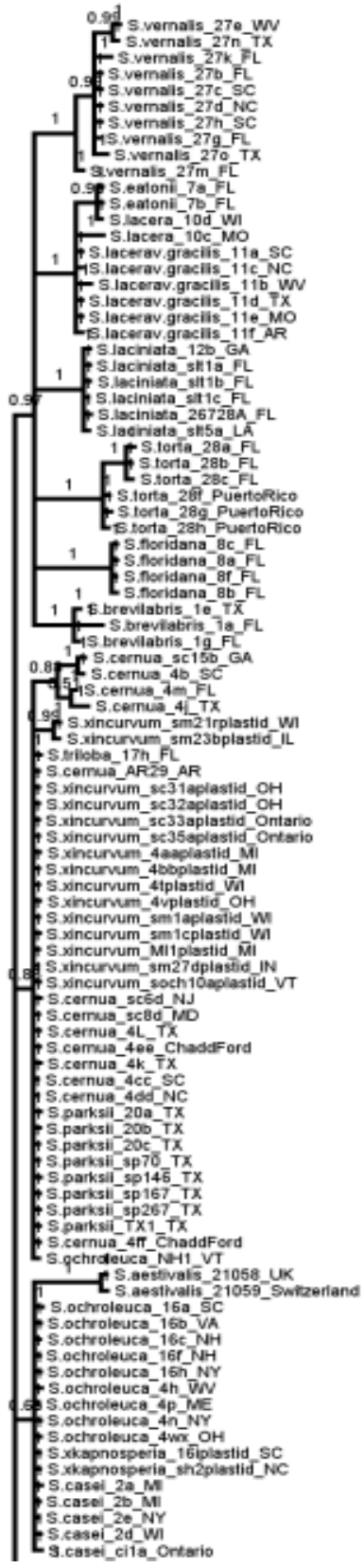


Fig Supp. 4(A)

Bayesian Majority Rule reconstruction of *mat*K in-part. (282 samples, sequence length = 1367). Posterior probabilities displayed on branches. Individual terminals represent individual samples.

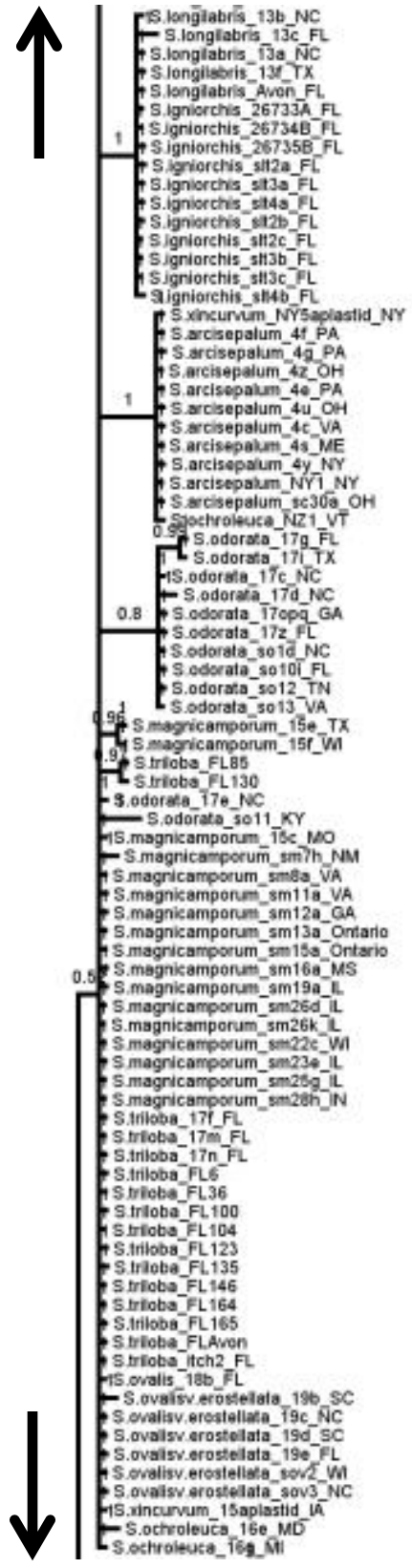


to fig. Supp. 4B

to fig. Supp. 4A

Fig Supp. 4(B)

Bayesian Majority Rule reconstruction of *mat*K in-part. (282 samples, sequence length = 1367). Posterior probabilities displayed on branches. Individual terminals represent individual samples.



to fig. Supp. 4C

to fig. Supp 4B

Fig Supp. 4(C)

Bayesian Majority Rule reconstruction of *mat*K in-part. (282 samples, sequence length = 1367). Posterior probabilities displayed on branches. Individual terminals represent individual samples.

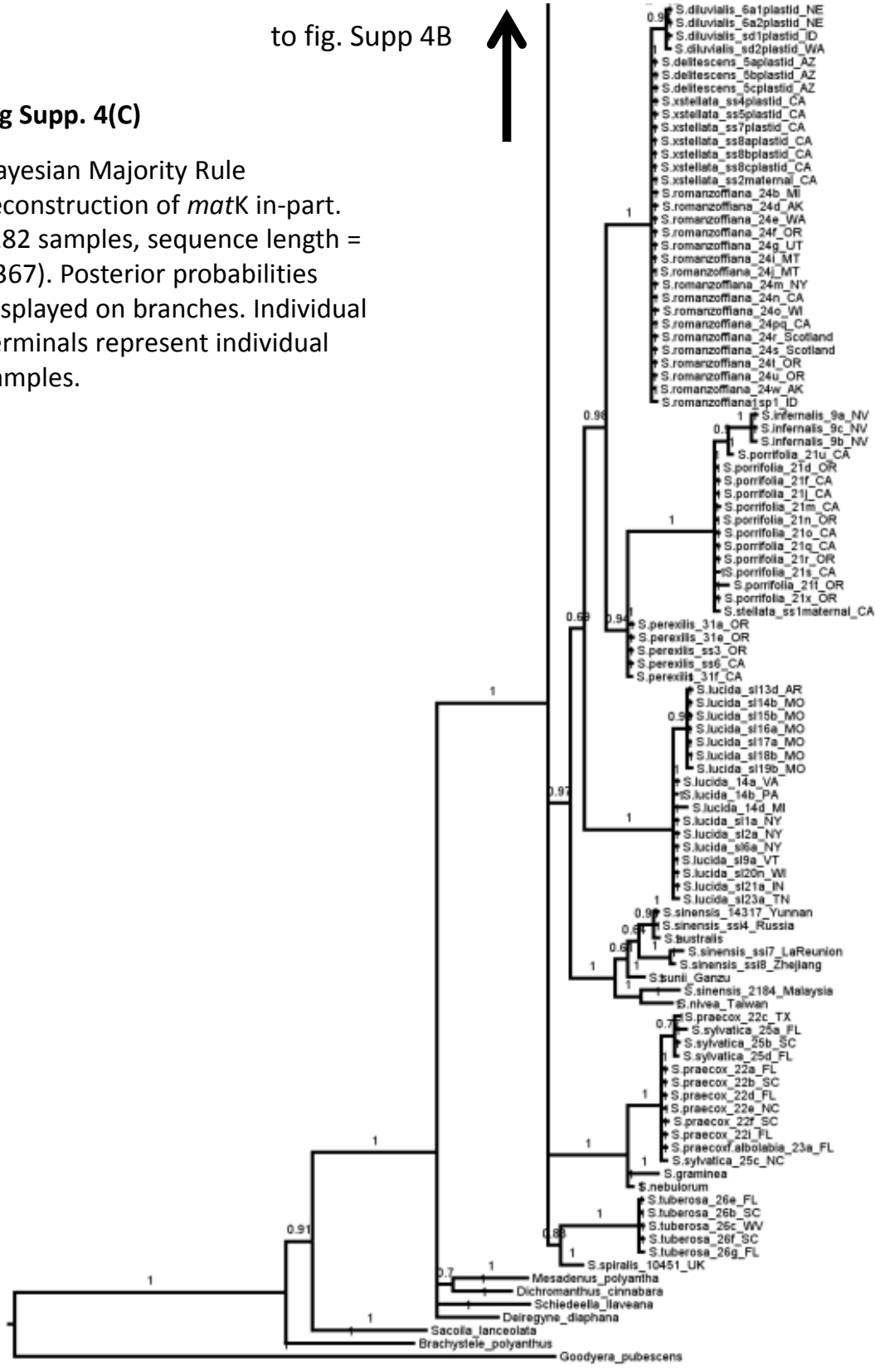
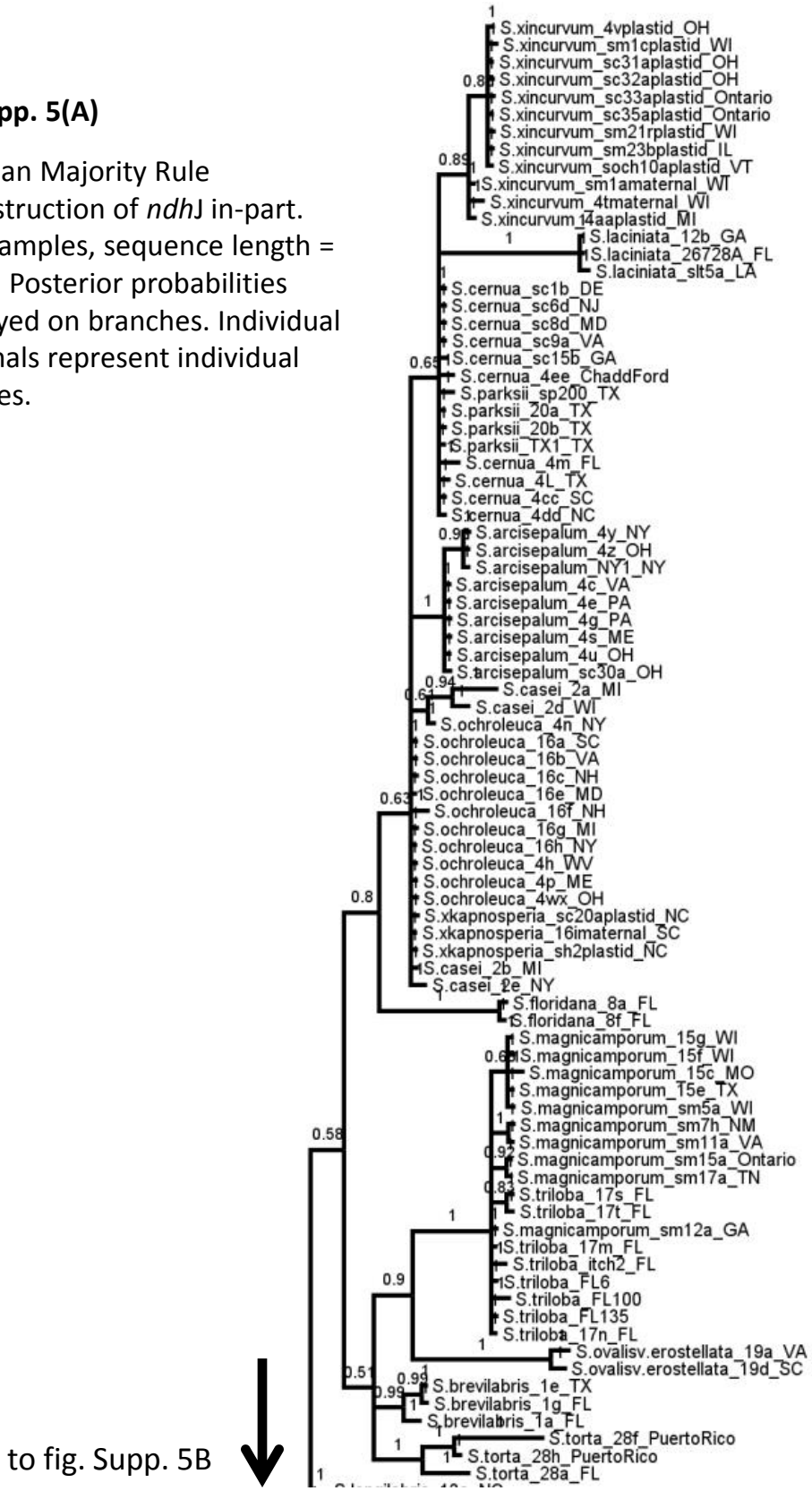


Fig Supp. 5(A)

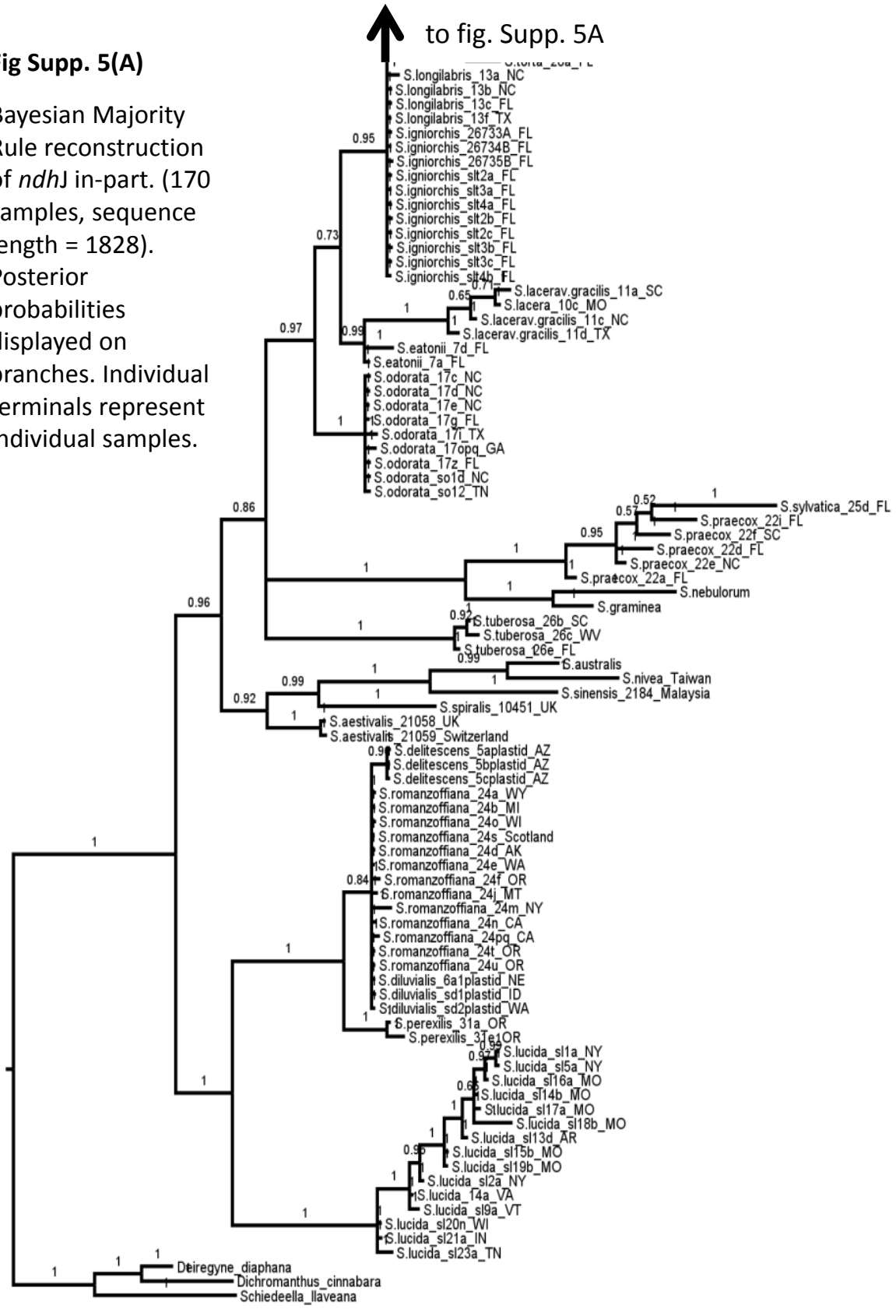
Bayesian Majority Rule reconstruction of *ndh*J in-part. (170 samples, sequence length = 1828). Posterior probabilities displayed on branches. Individual terminals represent individual samples.

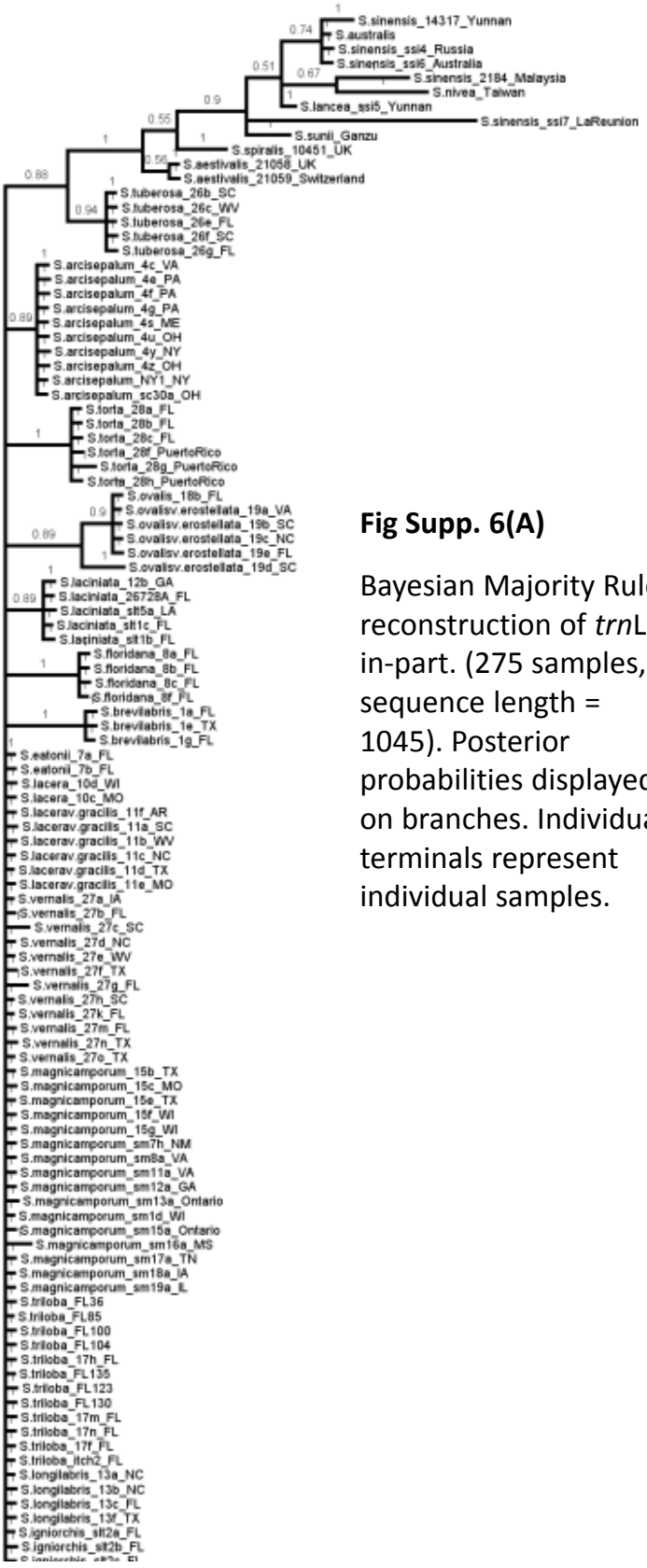


to fig. Supp. 5B

Fig Supp. 5(A)

Bayesian Majority Rule reconstruction of *ndh*J in-part. (170 samples, sequence length = 1828). Posterior probabilities displayed on branches. Individual terminals represent individual samples.





to fig. Supp. 6B

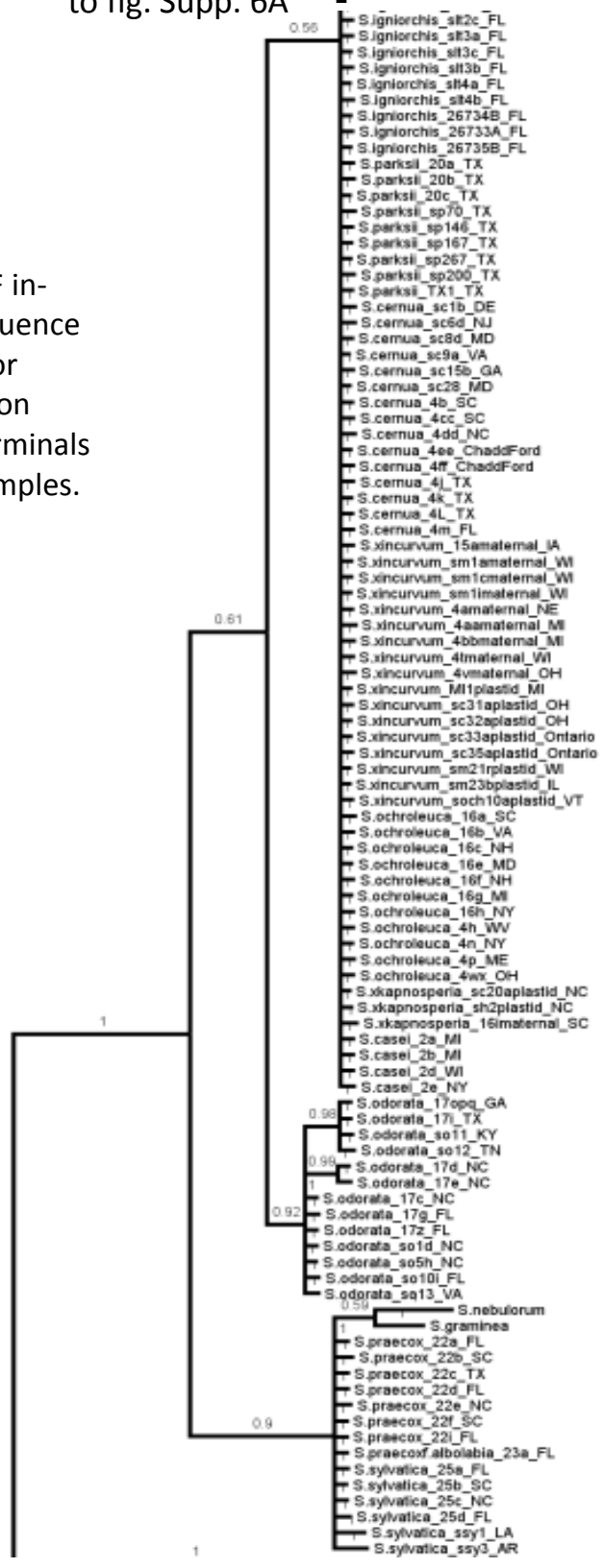
Fig Supp. 6(A)

Bayesian Majority Rule reconstruction of *trn*L-F in-part. (275 samples, sequence length = 1045). Posterior probabilities displayed on branches. Individual terminals represent individual samples.

to fig. Supp. 6A

Fig Supp. 6(B)

Bayesian Majority Rule reconstruction of *trn*L-F in-part. (275 samples, sequence length = 1045). Posterior probabilities displayed on branches. Individual terminals represent individual samples.



to fig. Supp. 6C

to fig. Supp. 6A

Fig Supp. 6(B)

Bayesian Majority Rule reconstruction of *trn*L-F in-part. (275 samples, sequence length = 1045). Posterior probabilities displayed on branches. Individual terminals represent individual samples.

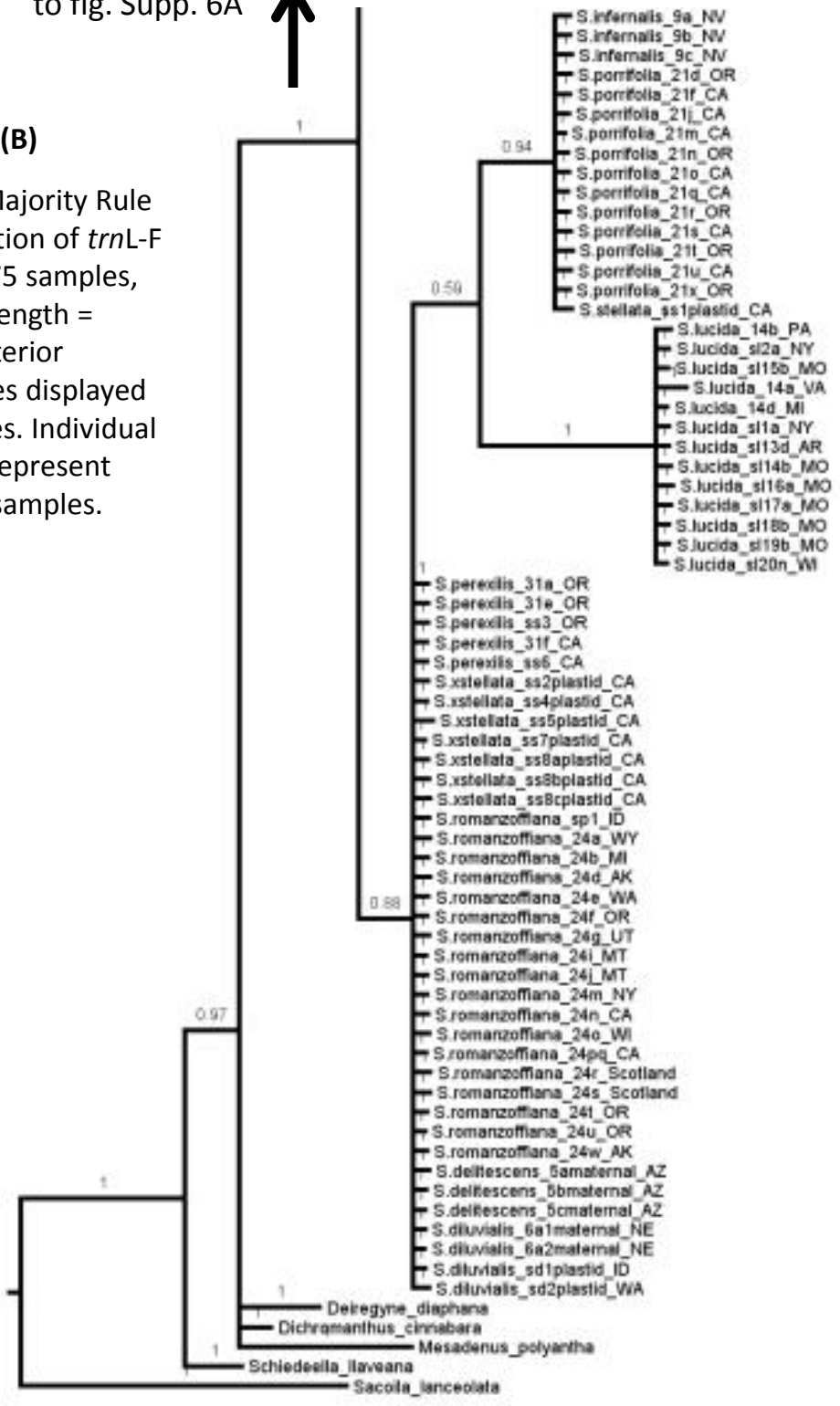
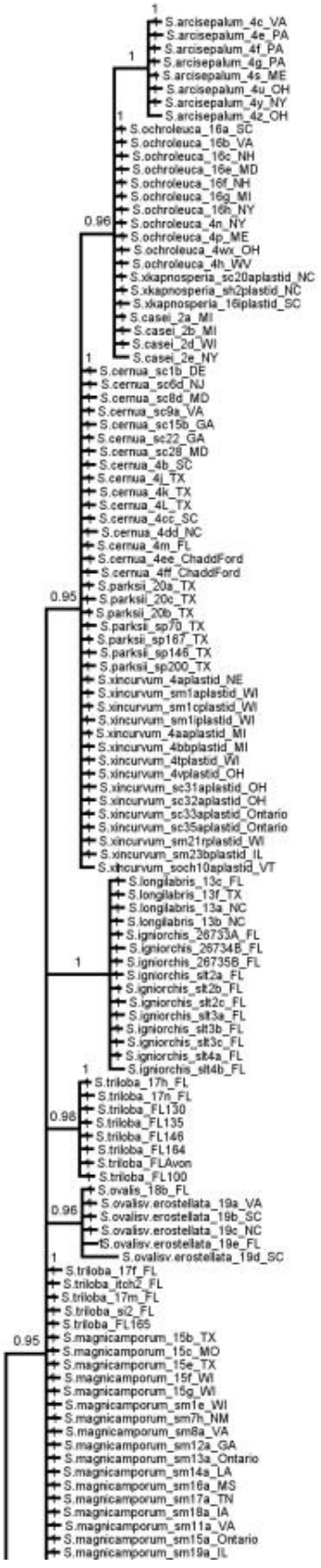


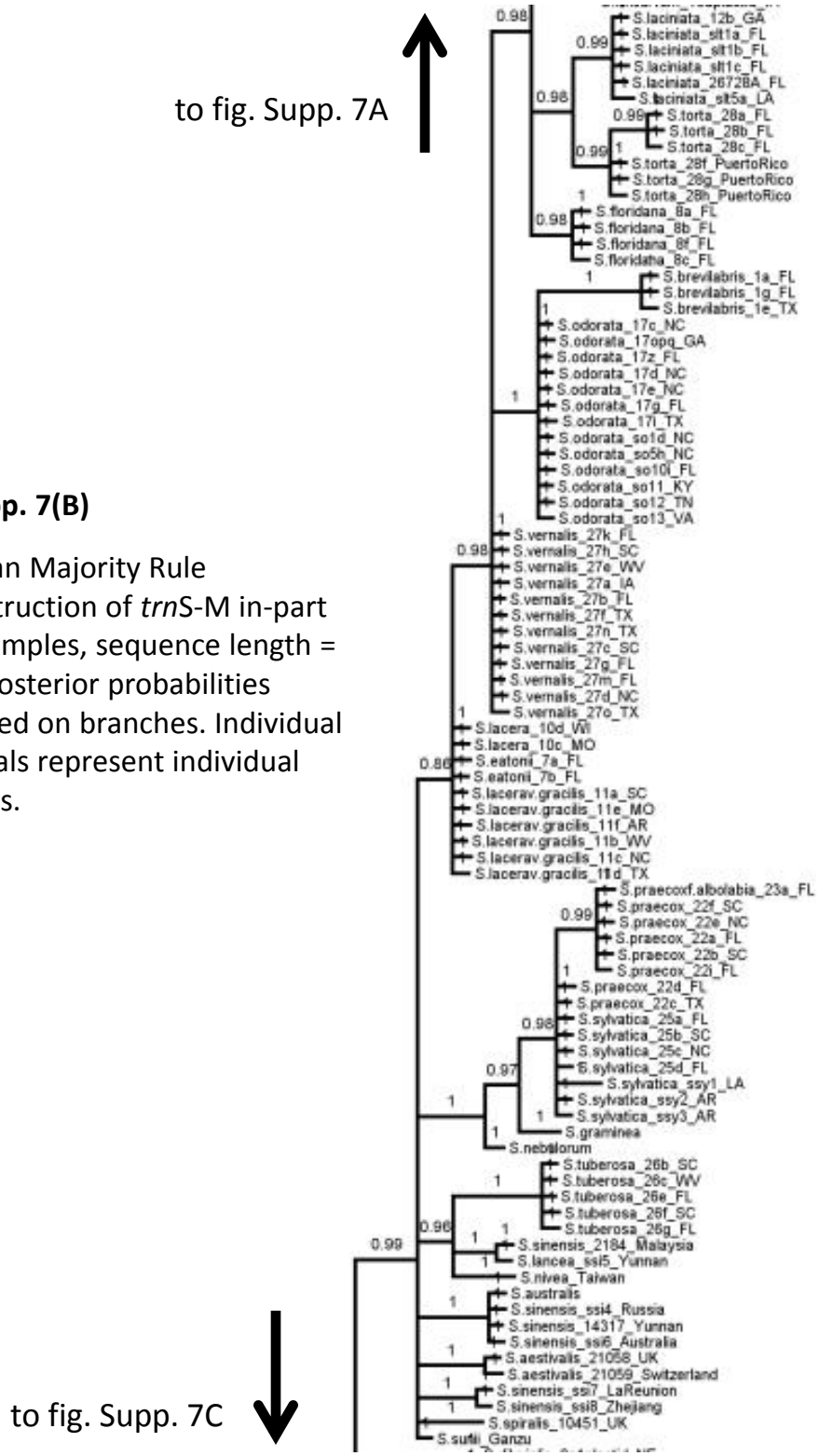
Fig Supp. 7(A)

Bayesian Majority Rule reconstruction of *trn*S-M in-part. (280 samples, sequence length = 823). Posterior probabilities displayed on branches. Individual terminals represent individual samples.



to fig. Supp. 7B

to fig. Supp. 7A



to fig. Supp. 7C

Fig Supp. 7(B)

Bayesian Majority Rule reconstruction of *trn*S-M in-part (280 samples, sequence length = 823). Posterior probabilities displayed on branches. Individual terminals represent individual samples.

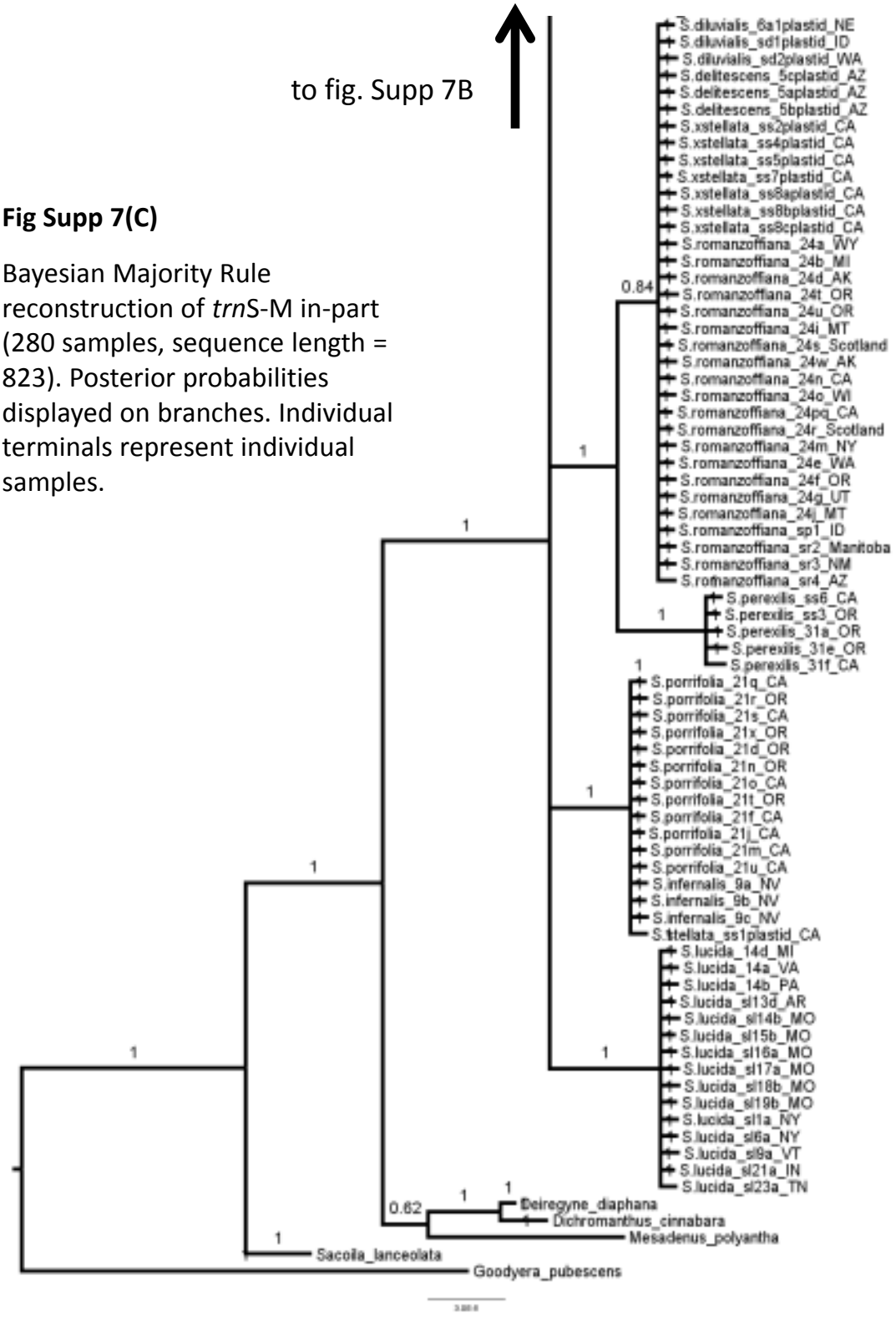
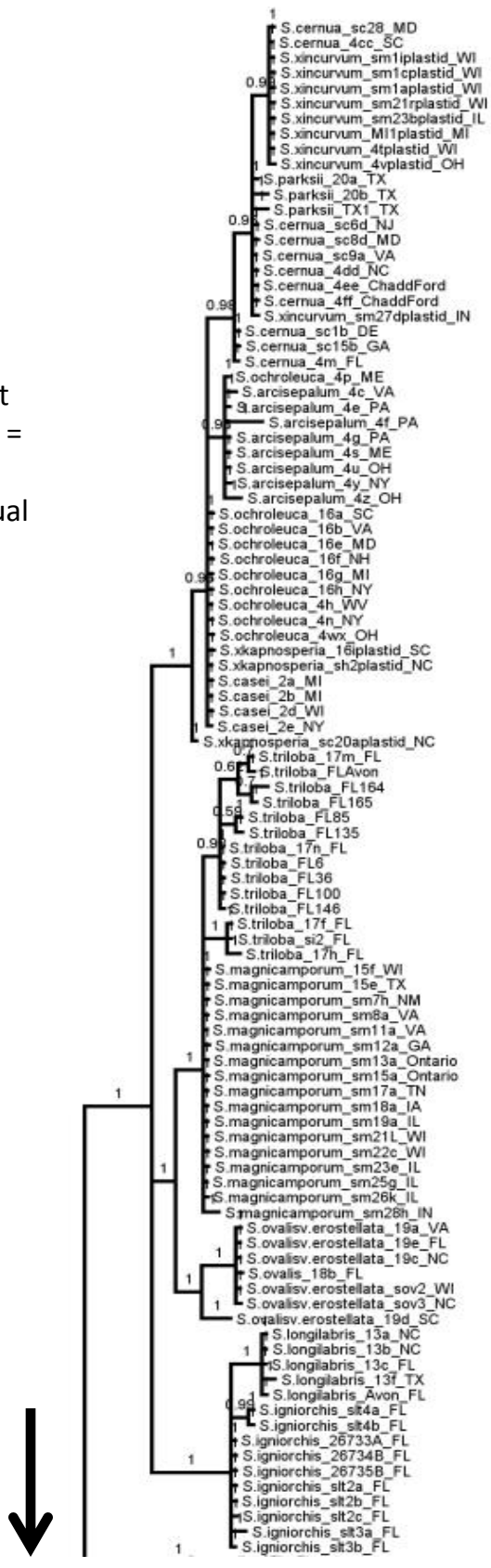


Fig Supp 7(C)

Bayesian Majority Rule reconstruction of *trn*S-M in-part (280 samples, sequence length = 823). Posterior probabilities displayed on branches. Individual terminals represent individual samples.

Fig Supp. 8(A)

Bayesian Majority Rule reconstruction of *ycf*1 3' in-part (229 samples, sequence length = 1918). Posterior probabilities displayed on branches. Individual terminals represent individual samples.



to fig. Supp 8 B

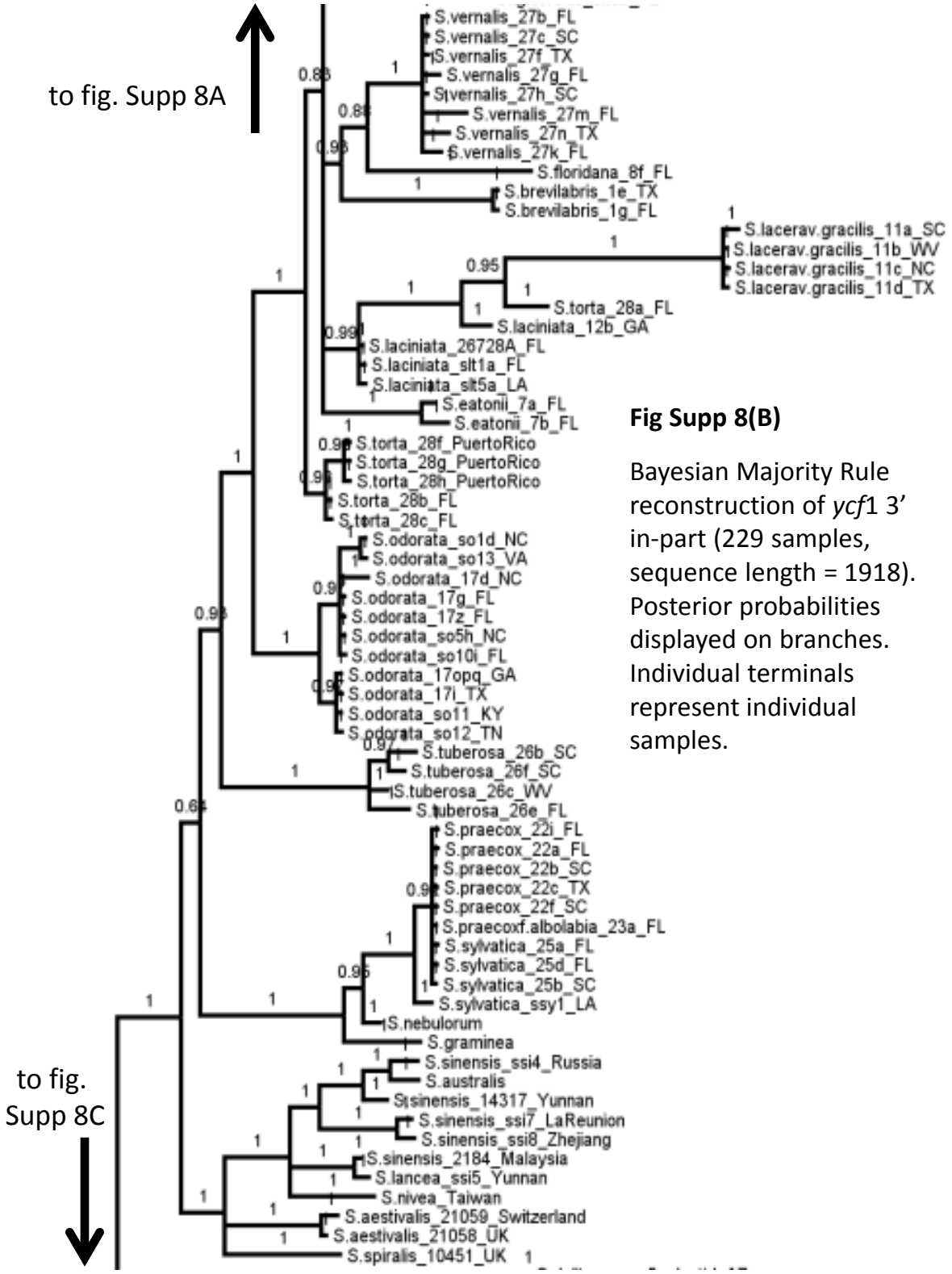
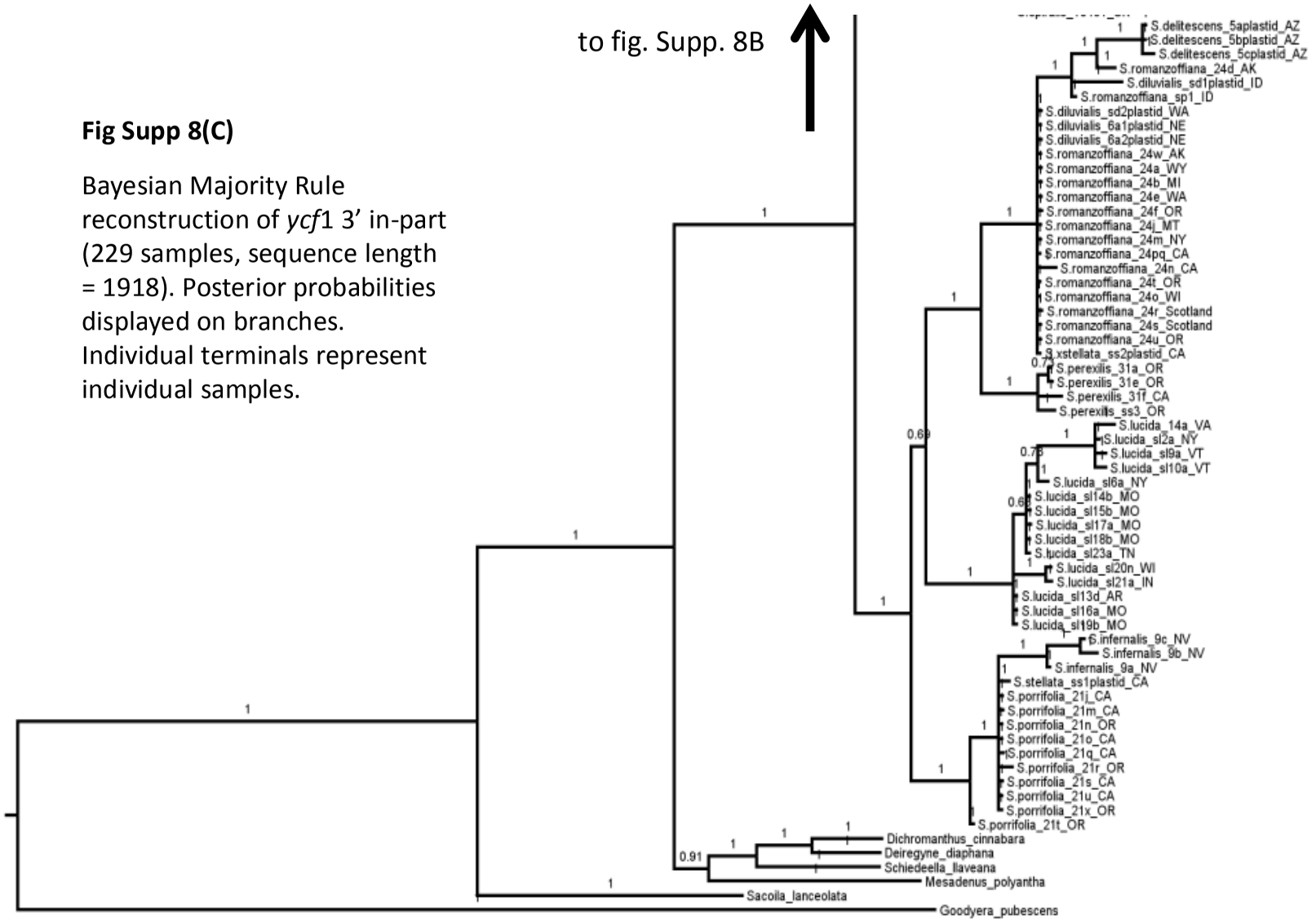


Fig Supp 8(B)

Bayesian Majority Rule reconstruction of *ycf*1 3' in-part (229 samples, sequence length = 1918). Posterior probabilities displayed on branches. Individual terminals represent individual samples.

Fig Supp 8(C)

Bayesian Majority Rule reconstruction of *ycf*1 3' in-part (229 samples, sequence length = 1918). Posterior probabilities displayed on branches. Individual terminals represent individual samples.



CHAPTER 6

The biogeographic evolutionary history of *Spiranthes* (Orchidaceae) reveals repeated speciation within and dispersal from the North American Geological Coastal Plain, an underappreciated biodiversity hotspot.

Matthew C. Pace & Kenneth M. Cameron

***Abstract*—**The biogeographic relationships of the flora of North America, both within the continent and among other regions of Earth, has been a topic of interest since European colonization. The Orchidaceae of North America, as exemplified by the genus *Spiranthes* represent an excellent case study in which to examine the movement of species across the planet, the role of North American physiographic regions as arks and/or cradles areas of speciation, and the influence that North American glacial cycles may have played in cladogenesis, the isolation of disjunct populations, and speciation events. Using a calibrated molecular phylogenetic reconstruction, we estimate divergence times and likely ancestral distributional ranges for the most recent common ancestors of extant taxa. We find that contrary to long standing hypotheses, at least in the case of *Spiranthes*, the North American Geologic Coastal Plain has been an important center of evolution for this genus. The North American Geologic Coastal Plain has repeatedly served as an ancestral region from which clades and individual species have repeatedly dispersed and migrated, including to Eurasia, the Caribbean, and Mexico and Central America.

***Key words*—**BioGeoBEARS; chronogram; North American Geologic Coastal Plain; population disjunctions; Quaternary Glaciation; Sierra Madre Occidental

Compared to neighboring Mexico, the orchid flora of the United States and Canada is relatively small in number –ca. 202 species in 69 genera in North America (21.978 million km^2) vs. ca. 1,231 species in 164 genera in Mexico (1.973 million km^2)– but is phylogenetically fairly diverse, with 4 out of 5 subfamilies, and 14 out of 27 tribes represented. North America excluding Mexico has two endemic orchid genera (*Isotria* Raf. and *Cleistesiopsis* Pansarin & F. Barros) and several orchid genera display the classic North American – East Asian biogeographic relationship (e.g., *Cypripedium* L., *Goodyera* R. Br., *Malaxis* Sol. ex Sw., *Platanthera* Rich., *Pogonia* Juss.) found in numerous plant groups (e.g., the *Adiantum pedatum* L. complex (Pteridaceae), *Hamamelis* L. (Hamamelidaceae), *Gymnocladus* Lam. (Fabaceae), *Polygonatum* Mill. (Asparagaceae), *Smilax* L. (Smilacaceae). Additionally, there is a clear biogeographic relationship between the orchid flora of North America and Mexico/Central America (e.g., subtribe Spiranthinae Lindl. ex Meisn.). North America, especially for taxa restricted to Florida, also shares elements with the Antillean and South American orchid floras, but the number of shared species is usually small (e.g., *Dendrophylax* Rchb.f. in W.G. Walpers: 1 spp. vs. 10 spp., *Epidendrum* L.: 7 spp vs. ca. 1,500 spp., *Pleurothallis* R. Brown: 1 spp vs. ca. 2,500 spp).

The North American Geological Coastal Plain [NAGCP], a long narrow region characterized by low rolling hills and flat alluvial deposits over sedimentary formations, stretches from Cape Cod, MA, and Long Island, NY, south through Florida and westward along the Gulf of Mexico to the southern tip of the Sierra Madre Oriental in Mexico. It is a hot spot of orchid species richness within North America, containing 125 species –including 23 endemic species, and 63 primarily Caribbean or South American species that reach their northern limit in central Florida. That the lowland NAGCP contains such diversity is in contrast to the

mountainous regions of the world typically considered as centers of orchid diversity –such as the northern Andes, New Guinea Highlands, and the Serrania de Tabasara and Darien Mountains of the Isthmus of Panama (Bogarín et al. 2013, Myers et al. 2000). Like other tropical and subtropical grassland and savanna biomes, the NAGCP has long been overlooked as a plant biodiversity hotspot or area of particular evolutionary significance (Sorrie and Weakley 2001, Noss et al. 2015, Parr et al. 2014). As exemplified by statements made by Gleason (1964) and Thorne (1993), the NAGCP has primarily been considered a 'recipient' of taxa from other physiographic regions –such as the Appalachian Mountains, Caribbean, or central Mexico, a hypothesized result of glacial oscillations– with little 'donation' of taxa to other regions. These ideas have persisted primarily due to the misconceptions that the NAGCP is geologically young and environmentally homogeneous, and that prairie and savanna communities are early successional phases resulting only from human-initiated fire. However, fossil evidence indicates that many extant plant communities or their close equivalents have existed in a relatively stable condition along the NAGP from the early Miocene onwards; these include *Taxodium–Nyssa* and broadleaf wet forests (Berry 1909, Stults and Axsmith 2011), marshes and wet pine forests (Hansen et al. 2001), and scrub oak dune communities (Berry 1937) from the Mid-Atlantic south to Florida, and oak-hickory forests near the northern limit of the NAGCP in New Jersey (Rachele 1976, Kotthoff et al. 2014). Additionally, naturally occurring lightning generated fire has been documented to have played a key role in the maintenance of grasslands and savannas in the NAGCP for periods greatly pre-dating the arrival of humans into the region (Graham 1999, He et al. 2012, Noss et al. 2015).

When contrasted against the size of the family, there are few published analyses of orchid biogeography considered in an explicit phylogenetic context. Furthermore, many of these

primarily focus on taxonomic relationships, with only a secondary emphasis on biogeography. An early study to place orchid biogeographic relationships in clear phylogenetic terms was Cameron and Chase (1999). Using Pogoniinae (Vanilloideae) as a case study for the classic East Asian–North America floristic disjunction, the authors recovered the origin of the tribe within northern Brazil and the Guiana Shield, with long distance dispersal into North America followed by subsequent migration across Beringia to Asia during the Tertiary – an 'out of the Americas' example that contrasts with the more common 'out of Asia' pattern. Within Epidendroideae, Van den Berg et al. (2005) discussed a biogeographic signal to the circumscription of the tribes Epidendreae (Neotropical) and Arethuseae (primarily Old World). Two cases of island biogeography have been examined within Orchidaceae: the Caribbean and the Western Indian Ocean. Trejo-Torres and Ackerman (2001), looking across the Caribbean orchid flora, found that orchid-defined groupings of islands correspond to their overall ecological features due to either common physiography or similar geomorphology, and that floristic affinities between island groups appeared to be determined by ecological characteristics, rather than by barriers to seed dispersal. Micheneau et al. (2008) supported the Madagascan origin of the Mascarene angraecoid orchids, however most species originated from independent colonizations of the Mascarene archipelago with subsequent radiation within the island system playing only a minor role. In Eastern Asia, *Holcoglossum* Schltr. spread across the Hengduan Mountains as a result of the Qinghai-Tibetan Plateau uplift after dispersing from tropical Asia (Fan et al. 2009). An unexpected relationship within the *Corallorhiza striata* Lindl. sensu lato complex between *C. striata* var. *involuta* (Greenm.) Freudenst. (Mexico) and *Corallorhiza bentleyi* Freudenst. (Virginia and West Virginia, U.S.) was discovered by Barrett and Freudenstein (2011). Additionally, the *C. striata* complex sensu stricto is composed of three well-supported and

geographically distinct monophyletic clades: California, Southwestern U.S. & Mexico, and Northeastern U.S. & Canada. In the Mediterranean Basin, Pavarese et al. (2011) found that Sardinian populations of *Platanthera biflora* var. *kuenkelei* (H. Baumann) P. Delforge, despite being geographically closer to populations in Italy, appear to be most closely related to Tunisian plants, and that this is not a recent dispersal. Guo et al. (2012) examined the biogeographic origins and role of vicariance in the subfamily Cypripedioideae and found that 1) the subfamily originally evolved in the New World and 2) at least two vicariance events have led to the disjunct distribution of conduplicate slipper orchids between the New and Old World. Most recently, an expansive phylogeographic study of subtribe Acianthinae (Pimm Lyon 2014) revealed that *Corybas* Salisb. has radiated multiple times from Australia to the Malay Archipelago, New Caledonia, and New Zealand, with secondary dispersals originating from Papua New Guinea to Southeast Asia, the rest of the Malay Archipelago, and Taiwan.

Within North America many plant and animal taxa have had their biogeographic relationships and histories estimated (see Soltis et al. 2006 for a review). Although its orchid flora is essentially wholly known from a biodiversity/species-richness point of view, only Cameron and Chase (1999), Barrett and Freudenstein (2011), and Guo et al. (2012) have investigated the biogeographic history of North American Orchidaceae objectively, and each of those studies focused only on a small clade. Sheviak (2013) attempted to put cytological variation within *Spiranthes romanzoffiana* into a geographic framework, but his results must be interpreted cautiously as they were presented without a phylogenetic perspective and included some misidentifications and hybrids (Chapter 4). Sheviak (2013) himself acknowledged some of these limitations, ultimately attributing these difficulties to his idea that "*Spiranthes romanzoffiana* is an abstraction." Although based on a molecular phylogeny, the many

biogeographic inferences discussed in Dueck et al. (2014) are mostly speculative, untested, and based on incomplete taxon sampling. It is remarkable that a charismatic group such as the orchids, particularly from a floristically well-characterized region such as North America, have been largely ignored from a biogeographic standpoint.

Spiranthes (Orchidoideae: Cranichideae: Spiranthinae) is unique among the orchids in that it occurs in six global-scale regions –Southern and Eastern Asia, Australasia/Oceania, the Caribbean, Europe & North Africa, Mesoamerica, and North America– and its closest relatives are Meso- or South American, yet it is most species-rich in North America (fig. 1). Furthermore, *Spiranthes* also contains some of the most widely and narrowly distributed plants on Earth: *Spiranthes sinensis* (Pers.) Ames spans approx. 107° of latitude and 135° of longitude; *S. delitescens* Sheviak is endemic 2–4 ciénegas in southern Arizona. It is remarkable that *Spiranthes* is one of a handful of orchid genera to display a transcontinental distribution. *Cypripedium*, *Goodyera*, *Liparis*, *Malaxis*, and *Vanilla* Mill. share similar mulit-biogeographic and transcontinental distributions, but are most species-rich in areas outside of North America. In this regard *Spiranthes* is comparable to *Trillium* L. (Melanthiaceae) which is found in both North America and East Asia, yet is far more species-rich in North America (ca. 42 spp. vs. 5–6 spp.; Case and Case 1997). *Spiranthes* is the second largest orchid genus in North America after *Platanthera* sensu Bateman et al. (2009) (ca. 26 spp. vs. ca. 42 spp.), with an additional 7–10 spp. found wholly outside of the United States and Canada (fig. 1). One species –*S. romanzoffiana*– is the most widely distributed, being found across boreal and montane North America in addition to the northern British Isles, and *Spiranthes torta* (Thunb.) Garay & H.R. Sweet grows throughout the Antilles, reaching its northern limit in southern Florida, with

peripheral populations along the eastern coast of Mesoamerica (Yucatan Mexico, Belize, and Nicaragua).

Since the reestablishment of *Spiranthes* s.s. (Balogh 1982), the genus had traditionally been divided into three informal biological and biogeographical associations: a Western North American Cordillera and Mesoamerican group, an Eastern North American group, and a Eurasian group (Sheviak 1982, 1984, 1989, 2012, 2013, Sheviak and Brown 2002). There is also a cytological division in the genus (n = 15 vs n = 22) (Sheviak 1982, 1984, 1989 1990, 2008). The phylogenetic work of Pace et al. (Chapter 5) supported the evolutionary validity of the cytological but not the biogeographic groupings; the present work seeks to address the historical biogeographic relationships recovered by Pace et al. (Chapter 5).

Due, in part, to a poor fossil record –just three undisputed orchid fossils (Ramírez et al. 2007, Conran et al. 2009) – few attempts have been made to date orchid phylogenetic reconstructions. Working before the publication of any verified orchid fossils, Sheviak (1984, 1989) invoked the interpluvial period of the Pleistocene as the probable period for the formation of the hybrid species *Spiranthes diluvialis* Sheviak and *S. delitescens*. Using the only verified orchid fossil at the time (*Meliorchis caribea*), the crown age for the subfamily Orchidoideae – which includes *Spiranthes*– was dated by Ramírez et al. (2007) to ca. 58 million years before present (mybp), near the boundary of the Paleocene and Eocene. Expanded and improved fossil calibration by Gustafsson et al. (2010) adjusted the origin of the Orchidoideae to ca. 53 mybp and timed the divergence of *Spiranthes* and *Sarcoglottis* C.Presl –a distantly related Spiranthinae (Salazar et al. 2003, Salazar and Jost 2012) – at ca. 17 mybp in the early mid-Miocene.

Broad-scale phylogenetic studies place *Spiranthes* as the only North American lineage within the otherwise Mesoamerican and South American Spiranthinae subtribe (Salazar et al.

2003, Álvarez-Molina & Cameron 2009, Salazar et al. 2009, Salazar and Jost 2012). The most inclusive genus-level sampling (Salazar and Jost 2012) recovers *Spiranthes* as embedded within a primarily Mexican and Mesoamerican clade of *Aulosepalum* Garay, *Beloglottis* Schltr., *Deiregyne* Schltr., *Dichromanthus* Garay, *Funkiella* Schltr., *Mesadenus* Schltr., *Microthelys* Garay, *Schiedeella* Schltr., *Sotoa* Salazar, and *Svenkoeltzia* Burns-Bal. Specifically, *Spiranthes* is the sister genus to a Northern Mexican clade of (*Mesadenus*, (*Schiedeella*, (*Dichromanthus*, *Deiregyne*))).

The distinctive biogeographic and phylogenetic attributes of *Spiranthes* both globally and within North America make it an excellent group in which to examine the movement of species across the planet, the role of North American physiographic regions as arks and/or cradles areas of speciation, and the influence that North American glacial cycles may have played in cladogenesis, the isolation of disjunct populations, and speciation events. An understanding of *Spiranthes*' inverse diversity pattern can serve as a model for researching the biogeography of other north temperate orchid and plant groups. Additionally, as climate- and habitat-change lead to new hybridization events (e.g., Rutledge et al. 2012, Becker et al. 2013, Monzon et al. 2014) – placing a renewed focus on conservation (e.g., Muhlfeld et al. 2014) – an examination of the biogeographic history of *Spiranthes* can lead to the generation and testing of novel hypotheses regarding the reaction of Nearctic species to a changing biome.

MATERIALS AND METHODS

Phylogenetic analyses

We utilized the two separate data matrices of Pace and Cameron (Chapter 5) for our analyses: I) a complete phylogenetic sampling with multiple individual samples per species, and II) a reduced data set composed of one accession per taxon; this second data set was necessary

for analyses in BioGeoBEARS. The complete sampling is composed of 308 individual samples/terminals, whereas the reduced data matrix is represented by 51 terminals (one terminal per species). Both matrices resulted from concatenation of low copy nuclear *ACO* and *Xdh*, nrITS, and plastid *mat*K, *ndh*J, *trn*L-F, *trn*S-M, and *ycf*1 3' sequence data. Methods for DNA extraction, PCR amplification of loci, locus alignment, and phylogenetic analyses under Bayesian inference follow those described by Pace and Cameron (Chapter 5).

Dating

Our analyses were implemented under a Bayesian framework in BEAST v1.8.1. (Drummond et al. 2012) through the CIPRES web portal (Miller et al. 2010) under a lognormal relaxed molecular clock (Drummond et al. 2006) and Speciation: Birth-Death Process tree process. Based on the calibrated reconstructions of Ramírez et al. (2007) and Gustafsson et al. (2010), the MRCA of monophyletic subtribes Goodyerinae and Spiranthinae was offset to 38 mybp under lognormal priors with a standard deviation of 0.75. This calibration was used to produce a chronogram for both the full and reduced datasets. Subtribe Goodyerinae is represented in our dataset by *Goodyera pubescnes* (Willd.) R. Br. We assessed convergence among independent runs and confirmed effective sampling of our parameters by examining output log files in Tracer v1.6 (Rambaut et al. 2014). Fifty percent of samples were removed as burn-in, and we constructed a maximum clade credibility tree using TreeAnnotator v1.8.0 (Drummond et al. 2012).

Biogeographic Analyses and Ancestral Range Estimation (ARE)

We performed AREs using the reduced dated dataset as outlined above. Analyses using the BEAST chronogram were run in the R package BioGeoBEARS (Matzke 2014) under the DEC and DEC+J models. For the ARE analysis, regions where *Spiranthes* occur were

categorized as present or absent using the standard US Geological Survey Physiographic Divisions of North America: Coastal Plain, Appalachian Highlands, Interior Lowlands, Ozark Plateau & Ouachita Mountains, Rocky Mountains, Laurentian Uplands & Boreal North, Intermountain Basin & Range, and Pacific Mountains (fig. 2, fig. 4). With the additional categories "Old World" (representing Europe, Asia, and Australia) and "Mesoamerica" (representing Mexico, Central America, and the Caribbean), 10 areas were included to capture the full range of the included taxa. The computational limitations of BioGeoBEARS necessitated the grouping of the non-North American regions into broad categories. Given the limited number of in-group species that occur in these areas, we feel this approach was justified, and likely has negligible impact on our analyses. For our outgroup taxa, which are represented by one species/genus, we coded these tips to represent the full distributional limit of that genus. We set the maximum number of areas at a given node to five so as to avoid unmanageability of our analysis.

RESULTS

Dating and Biogeography

Using the reduced data chronogram (fig. 5), the branching event between *Spiranthes* and its sister group occurred during the mid-Miocene, ca. 14.5 mybp. The two subgroups within *Spiranthes* –the branching event that lead to the evolution of the Western Cordillera + *S. lucida* clade and the rest of the genus– occurred ca. 10.9 mybp. The sister outgroup clade was recovered as having evolved in Mexico, whereas the ancestral biogeographic regions for the evolution of *Spiranthes* and the initial subgrouping within the genus were not resolved, although one might speculate that it may have occurred in the general area of northern Mexico and southern Arizona, New Mexico, and Texas where the Intermountain Basin and Range, Interior Lowlands, and

NAGCP provinces converge. Within these subdivisions of *Spiranthes*, the ancestral biogeographic region for the Western Cordillera + *S. lucida* clade was likely the region covering the border of the Basin and Range and southern Interior Lowlands, whereas the ancestral region of the sister sub-division is confidently placed within the NAGCP, as is the backbone for the remainder of the genus.

In accordance with prior analyses (Chapter 5), both the Mesoamerican and Old World taxa were found to be embedded within the otherwise North America genus. The lineage containing Mesoamerican *S. graminea* and *S. nebulorum*, together with North American *S. praecox*/*S. sylvatica* appears to have diverged from the rest of the genus ca. 9.4 mybp, with a subsequent branching event at ca. 3.3 mybp evident between *S. praecox*/*S. sylvatica* and the Mesoamerican species. Our ARE show these Mesoamerican species evolved from a common ancestor that likely lived in the NAGCP, possibly in Northern Mexico and/or southern Texas (fig. 14). The Old World taxa were recovered in a moderately supported clade with *S. tuberosa* (pp = 0.85; recovering the position of *S. tuberosa* in relation to the Old World taxa has remained challenging, see Chapter 5, and this topology is contrary to that of Dueck et al. (2014)). If taken as a clade, then the MRCA of *S. tuberosa* and the Old World taxa split from the rest of the genus ca. 7.4 mybp, most likely dispersing from the NAGCP or the southern Appalachian Mountains (fig. 13). The Old World taxa dispersed to Europe ca. 5.9 mybp during the Messinian Age of the late Miocene. The divergence between the primarily European species (*S. aestivalis* and *S. spiralis*) and the primarily Asian/Australian species (*S. sinensis* s.l., *S. nivea,* and *S. sunii*) occurred ca. 5.3 mybp.

Spiranthes odorata split from the tree ca. 5.5 mybp and is sister to the rest of the North American species. The remaining major subdivision within *Spiranthes* –between the *S. lacera*

complex and the *S. cernua* species complex sensu Pace (see Chapter 5)– occurred within the NAGCP ca. 4.5 mybp (fig. 5, fig. 15). Within these two sister complexes, MRCA's of several lineages and individual species migrated independently from the NAGCP northward into the Interior Lowlands, Appalachian Highlands, and boreal north beginning around 4.2 mybp and continuing into the Quaternary Glaciation (fig. 5, fig. 15).

DISCUSSION

Challenges in dating and ARE

As noted above, the most likely ancestral biogeographic ranges for a few clades (e.g., the ancestor of the entire genus, the Western Cordillera + *S. lucida* clade) could not be estimated with much precision (fig. 10). We attribute this to two factors: I) the extant geographic overlap between *Spiranthes* and its sister clade is limited, particularly in the absence of *Spiranthes* from Mexico (aside from *S. graminea* and *S. nebulorum*, which are confidently placed as embedded within the genus). II) The Western Cordillera + *S. lucida* clade, as indicated by its name, is a widely encompassing assemblage, composed of three non-monophyletic groupings. The first are species exclusive to the Intermountain Basin and Range, and Western North American Cordillera systems (*S. delitescens, S. diluvialis, S. infernalis, S. perexilis*, *S. porrifolia,* and *S.* ×*stellata*); second is enigmatic *S. lucida* which displays a highly fragmented distribution of disjunct population centers throughout Eastern North America; third is *S. romanzoffiana*, which is one of the most widely dispersed orchid species on Earth. Together, these three groupings incorporate every biogeographic region included in our analyses except the NAGCP and Mexico. We think this expansive representation of biogeographic regions is the main factor limiting the resolution of the most likely ancestral region of origin for both the genus and the Western Cordillera + *S. lucida* clade.

Evolutionary origin of *Spiranthes*. We place the evolution of *Spiranthes* within the mid-Miocene, a period of moderately warm climates. Grasslands and grass dominated habitats –the preferred habitat type of many extant *Spiranthes*– had already developed in North America (fig. 6; Manchester 2001, Thomasson 1990, Strömberg 2004, 2005) and expanded from the mid- to early-Miocene. As many extant species (e.g., *S. porrifolia*, *S. praecox*, *S. spiralis*, *S. triloba*, *S. vernalis*) typically occur in moist graminoid communities, we feel confident in asserting that ancestral *Spiranthes* taxa also preferred moist graminoid habitats such as wet swales, wet meadows, and short statured prairies and open savannas; however it is unknown if the expansion of grasslands directly influenced the evolution of *Spiranthes*. The taxa of *Spiranthes*' sister clade primarily occur in open dry pine-oak forests and xerophilous scrub, but do occasionally occur in grasslands as well. The mid-Miocene was also the time period during which *Bombus* –the major or sole know pollinators of every extant North American *Spiranthes* except *S. lucida* (Catling 1983) – migrated from Asia across Beringia into North America (fig. 6; Hines 2008). Most taxa in Spiranthinae are bee pollinated (or presumed to be so), yet only in the *Spiranthes* clade sensu Salazar and Jost (2012) of this subtribe is *Bombus* dominant. The appearance of a novel pollinator in the New World may have led to the evolution of this wider group of orchids, whereas the move towards North American grasslands and other graminoid-dominated habitats of the Basin and Range, Interior Lowlands, and NAGCP during the mid-Miocene may have been the key factors leading to the divergence between temperate *Spiranthes* s.s. and its Neotropical sister clade.

Importance of the Coastal Plain. Excluding The Western Cordillera + *S. lucida* clade, our analyses indicate the MRCA of all extant *Spiranthes* most likely evolved in the NAGCP ca. 9 mybp (fig. 9). Furthermore, the MRCAs of all individual lineages within this clade occurred

within the NAGCP. Thus it appears that the NAGCP is the main center of evolutionary diversity for North Americas' second largest orchid genus, and contrary to long held ideas concerning the NAGCP, ancestral species from within this region have repeatedly given rise to lineages and species of *Spiranthes* that have migrated and dispersed to other North American physiographic areas, the Caribbean, Mesoamerica, and even across the Atlantic to the Old World. This conclusion is strengthened by the current endemism to the NAGCP displayed by 8 of 26 North American extant *Spiranthes*. That the NAGCP has been the source of lineage evolution for *Spiranthes*, rather than a sink, is in stark contrast to the traditional thinking regarding this physiographic division (Gleason 1964, Thorne 1993).

The novel association between the NAGCP and montane Mexico and Central America. Our ARE strongly suggests that the NAGCP is the ancestral range of the ancestors of a montane Mexican and a Central American species (fig. 14). To our knowledge, this is a previously undescribed or little known pattern, and is based on our molecular phylogenetic evidence for the sister relationship between *S. praecox*/*S. sylvatica* (NAGCP endemic; the taxonomic status of *S. sylvatica* is in doubt and it may represent morphological variation within a polytypic *S. praecox*; Chapter 5) and *S. graminea* (Sierra Madre Occidental endemic) and *S. nebulorum* (eastern Eje Volcánico Transversal, Sierra Madre Occidental, Sierra Madre di Chiapas). Other instances of this biogeographic pattern whereby Mexican taxa are supported as sister to and derived from NAGCP taxa based on molecular phylogenetic data are unknown, making the relationship and evolutionary history of *S. graminea*, *S. nebulorum*, and *S. praecox* a novel exemplar. An analogous situation occurs with *Hamamelis mexicana* (Sierra Madre Oriental), which is very closely related to the Ozark Plateau endemic *Hamamelis vernalis* and is likely derived from a North American MRCA (the exact topological relationship is unresolved and the two taxa may in

fact be synonyms, Xie et al. 2010). Similarly, Calviño et al. (2008) found Mexican *Eryngium* to be derived from an Eastern North American MRCA, however the authors did not specify from where in Eastern North America the MRCA of this clade dispersed.

The only other example supported by molecular phylogenetic data of which we are aware in which sister taxa display a biogeographic relationship between endemism in the NAGCP and montane Mexico and Central America is *Magnolia grandiflora* (NAGCP endemic) which is sister to *Magnolia tamaulipana* (Sierra Madre Oriental). Possible instances of sister-relationships between non-endemic NAGCP and Mexican and Central America taxa include *Magnolia virginana* (a near NAGCP endemic) which is embedded within an unresolved clade of several Mexican and Central American species, and *Magnolia macrophylla* (NAGCP, southern Appalachian Highlands) and *Magnolia asheii* (NAGCP endemic), which together are sister to *Magnolia dealbata* (Eje Volcánico Transversal, Sierra Madre del Sur) (Azuma et al. 2001). More broadly, *Liquidambar styraciflua* displays a disjunct distribution between the NAGCP, Piedmont, and Cumberland Plateau, and the Eje Volcánico Transversal, Sierra Madre Occidental, and Sierra Madre di Chiapas (Morris et al. 2008, Ruiz-Sanchez & Ornelas 2014). However these North American taxa and populations are likely derived from Mexican and/or Central American MRCAs.

A biological relationship between wider areas of Eastern North America (including broad distributional combinations of the Appalachian Highlands, Cumberland Plateau, NAGCP, and Piedmont) and montane Mexico and Central America has long been appreciated and recently expanded: e.g., the migration of *Danaus plexippus* L., and the distributions of *Carpinus* L. (Furlow 1997), *Conopholis* (Rodrigues et al. 2011, 2013), *Corallorhiza* (Barrett and Freudenstein 2011), and *Juglans* (Stone et al. 2009).

Prior to the our earlier work (Chapter 5) and the work we present here, based on their floral morphology, habitat, and distribution, *S. graminea* and *S. nebulorum* were assumed to be most closely related to the Western Cordillera taxa of *S. perexilis*, *S. porrifolia*, *S. romanzoffiana*, and *S.* ×*stellata* (Sheviak 1989, 2012, 2013). Catling & Catling (1988) proposed a possible relationship between *S. nebulorum*, *S. laciniata* (a distantly related NAGCP endemic (fig. 5)), and *S. praecox* based on floral morphology. Although Catling & Catling (1988) did briefly discuss the biogeographic disjunction among these species, they failed to recognize the distinctive relationship between the NAGCP and montane Mexico and Central America, instead merging it under a broad concept of "eastern North America." Curiously, the preferred habitats of *S. graminea*, *S. nebulorum*, and *S. praecox* are quite dissimilar: the Mexican and Central American species grow in cool, foggy, montane bogs and springs surrounded by dry *Quercus-Pinus-Abies* forests, whereas *S. praecox* inhabits hot and humid open wet meadows, savannas, and flatwoods at or near sea-level.

Spiranthes triloba from the NAGCP and *S. magnicamporum*: a possible example of Quaternary Glaciation induced allopatric speciation. *Spiranthes triloba*, a recently reinstated Florida endemic (Chapter 1) is the sister species to *S. magnicamporum* which is primarily distributed throughout the Interior Lowlands with peripheral disjunct populations in the Appalachian Highlands, the NAGCP, and New Mexico. Both taxa require frequently burned, often calcareous prairie habitat; the closest extant populations of these sister species are separated by ca. 342 km: the southernmost extant populations of *S. magnicamporum* are located in the endangered Black Belt Prairies of Alabama and Mississippi, the Louisiana Keiffer Prairie complex, and the Terre Noire prairie systems of central Arkansas, whereas the northernmost extant populations of *S. triloba* occur in flatwood prairies of the Florida Panhandle near the

Aucilla and Ochlockonee Rivers; the two are fully allopatric. Phenologically the pair forms an overlapping continuum over their collective distribution, flowering from mid-August at the northern limit of *S. magnicamporum* in southern Manitoba, to early-February at the southern limit of *S. triloba* in southern Florida, and the flowers share many potential *S. cernua* complex-specific synapomorphies (e.g., papillate labellum, strongly fragrant flowers; Chapter 1).

The branching event between these sister species occurred ca. 1.7 mybp, near the mid-point of the Quaternary Glaciation (figs. 7-9). Our ARE analyses place their MRCA as being distributed across a wide area, including the Appalachian Highlands (probably the southern portion), Intermountain Basin and Range, Interior Lowlands, and NAGCP. It appears likely that the immediate ancestor of *S. magnicamporum* retreated westward in response to the drying, cooling, and glacial advance of the Calabrian, (possibly leaving relictual disjunct populations in the foothills of the western Appalachian Mountains, Cumberland Plateau, and NAGCP) whereas the direct ancestor of *S. triloba* remained in the NAGCP. Eventually, the ancestor of *S. triloba* might have reacted to glacial cycles in a similar fashion to other NAGCP endemics –such as *Pyxidanthera* (Wall et al. 2010)– and may have reached an approximation of its present distribution in Florida by the mid- to early Pleistocene, remaining there during the ensuing glacial period with only slight changes in response to rising or falling ocean levels.

Phylogeographic structure of *S. odorata*. *Spiranthes odorata* is one of just a few orchids to occur in permanently inundated habitats (often the inflorescence is the only structure above water at anthesis), and is primarily found along the NAGCP in *Taxodium-Nyssa-Acer* dominated flooded forests from the mouth of the James River, Virginia, south to the southern Everglades, and west to Piney Woods of east Texas. Unusually, this near NAGCP endemic also has three known disjunct populations located in the Cumberland Plateau of Kentucky and the Interior

Lowlands of Tennessee (Chapter 1). Disjunctions between the NAGCP and the Cumberland-Plateau/Ohio River Valley lobe of the Interior Lowland is a little known and understudied biogeographic pattern, despite the occurrence of at least 59 species –now including *S. odorata*– which fit this distribution (e.g., *Agalinis obtiusifolia* (Orobanchaceae), *Platanthera integra* (Orchidaceae), *Rhynchospora chalarocephala* (Cyperaceae), and *Xyris fimbriata* (Xyridaceae); Sorrie & Weakley 1999).

As *S. odorata* is predominantly fluvial, we expected any possible phylogeographic signal to reflect the geography and isolating effects of different river basins. Alternatively, we hypothesized that the disjunct populations might cluster distinctly from the remainder of the species. However, we recovered a fully supported (pp = 1) dichotomization between samples from not only Kentucky and Tennessee, but also east Texas and eastern Georgia vs. samples from Florida, North Carolina, and Virginia. Our full-sample chronogram (fig. 8) recovered a date of ca. 1.1 mybp for this divergence between these two clades. This places the split within *S. odorata* during the glacial advances of the Calabrian stage. This may be an example of the Appalachian Mountain discontinuity (Soltis et al. 2006), however the inclusion of the Georgia population in the grouping is anomalous, and we lack samples from Alabama, Arkansas, Louisiana, Mississippi, and South Carolina; therefore a definitive conclusion cannot be drawn at this time. One possible explanation for the formation of the disjunct populations is as follows: The northwestern limit of the NAGCP is approximately at the confluence of the Mississippi and Ohio Rivers; the northwestern limit of *S. odorata* is located in south-central Arkansas (but absent from the Mississippi Embayment north of Louisiana). At an earlier period during the Calabrian stage the Mississippi Embayment may have presented suitable habitat for *S. odorata* –both *Nyssa aquatic* and *Taxodium distichum*, the primary dominants of extant *S. odorata* habitat, are still

found north to the confluence– and the species may have continued northeasterly long the Ohio River Valley and across the Interior Lowlands lobe and Cumberland Plateau, forming a crescent along the southern region of the Appalachian Mountains. As the glacial cycles of the Quaternary unfolded, the majority of *S. odorata* populations within this northern region died out, leaving the current disjunction as relicts restricted to a few *Acer–Celtis–Fraxinus–Ulmus* dominated flooded forests. An alternative explanation of long distance dispersal cannot be ruled out, and has occurred elsewhere in the genus (e.g., *S. romanzoffiana*, the Old World Clade), but one would need to invoke at least three independent dispersal events to the same location to explain this pattern, a scenario we consider unlikely. Further population based analyses to understand the phylogeographic pattern exhibited by *S. odorata* and other taxa with similar NAGCP/Tennessee/Kentucky disjunctions is an area ripe for continued research.

Hybrid origins of *Spiranthes diluvialis* in the Pleistocene. *Spiranthes diluvialis* is an amphiploid species of hybrid origin known from disjunct populations scattered throughout the Great Basin of the USA. Our sampled populations of *S. diluvialis* from Idaho, Nebraska, and Washington share a MRCA with its paternal parent *S. romanzoffiana* ca. 230,000 ybp, and a MRCA with *S. magnicamporum* (its maternal parent) ca, 270,000 ybp, indicating a relatively recent hybrid origin of *S. diluvialis* within the last 300,000 years (fig. 8). Sheviak (1984) hypothesized *S. diluvialis* formed during a Pleistocene Pluvial Period, but did not indicate during what approximate timeframe this may have occurred. Our results place the evolutionary origin of *S. diluvialis* in the Gelasian stage of the Pleistocene Epoch near the onset of the Quaternary Glacial Episode, although it is unclear if glaciation directly influenced or caused the hybridization between *S. romanzoffiana* and *S. magnicamporum*. The probable ARE for *S. diluvialis* was located across the Interior Lowlands and Intermountain Basin & Range.

Most of the middle North American Western Cordillera and Intermountain Region – including areas where *S. diluvialis* is extant in Colorado, Idaho, Montana, and Utah– was ice-free during the Pleistocene Epoch (Dyke et al. 2003), and mountain lake pollen records indicate an apparent increase in cool-moist plant taxa (Jiménez-Moreno et al. 2007). Although the geographic heterogeneity of the region and cyclical nature of glacial expansion and retreat makes any broad-scale statements regarding environmental conditions in the western mountain systems of North America difficult, additional lake pollen and rodent midden records indicate a fairy wet climate for many portions of the Rocky Mountains, the Sonoran and Chihuahuan Deserts, and Great Salt Lake region into the Younger Dryas Period (Cole et al. 2013, Davis 1998, Holmgren et al. 2010). This indicates that suitable habitat for *S. magnicamporum* may have occurred throughout the Cordillera for tens of thousands of years after the initial period of hybridization with *S. romanzoffiana*, lending support to the likelihood of multiple origins for *S. diluvialis*.

Evidence toward understanding the evolution of enigmatic Spiranthes lucida.

Spiranthes lucida is distinct from both its most closely related relatives (*S. romanzoffiana, S. porrifolia*, and *S. perexilis*) and the entire genus by several unusual characters, including its non-protandrous flowers (all other *Spiranthes* are protandrous), a white column (all other *Spiranthes* display a green column), and its exclusive pollination relationship with short lounged Halictid bees (as opposed to *Bombus*, Catling 1983). *Spiranthes lucida* also displays an unusual distribution composed of several disjunct population centers: 1) the Appalachian Highlands from Tennessee to Newfoundland (a historic population in Alabama may have recently become extirpated); 2) scattered populations in the Great Lakes Basin including Michigan, northern Illinois and Indiana, northern Ohio, and northeastern WI; 3) the Ozark Plateau; and 4) very small historical populations in Nebraska and Iowa that may now be extirpated. Even within relatively

large distributional areas such as the Appalachian Highlands, populations of *S. lucida* are typically small and rare on the landscape. As the result of extensive fieldwork focused on this species, we were able to incorporate samples of *S. lucida* from throughout its range, including AR, IN, MI, NY, PA, TN, VT, WI, thus offering an opportunity to examine any possible phylogeographic structure to this unusual and intriguing species.

Spiranthes lucida has consistently been recovered as a member of/sister to the Western Cordillera complex of *S. perexilis*, *S. porrifolia*, and *S. romanzoffiana*, and is distantly related to the other *Spiranthes* distributed throughout Eastern North America (fig. 5) (Chapter 5, Dueck et al. 2014). The distant ancestor of this species appears to have diverged from a MRCA with the western species ca. 8.5 mya during the Tortonian stage of the Miocene. Although the likely geographic region of this shared MRCA is unresolved, the ancestor of *S. lucida* likely migrated to the Interior Lowlands from a more western or southwestern place of origin (fig. 11). From there *S. lucida* continued to migrate eastward, although in contrast to the other Eastern North American species, there is no evidence for this occurring via the NAGCP. A split within *S. lucida* between the Ozark Plateau populations and the Great Lakes and Appalachian Highland populations appears to have occurred ca. 1.1 mya – 840,000 ybp (the subclade for the Ozark Pleateau populations is fully supported (pp = 1); whereas the support values for the other clades are very weak (pp < 0.6)). Glacial cycles during the Calabrian stage –as in other species or clades– may have played a role in the formation of this disjunction of the Ozark vs. eastern populations, however population genetic analyses incorporating wider geographic sampling and Next Generation Sequencing, are needed to test this hypothesis. Although it remains to be tested, based the topology we recovered (fig. 8 [third section of the tree]; Chapter 5), in which the Ozark and eastern populations appear to display a possible sister relationship –as opposed to a clade of

eastern populations embedded within an Ozark Plateau clade– we hypothesize that the Ozark populations are not glacial relicts that served as a source for populations to disperse or migrate eastward, but instead are an example of glacially induced population disjunction.

The Spiranthes cernua species complex s.s. recently evolved in the NAGCP and likely diversified in the Appalachian Mountains. The *Spiranthes cernua* species complex s.s. is characterized by morphologically variable species recovered within clades subtended by short but strongly to fully support branches (pp = 0.9 – 1; Chapter 3). The complex includes four non-hybrid species (*S. arcisepalum*, *S. casei*, *S. cernua* s.s., and *S. ochroleuca*), in addition to two known taxa of hybrid origin (*S.* ×*incurvum* [*S. cernua* s.s. × *S. magnicamporum*] and *S.* ×*kapnosperia* [*S. cernua* × *S. ochroleuca*]). All species within the *S. cernua* species complex are exclusive to eastern North America, from eastern Texas to eastern Minnesota, northern Florida, the Atlantic Coast, and southern Ontario, Quebec, and Newfoundland. Within-species phenotypic variability and wide geographic sympatry (with species frequently growing within cm of each other) has caused this complex to be regarded as particularly challenging to identify (e.g., Sheviak 1982). The 'when' and 'where' of the complex's evolution, prior to this study, has long remained unknown.

Our calibrated chronogram and ARE analyses suggest the common ancestor of the extant members of this complex evolved ca. 1–2 mybp, most likely within the NAGCP, or a shared area covering the NAGCP and the Appalachian Highlands (fig. 15). Within this complex, only *S. cernua* s.s. is still found within the NAGCP, from Texas to northern Florida, and north to Massachusetts, and the ancestor of *S. cernua* (including the maternal parent of *S.* ×*incurvum*) occurred across the NAGCP and the Appalachian Highlands. The MRCA of *S. arcisepalum* and *S. ochroleuca* likely migrated from the NAGCP into the Appalachian Highlands, followed by

extinction in the former area. In contrast to MRCAs of most extant species in *Spiranthes*, the divergence between *S. arcisepalum*, *S. casei*, and *S. ochroleuca* occurred within the Appalachian Highlands (as opposed to the NAGCP), followed by separate migrations of each species northward and westward into the Interior Lowlands surrounding the Great Lakes, and the boreal zone of southern Canada. Areas of hybridization within the *Spiranthes cernua* species complex include the Appalachian Highlands for *S.* ×*kapnosperia* (to which it is endemic), and the combined NAGCP, Appalachian Highlands, and Interior Lowlands regions for *S.* ×*incurvum*.

CONCLUSIONS

Spiranthes, owing to its unusual and expansive distributional patterns, serves as an interesting and powerful example through which to explore biogeographic relationships at both global and North American scales. Evidence we presented here support the NAGCP as an evolutionarily important area, having served as a reservoir in which multiple species and lineages likely evolved, and from which multiple species and lineages dispersed or migrated from. It is our hope that *Spiranthes* can be used to help support further research centered on the biogeographic relationships of North American taxa, particularly within the NAGCP, and the disjunct-floristic relationships between eastern North America and Central America, and Eastern Asia. Although *Spiranthes* is a systematically challenging genus that has long perplexed and intrigued botanists from Darwin to Ames, in addition to engaged citizen scientists and local orchid enthusiasts, we maintain that efforts to unravel the evolutionary and biogeographic relationships among its species are profoundly rewarding, serving to illuminate evolutionary processes that may be applied to other genera, both within North America and across the globe, and recalibrate attitudes toward underappreciated biodiversity hotspots.

Álvarez-Molina, A., and K.M. Cameron. 2009. Molecular phylogenetics of Prescottiinae s.l. and their close allies (Orchidaceae, Cranichideae) inferred from plastid and nuclear ribosomal DNA sequences. American Journal of Botany, 96(5): 1020–1040.

Azuma, H., J.G. García-Franco, V. Rico-Gray, and L. B. Thien. Molecular Phylogeny of the Magnoliaceae: The Biogeography of Tropical and Temperate Disjunctions. American Journal of Botany, 88(12): 2275–2285.

Bateman, R.M., K.E. James, Y. Luo, R.K. Lauri, T. Fulcher, P.J. Cribb, and M.W. Chase. 2009. Molecular phylogenetics and morphological reappraisal of the *Platanthera* clade (Orchidaceae: Orchidinae) prompts expansion of the generic limits of *Galearis* and *Platanthera*. Annals of Botany, 104: 431–445.

Becker, M., N. Gruenheit, M. Steel, C. Voelckel, O. Deusch, P.B. Heenan, P.A. McLenachan, et al. 2013. Hybridization may facilitate in situ survival of endemic species through periods of climate change. Nature Climate Change, 3(12): 1039-1043.

Berry, E.D. 1909. A Miocene flora from the Virginia Coastal Plain. The Journal of Geology, 17(1): 19–30.

Berry, E.D. 1937. Tertiary floras of Eastern North America. Botanical review, 3(1): 31–46.

Bogarín, D., F. Pupulin, C. Arrocha, and J. Warner. 2013. Orchids without borders: studying the hotspot of Costa Rica and Panama. Lankesteriana, 13: 13-26.

Calviño, C.I., S.G. Martínez, and S.R. Downie. 2008. The evolutionary history of *Eryngium* (Apiaceae, Saniculoideae): Rapid radiations, long distance dispersals, and hybridizations. Molecular Phylogenetics and Evolution, 46: 1129–1150.

Cameron, K.M., and M.W. Chase. 1999. Phylogenetic relationships of Pogoniinae (Vanilloideae, Orchidaceae): An herbaceous example of the Eastern North America-Eastern Asia phytogeographic disjunction. Journal of Plant Research, 112(1107): 317–329.

Case, F.W., and R.B. Case. 1997. *Trilliums*. Timber Press, Portland, OR, USA.

Catling, P.M. 1983. Pollination of northeastern North American *Spiranthes* (Orchidacea). Canadian Journal of Botany, 61(4): 1080–1093.

Catling, P.M., and V.R. Catling. 1988. *Spiranthes nebulorum* (Orchidaceae), a new species from southern Mexico and Guatemala. Rhodora, 90(862): 139–148.

Cole, K.L., J.F. Fisher, K. Ironside, J.I. Mead, and P. Koehler. 2013. The biogeographic histories of *Pinus edulis* and *Pinus monophylla* over the last 50,000 years. Quaternary International, 310: 96–110.

Conran, J.G., J.M. Bannister, and D.E. Lee. 2009. Earliest orchid macrofossils: Early Miocene *Dendrobium* and *Earina* (Orchidaceae: Epidendroideae) from New Zealand. American Journal of Botany, 96(2): 466–474.

Davis, O.K. 1998. Palynological ecidence for vegetation cycles in a 1.5 million year pollen record from the Great Salt Lake, Utah, USA. Palaeogeography, Palaeoclimatology, Palaeoecology, 138: 175–185.

Drummond, A.J., M.A. Suchard, D. Xie, and A. Rambaut. 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7 Molecular Biology and Evolution, 29: 1969–1973.

Drummond, A.J., S.Y.W. Ho, M.J. Phillips, and A. Rambaut. 2006. Relaxed phylogenetics and dating with confidence. PLoS Biology 4:e88.

Dueck, L.A., D. Aygoren, and K.M. Cameron. 2014. A molecular framework for understanding the phylogeny of *Spiranthes* (Orchidaceae), a cosmopolitan genus with a North American center of diversity. American Journal of Botany, 101(9):1551–1571.

Dyke, A.S., A. Moore, and L. Robertonson. 2003. Deglaciation of North America, Open file 1574. Geological Survey of Canada.

Furlow, J.J. 1997. Betulaceae. *In*: Flora of North America Editorial Committee (eds.). 1993+. Flora of North America north of Mexico. Volume 3. Oxford University Press, New York.

Gleason, H.A. 1964. The natural geography of plants. New York, Columbia University Press, 420 pp.

Górniak, M., O. Paun, and M.W. Chase. 2010. Phylogenetic relationships within Orchidaceae based on a low-copy nuclear coding gene, *Xdh*: Congruence with organellar and nuclear ribosomal DNA results. Molecular Phylogenetics and Evolution, 56(2): 784–789.

Graham, A. 1999. Late Cretaceous and Cenozoic history of North American vegetation, north of Mexico. Oxford University Press, New York.

Guo Y-Y., Y-B. Luo, Z-J. Liu, X-Q. Wang. 2012. Evolution and Biogeography of the Slipper Orchids: Eocene vicariance of the conduplicate Genera in the Old and New World tropics. PLoS ONE 7(6): e38788. doi:10.1371/journal.pone.0038788

Gustafsson, A.L., C.F. Verola, and A. Antonelli. 2010. Reassessing the temporal evolution of orchids with new fossils and a Bayesian relaxed clock, with implications for the diversification of the rare South American genus *Hoffmannseggella* (Orchidaceae: Epidendroideae). BMC Evolutionary Biology, 10:177. http://www.biomedcentral.com/1471-2148/10/177

Hansen, B.C.S., E.C. Grimm, and W.A. Watts. 2001. Palynology of the Peace Creek Site, Polk County, Florida. Geological Society of America Bulletin, 113(6): 682–692.

He, T., J.G. Pausas, C.M. Belcher, D.W. Schwilk, D.W., and B.B. Lamont. 2012. Fire-adapted traits of *Pinus* arose in the fiery Cretaceous. New Phytologist, 194, 751–759.

Hines, H.M. 2008. Historical biogeography, divergence times, and diversification patterns of bumble bees (Hymenoptera: Apidae: *Bombus*). Systematic Biology, 57(1): 58–75.

Holmgren, C. A., J.L. Betancourt, and K.A. Rylander. 2010. A long-term vegetation history of the Mojave–Colorado Desert ecotone at Joshua Tree National Park. Journal of Quaternary Science, 25: 222–236.

Jiménez-Moreno, G., R.S. Anderson, and P.J. Fawcett. 2007. Orbital- and millennial-scale vegetation and climate changes of the past 225 ka from Bear Lake, Utah–Idaho (USA). Quaternary Science Reviews, 26: 1713–1724.

Kotthoff, U., D.R. Greenwood, F.M.G. McCarthy, K. Müller-Navarra, S. Prader, and S>P. Hesselbo. 2014. Late Eocene to middle Miocene (33 to 13 million years ago) vegetation and climate development on the North American Atlantic Coastal Plain (IODP Expedition 313, Site M0027). Climate of the Past, 10, 1523-1539, doi:10.5194/cp-10-1523–2014.

Manchester SR. 2001. Update on the megafossil flora of Florissant, CO. In *Fossil Flora and Stratigraphy of the Florissant Formation, Colorado*, ed. E Evanoff, KM Gregory-Wodzicki, KR Johnson, pp. 137–62. Denver, CO: Denver Museum of Natural Scoence.

Matzke, N.J. 2014. "Stochastic mapping under biogeographical models." PhyloWiki BioGeoBEARS website, May 2014, http://phylo.wikidot.com/biogeobears#stochastic_mapping . Code at: http://phylo.wikidot.com/local--files/biogeobears/code_data_files.zip

Micheneau, C., B.S. Carlsward, M.F. Fay, B. Bytebier, T. Pailler, and M.W. Chase. 2008. Phylogenetics and biogeography of Mascarene angraecoid orchids (Vandeae, Orchidaceae). Molecular Phylogenetics and Evolution, 46: 908–922.

Miller, M.A., W. Pfeiffer, and T. Schwartz. 2010. “Creating the CIPRES Science Gateway for inference of large phylogenetic trees" in Proceedings of the Gateway Computing Environments Workshop (GCE), 14 Nov. 2010, New Orleans, LA pp 1 - 8.

Monzon, J., R. Kays, and D.E. Dykhuizen. 2012. Assessment of coyote-wolf-dog admixture using ancestry-informative diagnostic SNPs. Molecular Ecology, 23(1): 182–197.

Muhlfeld, C.C., R.P. Kovach, L.A. Jones, R. Al-Chokhachy, M.C. Boyer, R.F. Leary, W.H. Lowe, G. Luikart, and F.W. Allendorf. Invasive hybridization in a threatened species is accelerated by climate change. Nature Climate Change, 4(7): 620–624.

Myers, N., R.A. Mittermeter, C.G. Mittermeter, G.A.B. da Fonseca, and J. Kent. 2000. Biodiversity hotspots for conservation priorities. Nature, 403: 853–858.

Noss, R.F., W.J. Platt, B.A. Sorrie, A.S. Weakley, D.B. Means, J. Costanza, and R.K. Peet. How global biodiversity hotspots may go unrecognized: lessons from the North American Coastal Plain. Diversity and Distributions, 21(2): 236–244.

Parr, C.L., C.E.R. Lahmann, W.J. Bond, W.A. Hoffmann, and A.N. Andersen. 2014. Tropical grassy biomes: misunderstood, neglected, and under threat. Trends in Ecology and Evolution, 29(4): 205–213.

Rachele, L.D. 1976. Palynology of the Legler Lignite; a deposit in the Tertiary Cohansey Formation of New Jersey, U.S.A. Review of Palaeobotany and Palynology, 22(3): 225–252.

Rambaut, A., M.A. Suchard, D. Xie, and A.J. Drummond. 2014. Tracer v1.6, Available from http://beast.bio.ed.ac.uK/Tracer

Ramírez, S.R., B. Gravendeel, R.B. Singer, C.R. Marshall, and N.E. Pierce. 2007. Dating the origin of the Orchidaceae from a fossil orchid with its pollinator. Nature, 448: 1042–1045.

Rodrigues, A.G., A.E.L. Colwell, and S. Stefanovic. 2011. Molecular systematics of the parasitic genus *Conopholis* (Orobanchaceae) inferred from plastid and nuclear sequences. American Journal of Botany, 98(5): 896–908.

Rodrigues, A.G., S. Shaya, T.A. Dickinson, and S. Stefanovic. 2013. Morphometric analyses and taxonomic revision of the North American holoparasitic genus *Conopholis* (Orobanchaceae). Systematic Botany, 38(3):795–804.

Rutledge, L.Y., B.N. White, J.R. Row, B.R. Patterson. 2012. Intense harvesting of eastern wolves facilitated hybridization with coyotes. Ecology and Evolution, 2(1): 19–33.

Salazar, G.A., M.W. Chase, M.A. Soto, and M. Ingrouille. 2003. Phylogenetics of Cranichideae with emphasis on Spiranthinae (Orchidaceae, Orchidoideae): evidence from plastid and nuclear DNA sequences. American Journal of Botany, 90: 777–795.

Salazar, G.A., L.I. Cabrera, S. Madriñán, M.W. Chase. 2009. Phylogenetic relationships of Cranichidinae and Prescottiinae (Orchidaceae, Cranichideae) inferred from plastid and nuclear DNA sequences. Annals of Botany, 104(3): 403–416.

Salazar, G.A., C. van den Berg, and A. Popovkin. 2014. Phylogenetic relationships of *Discyphus scopulariae* (Orchidaceae, Cranichideae) inferred from plastid and nuclear DNA sequences: evidence supporting recognition of a new subtribe, Discyphinae. Phytotaxon, 173(2): 127–139.

Salazar, G.A., and L. Jost. 2012. *Quechua*, New Monotypic Genus of Andean Spiranthinae (Orchidaceae). Systematic Botany, 37(1): 78–86.

Sheviak, C.J. 1982. Biosystematic study of the *Spiranthes cernua* complex. New York State Museum Bull. 448: 1–73.

Sheviak, C.J. 1984. *Spiranthes diluvialis* (Orchidaceae): a new species from the western United States. Brittonia 36(1): 8–14.

Sheviak, C.J. 1989. A new *Spiranthes* (Orchidaceae) from Ash Meadows, Nevada. Rhodora, 91:225–234.

Sheviak, C.J. 2012. A new and rare *Spiranthes* from the Sierra Nevada. The Native Orchid Conference Journal, 9(4): 1–6.

Sheviak, C.J. 2013. A cytogeographic survey of *Spiranthes romanzoffiana* Cham. and its taxonomic implications. The Native Orchid Conference Journal, 10 (2): 1–22.

Sorrie, B.A., and A.S. Weakley. 2001. Coastal Plain Vascular Plant Endemics: Phytogeographic Patterns. Castanea, 66(1–2): 50–82.

Stone, D.E., S.H. Oh, E.A. Tripp , L.E. Ríos G , and P.S. Manos. 2009. Natural history, distribution, phylogenetic relationships, and conservation of Central American black walnuts (*Juglans* sect. *Rhysocaryon*). The Journal of the Torrey Botanical Society, 136(1): 1–25.

Strömberg, C.A.E. 2004. Using phytolith assemblages to reconstruct the origin and spread of grass-dominated habitats in the Great Plains during the late Eocene to early Miocene. Palaeogeography, Palaeoclimatology, Palaeoecology, 207:239–75

Strömberg, C.A.E. 2005. Decoupled taxonomic radiation and ecological expansion of open-habitat grasses in the Cenozoic of North America. Proceeding of the national Acedemy of Sciences, 102:11980–11984.

Stults, D.Z., and B.J. Axsmith. 2011. Filling the gaps in the Neogene plant fossil record of eastern North America: New data from the Pliocene of Alabama. Review of Palaeobotany and Palynology, 167(1–2): 1–9.

Thomasson, J.R. 1990. Fossil plants from the Late Miocene Ogallala Formation of central North America: possible paleoenvironmental and biostratigraphic significance. In *Geologic Framework and Regional Hydrology: Upper Cenozoic Blackwater Draw and Ogallala Formations, Great Plains*, ed. T.C. Gustavson, pp. 99–114. Austin, TX: Bur. Econ. Geol. Univ. Texas Austin

Thorne, R.F. 1993. Phytogeography. p. 132-153. *In*: Flora of North America Editorial Committee (eds.). 1993+. Flora of North America north of Mexico. Volume 1. Introduction. Oxford University Press, New York.

Trejo-Torres, J.C., and J.D. Ackerman. 2001. Biogeography of the Antilles based on a parsimony analysis of orchid distributions. Journal of Biogeography, 28(6): 775–794.

Van den Berg, C., D.H. Goldman, J.V. Freudenstein, A.M. Pridgeon, K.M. Cameron, and M.W. Chase. 2005. An Overview of the Phylogenetic Relationships within Epidendroideae Inferred from Multiple DNA Regions and Recircumscription of Epidendreae and Arethuseae (Orchidaceae). American Journal of Botany, 92(4): 613–624.

Xi, L., T.S. Yi, R. Li, D.Z. Li, and J. Wen. 2010. Evolution and biogeographic diversification of the witch-hazel genus (*Hamamelis* L., Hamamelidaceae) in the Northern Hemisphere. Molecular Phylogenetics and Evolution, 56: 675–689.

Wall, W.A., N.A. Douglas, Q.Y. Xiang, W.A. Hoffmann, T.R. Wentworth, and M.G. Hommann. 2010. Evidence for range stasis during the latter Pleistocene for the Atlantic Coastal Plain endemic genus, *Pyxidanthera* Michaux. Molecular Ecology, 19: 4302–4314.



Fig. 1.



Fig. 1. [Legend to preceding page/map] Global distribution map of *Spiranthes*. Colors denote broad geographic regions that correspond to groupings of species [**colors do NOT match the colors of the BioGeoBEARS output pie-charts**].

Green: North America: *S. arcisepalum*, *S. brevilabris*, *S. casei*, *S. cernua*, *S. delitescens*, *S. diluvialis*, *S. floridana*, *S. igniorchis*, *S. ×incurvum*, *S. ×kapnosperia*, *S. lacera*, *S. laciniata*, *S. longilabris*, *S. lucida*, *S. magnicamporum*, *S. ochroleuca*, *S. odorata*, *S. ovalis*, *S. perexilis*, *S. porrifolia*, *S. praecox*, *S. romanzoffiana*, *S. ×stellata*, *S. triloba*, *S. tuberosa*, *S. vernalis*.

Pink: Caribbean Basin (The Antilles, Bermuda, and coastal Yucatan, Belize, and Nicaragua): *S. torta*.

Orange: Central America (Southern Mexico & Guatemala): *S. graminea*, *S. nebulorum*.

Yellow: Eurasia: *S. aestivalis*, *S. spiralis*.

White: Asia, Australia, and western Oceania: *S. alticola*, *S. hongkongensis*, *S. nivea*, *S. sinensis*, *S. sunii*.

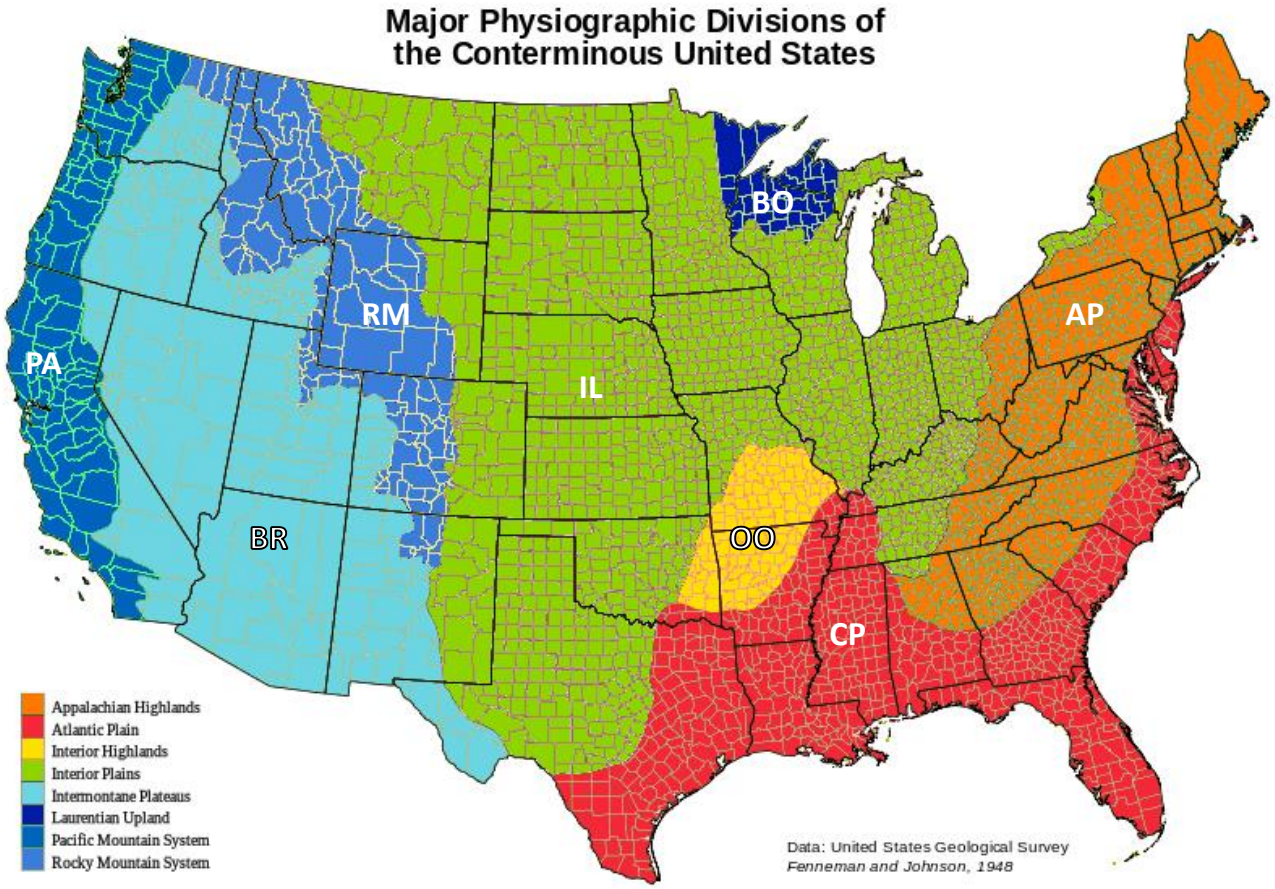


Fig. 2. Physiographic Divisions of The United States (USGS) used to code the BioGeoBEARS ARE analysis. Acronyms are the same as in the BioGeoBEARS data matrix. Colors on the map represent the base colors used in the BioGeoBEARS analysis. Regions and Iiage from the United States Geological Survey, Fenneman & Johnson, 1948.

Red: Atlantic Geological Coastal Plain [CP]

Orange: Appalachian Highlands [AP]

Green: Interior Lowlands [IL]

Dark Blue: Laurentian Uplands & Boreal North [BO]

Yellow: Ozark Plateau & Ouachita Mountains [OO]

Gray-blue: Rocky Mountains [RM]

Bright-blue: Basin & Range, & Intermountain Region [BR]

Blue: Pacific Mountain systems [PA]

51 10	CP	AP	OO	IL	RM	BR	PA	BO	MX	OW
Goodyera_pubescens	1	1	1	1	0	0	0	1	0	0
Sacoila_lanceolata	1	0	0	0	0	0	0	0	1	0
Dichromanthus_cinnabara	0	0	0	0	0	1	0	0	1	0
Mesadenus_polyantha	1	0	0	0	0	0	0	0	1	0
Deiregyne_diaphana	0	0	0	0	0	0	0	0	1	0
Schiedeella_llaveana	0	0	0	0	0	1	0	0	1	0
S.lucida_sl13d_AR	0	1	1	1	0	0	0	1	0	0
S.romanzoffiana_24d_AK	0	0	0	1	1	1	1	1	0	0
S.perexilis_31a_OR	0	0	0	0	0	0	1	0	0	0
S.xstellata_ss2paternal_CA	0	0	0	0	0	0	1	0	0	0
S.xstellata_ss2maternal_CA	0	0	0	0	0	0	1	0	0	0
S.porrifolia_21u_CA	0	0	0	0	0	1	1	0	0	0
S.infernalis_9a_NV	0	0	0	0	0	1	0	0	0	0
S.delitescens_5apaternal_AZ	0	0	0	0	0	1	0	0	0	0
S.delitescens_5amaternal_AZ	0	0	0	0	0	1	0	0	0	0
S.diluvialis_sd1paternal_ID	0	0	0	1	1	1	0	0	0	0
S.diluvialis_sd1maternal_ID	0	0	0	1	1	1	0	0	0	0
S.graminea	0	0	0	0	0	0	0	0	1	0
S.nebulorum	0	0	0	0	0	0	0	0	1	0
S.praecox_22i_FL	1	0	0	0	0	0	0	0	0	0
S.sylvatica_25d_FL	1	0	0	0	0	0	0	0	0	0
S.tuberosa_26c_WV	1	1	1	1	0	0	0	0	0	0
S.lacerav.gracilis_11a_SC	1	1	1	1	0	0	0	1	0	0
S.lacera_10c_MO	0	1	1	1	0	0	0	1	0	0
S.brevilabris_1g_FL	1	0	0	0	0	0	0	0	0	0
S.floridana_8f_FL	1	0	0	0	0	0	0	0	0	0
S.torta_28h_PuertoRico	1	0	0	0	0	0	0	0	1	0
S.laciniata_12b_GA	1	0	0	0	0	0	0	0	0	0
S.vernalis_27m_FL	1	1	1	1	0	0	0	0	0	0
S.aestivalis_21059_Switzerland	0	0	0	0	0	0	0	0	0	1
S.spiralis_10451_UK	0	0	0	0	0	0	0	0	0	1
S.australis	0	0	0	0	0	0	0	0	0	1
S.sinensis_2184_Malaysia	0	0	0	0	0	0	0	0	0	1
S.sinensis_ssi7_LaReunion	0	0	0	0	0	0	0	0	0	1
S.nivea_Taiwan	0	0	0	0	0	0	0	0	0	1
S.sunii_Ganzu	0	0	0	0	0	0	0	0	0	1
S.odorata_so1d_NC	1	0	0	1	0	0	0	0	0	0
S.longilabris_13a_NC	1	0	0	0	0	0	0	0	0	0
S.igniorchis_slt3a_FL	1	0	0	0	0	0	0	0	0	0
S.ovalis_18b_FL	1	0	1	0	0	0	0	0	0	0
S.ovalisv.erostellata_19d_SC	1	1	1	1	0	0	0	0	0	0
S.magnicamporum_sm7h_NM	1	1	0	1	0	1	0	0	0	0
S.triloba_FL135	1	0	0	0	0	0	0	0	0	0
S.cernua_sc15b_GA	1	1	1	1	0	0	0	0	0	0
S.arcisepalum_4y_NY	0	1	0	1	0	0	0	1	0	0
S.ochroleuca_16b_VA	0	1	0	1	0	0	0	1	0	0
S.casei_2a_MI	0	1	0	0	0	0	0	1	0	0
S.xincurvum_4tmaternal_WI	0	1	0	1	0	0	0	1	0	0
S.xincurvum_4tpaternal_WI	0	1	0	1	0	0	0	1	0	0
S.xkapnosperia_sh2maternal_NC	0	1	0	0	0	0	0	0	0	0
S.xkapnosperia_sh2paternal_NC	0	1	0	0	0	0	0	0	0	0

Fig. 4. Data matrix used for the BioGeoBEARS analysis. Acronyms at the top represent Physiographic regions of the United States used for analysis [CP = Coastal Plain, AP = Appalachian Highlands, OO = Ozark Plateau & Ouachita Mountains, IL = Interior Lowlands, BO = Laurentian Uplands & Boreal North, BR = Basin & Range, RM = Rocky Mountains, PA = Pacific Mountains]. Additional areas are: MX = Mesoamerican & the Caribbean, OW = Old World.

Fig. 5.

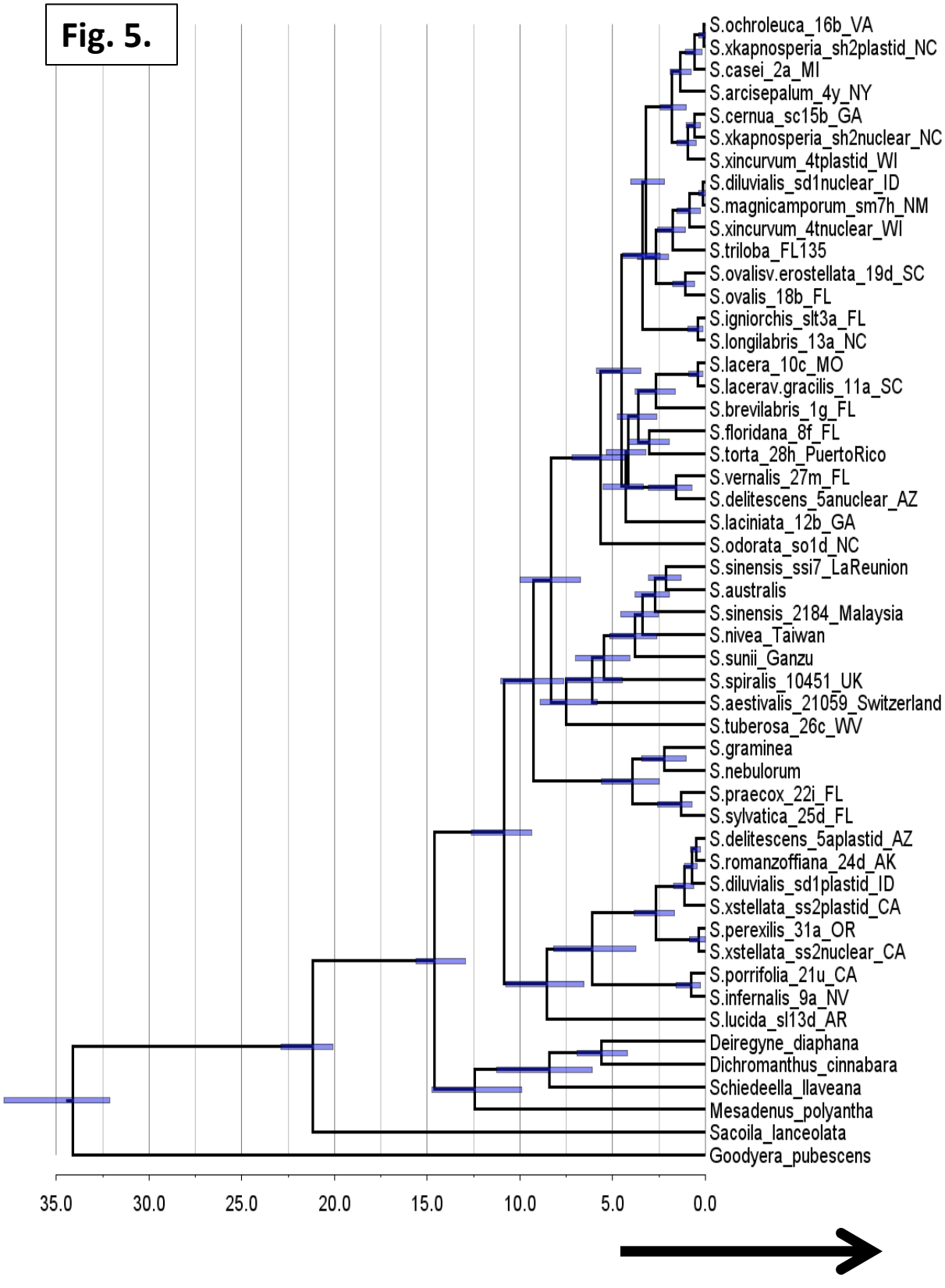

S.ochroleuca_16b_VA
S.xkapnosperia_sh2plastid_NC
S.casei_2a_MI
S.arcisepalum_4y_NY
S.cernua_sc15b_GA
S.xkapnosperia_sh2nuclear_NC
S.xincurvum_4tplastid_WI
S.diluvialis_sd1nuclear_ID
S.magnicamporum_sm7h_NM
S.xincurvum_4tnuclear_WI
S.triloba_FL135
S.ovalisv.erostellata_19d_SC
S.ovalis_18b_FL
S.igniorchis_slt3a_FL
S.longilabris_13a_NC
S.lacera_10c_MO
S.lacerav.gracilis_11a_SC
S.brevilabris_1g_FL
S.floridana_8f_FL
S.torta_28h_PuertoRico
S.vernalis_27m_FL
S.delitescens_5anuclear_AZ
S.laciniata_12b_GA
S.odorata_so1d_NC
S.sinensis_ssi7_LaReunion
S.australis
S.sinensis_2184_Malaysia
S.nivea_Taiwan
S.sunii_Ganzu
S.spiralis_10451_UK
S.aestivalis_21059_Switzerland
S.tuberosa_26c_WV
S.graminea
S.nebulorum
S.praecox_22i_FL
S.sylvatica_25d_FL
S.delitescens_5aplastid_AZ
S.romanzoffiana_24d_AK
S.diluvialis_sd1plastid_ID
S.xstellata_ss2plastid_CA
S.perexilis_31a_OR
S.xstellata_ss2nuclear_CA
S.porrifolia_21u_CA
S.infernalis_9a_NV
S.lucida_sl13d_AR
Deiregyne_diaphana
Dichromanthus_cinnabara
Schiedeella_llaveana
Mesadenus_polyantha
Sacoila_lanceolata
Goodyera_pubescens
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0



Fig. 5. [Legend to preceding page/tree] Calibrated BEAST chronogram of the reduced dataset. Node bars represent 95% HPD. Axis times in millions of years.

Fig. 6

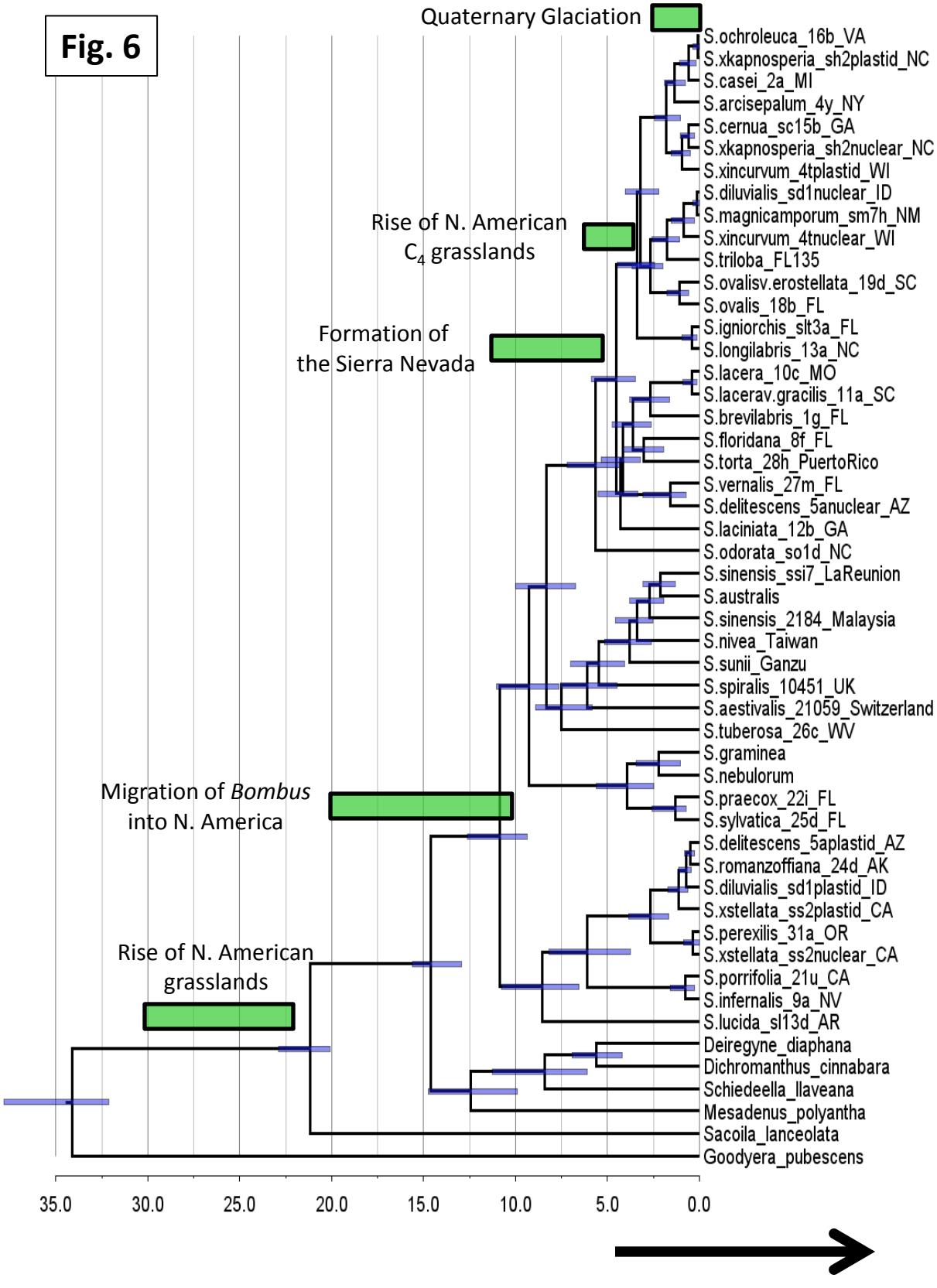

Quaternary Glaciation
Rise of N. American C4 grasslands
Formation of the Sierra Nevada
Migration of Bombus into N. America
Rise of N. American grasslands
S.ochroleuca_16b_VA
S.xkapnosperia_sh2plastid_NC
S.casei_2a_MI
S.arcisepalum_4y_NY
S.cernua_sc15b_GA
S.xkapnosperia_sh2nuclear_NC
S.xincurvum_4tplastid_WI
S.diluvialis_sd1nuclear_ID
S.magnicamporum_sm7h_NM
S.xincurvum_4tnuclear_WI
S.triloba_FL135
S.ovalisv.erostellata_19d_SC
S.ovalis_18b_FL
S.igniorchis_slt3a_FL
S.longilabris_13a_NC
S.lacera_10c_MO
S.lacerav.gracilis_11a_SC
S.brevilabris_1g_FL
S.floridana_8f_FL
S.torta_28h_PuertoRico
S.vernalis_27m_FL
S.delitescens_5anuclear_AZ
S.laciniata_12b_GA
S.odorata_so1d_NC
S.sinensis_ssi7_LaReunion
S.australis
S.sinensis_2184_Malaysia
S.nivea_Taiwan
S.sunii_Ganzu
S.spiralis_10451_UK
S.aestivalis_21059_Switzerland
S.tuberosa_26c_WV
S.graminea
S.nebulorum
S.praecox_22i_FL
S.sylvatica_25d_FL
S.delitescens_5aplastid_AZ
S.romanzoffiana_24d_AK
S.diluvialis_sd1plastid_ID
S.xstellata_ss2plastid_CA
S.perexilis_31a_OR
S.xstellata_ss2nuclear_CA
S.porrifolia_21u_CA
S.infernalis_9a_NV
S.lucida_sl13d_AR
Deiregyne_diaphana
Dichromanthus_cinnabara
Schiedeella_llaveana
Mesadenus_polyantha
Sacoila_lanceolata
Goodyera_pubescens
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0



Fig. 6. [Legend to preceding page/tree] Calibrated BEAST chronogram. Node bars represent 95% HPD. Green bars represent major North American geological and biological events within North America. Axis times in millions of years.

Fig. 7.

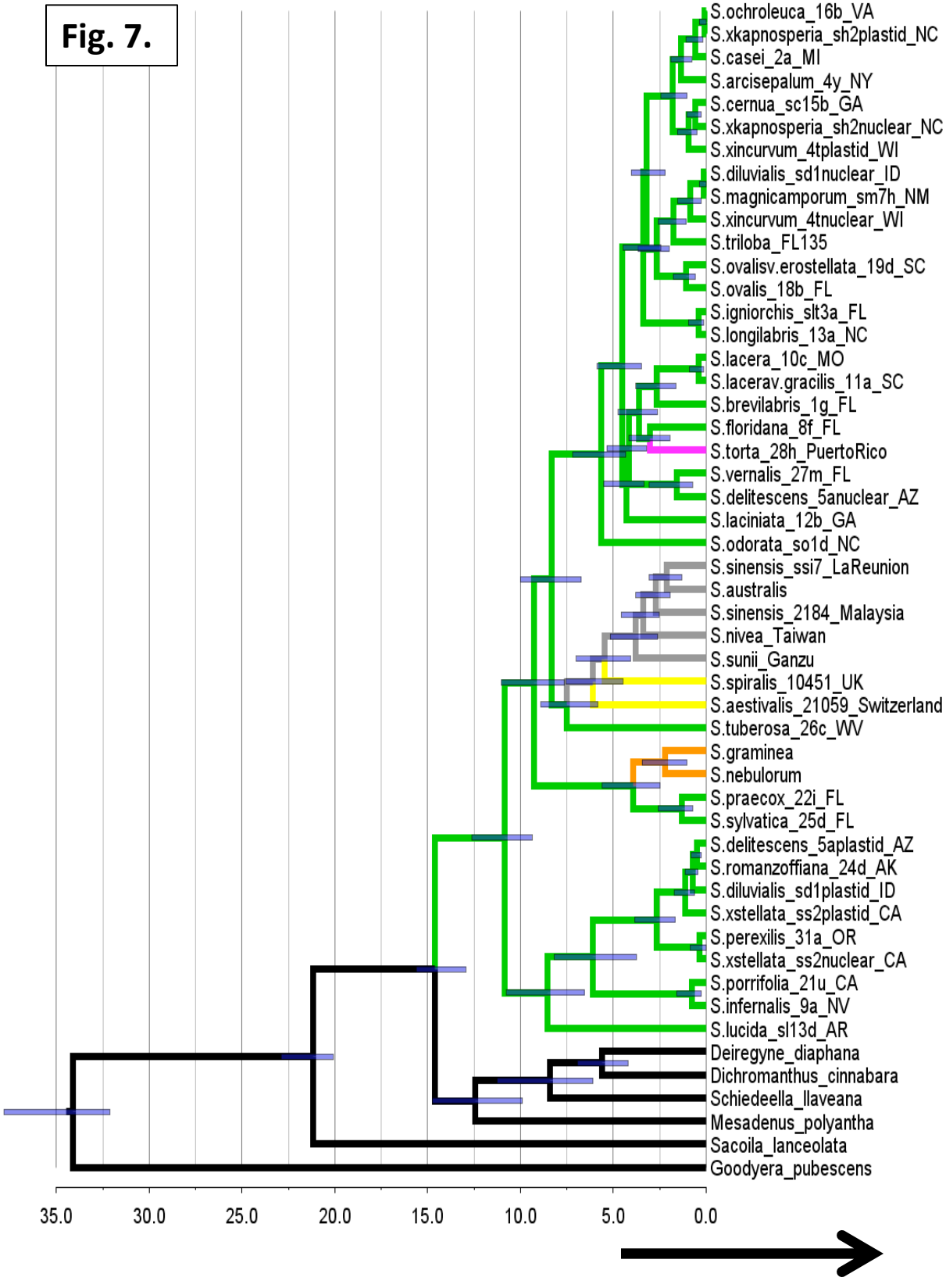

S.ochroleuca_16b_VA
S.xkapnosperia_sh2plastid_NC
S.casei_2a_MI
S.arcisepalum_4y_NY
S.cernua_sc15b_GA
S.xkapnosperia_sh2nuclear_NC
S.xincurvum_4tplastid_WI
S.diluvialis_sd1nuclear_ID
S.magnicamporum_sm7h_NM
S.xincurvum_4tnuclear_WI
S.triloba_FL135
S.ovalisv.erostellata_19d_SC
S.ovalis_18b_FL
S.igniorchis_slt3a_FL
S.longilabris_13a_NC
S.lacera_10c_MO
S.lacerav.gracilis_11a_SC
S.brevilabris_1g_FL
S.floridana_8f_FL
S.torta_28h_PuertoRico
S.vernalis_27m_FL
S.delitescens_5anuclear_AZ
S.laciniata_12b_GA
S.odorata_so1d_NC
S.sinensis_ssi7_LaReunion
S.australis
S.sinensis_2184_Malaysia
S.nivea_Taiwan
S.sunii_Ganzu
S.spiralis_10451_UK
S.aestivalis_21059_Switzerland
S.tuberosa_26c_WV
S.graminea
S.nebulorum
S.praecox_22i_FL
S.sylvatica_25d_FL
S.delitescens_5aplastid_AZ
S.romanzoffiana_24d_AK
S.diluvialis_sd1plastid_ID
S.xstellata_ss2plastid_CA
S.perexilis_31a_OR
S.xstellata_ss2nuclear_CA
S.porrifolia_21u_CA
S.infernalis_9a_NV
S.lucida_sl13d_AR
Deiregyne_diaphana
Dichromanthus_cinnabara
Schiedeella_llaveana
Mesadenus_polyantha
Sacoila_lanceolata
Goodyera_pubescens
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0



Fig. 7. [Legend to preceding page/tree] Calibrated BEAST chronogram, with global geographic regions manually mapped onto the tree. Node bars represent 95% HPD. Axis times in millions of years. Colors are the same as the global regions in Fig. 1. [**colors do NOT match the colors of the BioGeoBEARS output pie-charts**].

Green: North America

Pink: Caribbean Basin (The Antilles, Bermuda, and coastal Yucatan, Belize, and Nicaragua)

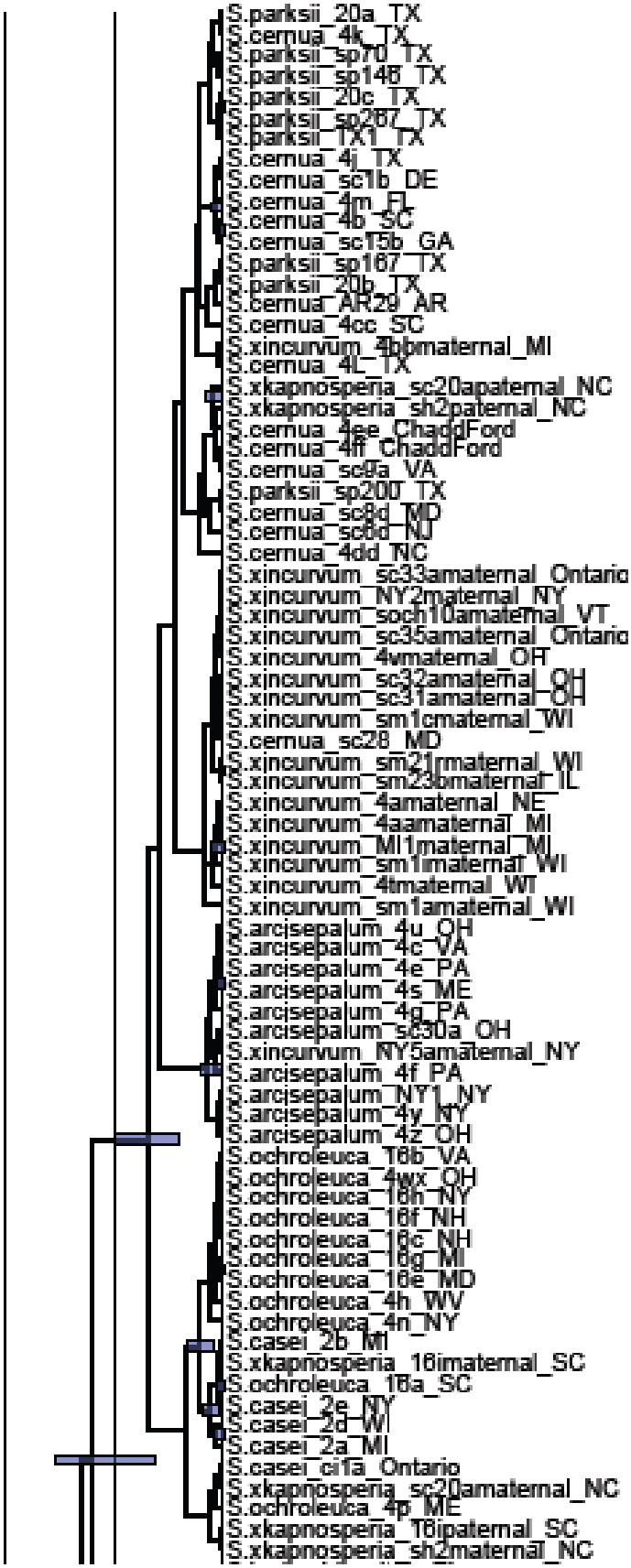
Orange: Central America (Southern Mexico & Guatemala)

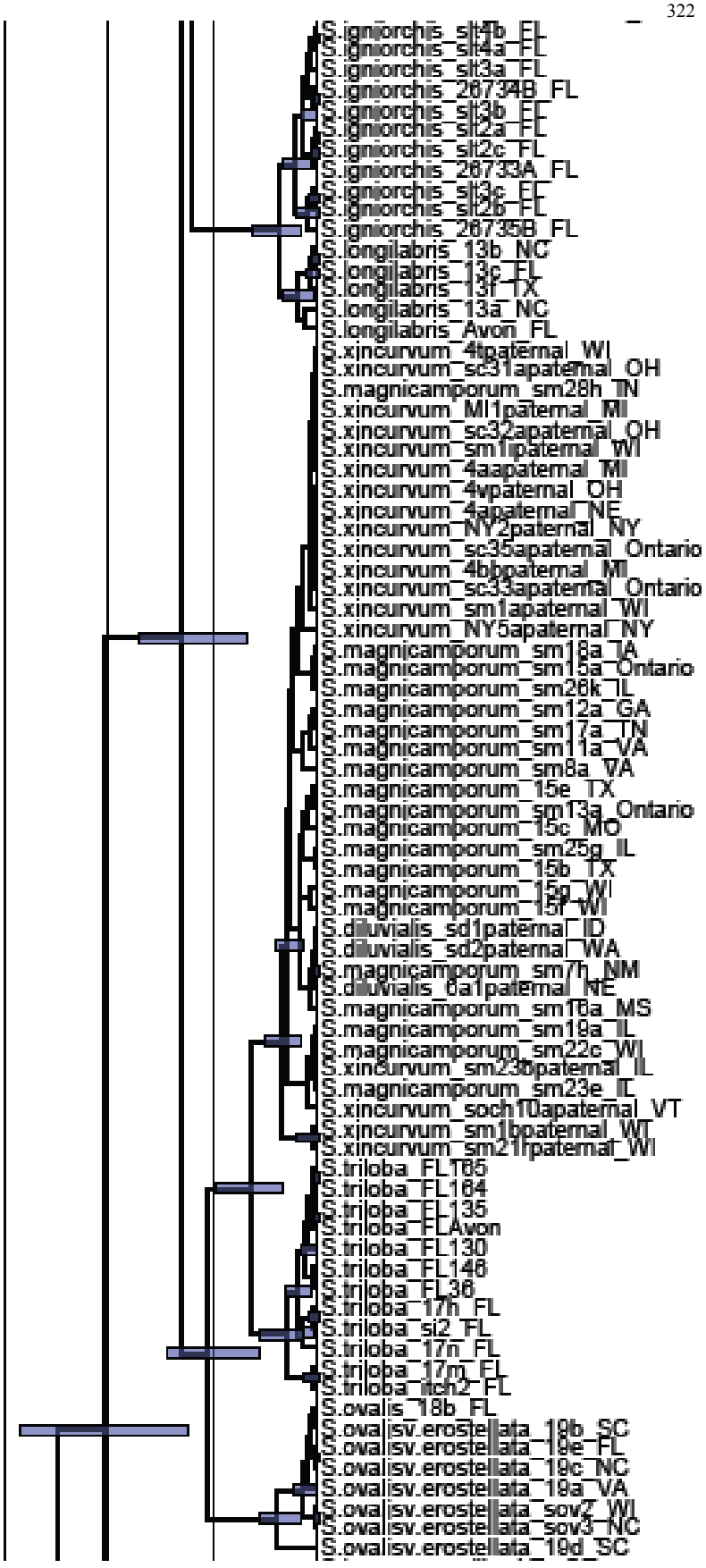
Yellow: Eurasia

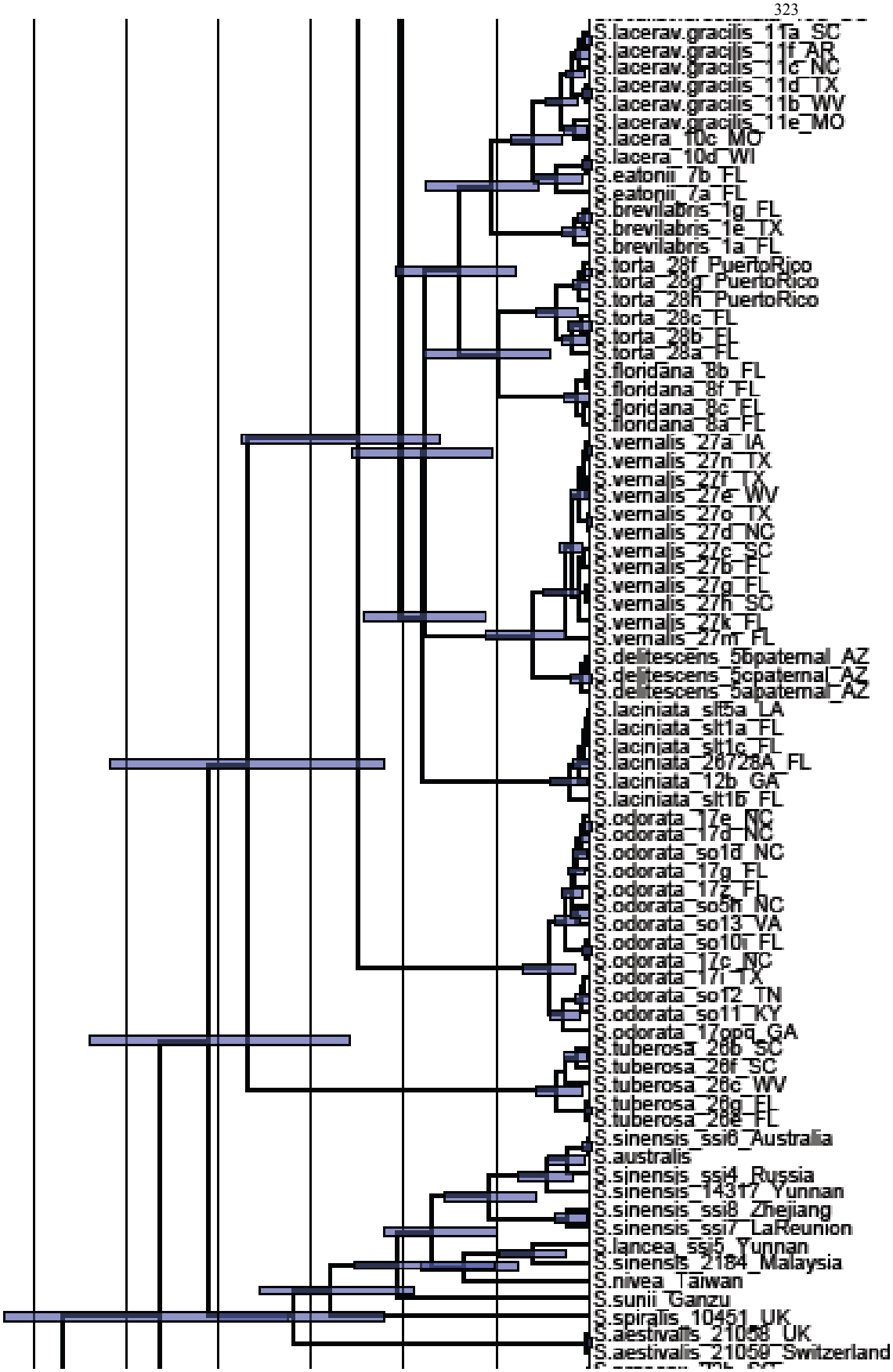
White [gray]: Asia, Australia, and western Oceania

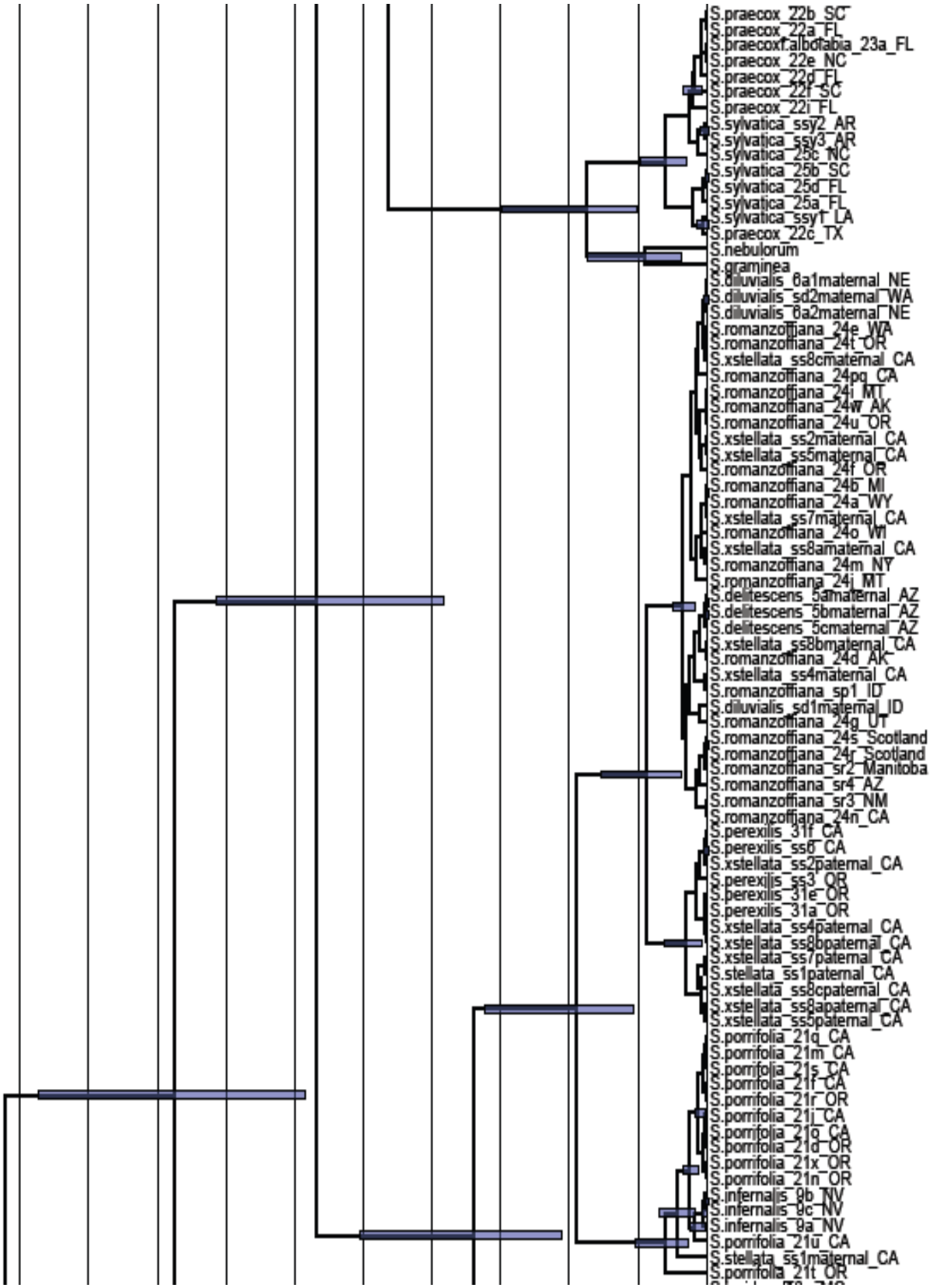
Black: Outgroup taxa (Northern & Central Mexico, Caribbean Basin; *Goodyera pubescens* is North American).

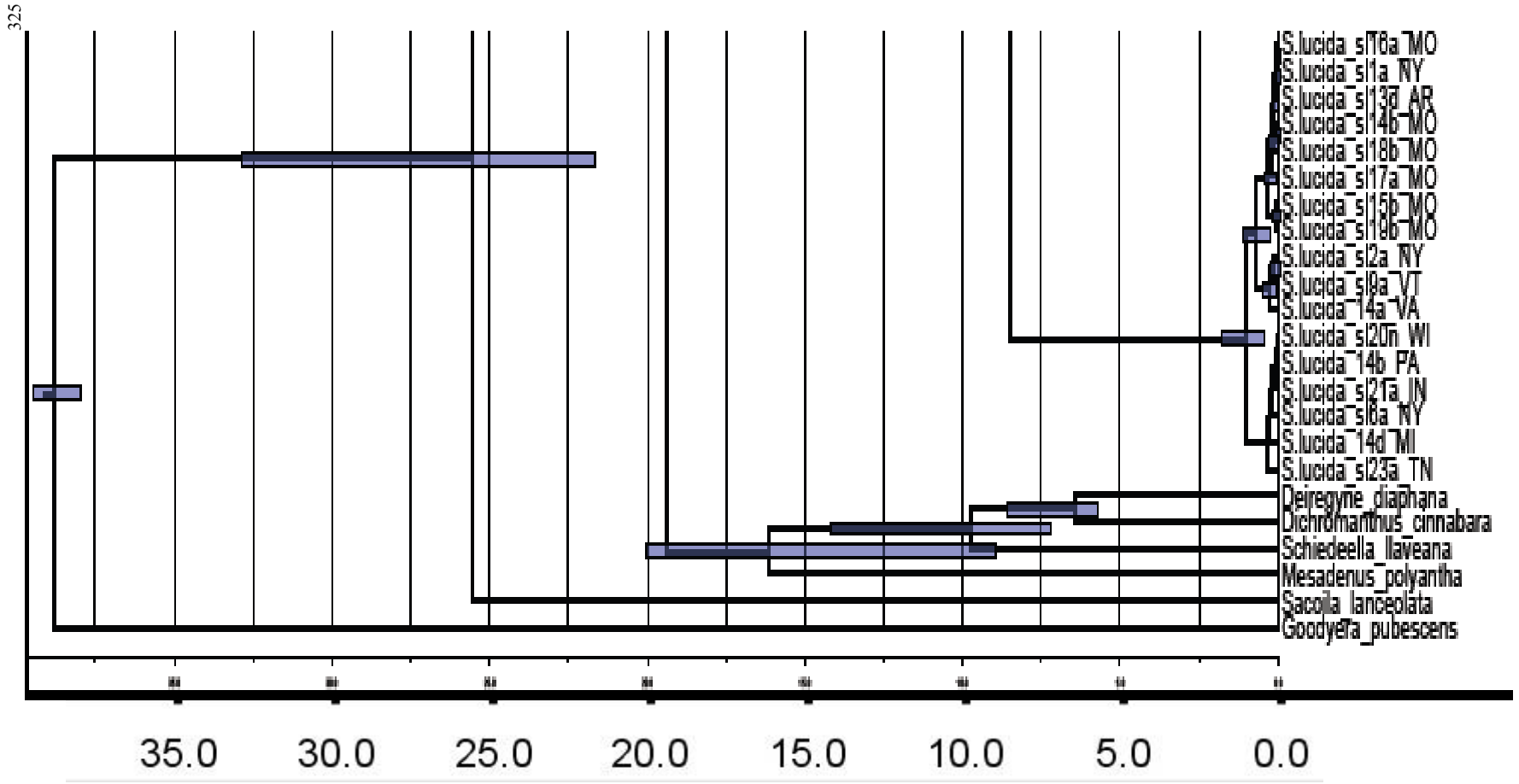
Fig. 8. [Legend for the next five pages fig 8 I-V]. Fossil calibrated BEAST chronogram for the full, multiple individual per species combined nuclear + plastid dataset. Vertical bars indicate 2.5 million year internals. Gray horizontal bars at nodes indicate 95% Highest Posterior Density (HPD) intervals.




S.igniorchis_slt4b_FL
S.igniorchis_slt4a_FL
S.igniorchis_slt3a_FL
S.igniorchis_26734B_FL
S.igniorchis_slt3b_FL
S.igniorchis_slt2a_FL
S.igniorchis_slt2c_FL
S.igniorchis_26733A_FL
S.igniorchis_slt3c_FL
S.igniorchis_slt2b_FL
S.igniorchis_26735B_FL
S.longilabris_13b_NC
S.longilabris_13c_FL
S.longilabris_13f_TX
S.longilabris_13a_NC
S.longilabris_Avon_FL
S.xincurvum_4tpaternal_WI
S.xincurvum_sc31apaternal_OH
S.magnicamporum_sm28h_TN
S.xincurvum_MI1paternal_MI
S.xincurvum_sc32apaternal_OH
S.xincurvum_sm1tpaternal_WI
S.xincurvum_4aapaternal_MI
S.xincurvum_4vpaternal_OH
S.xincurvum_4apaternal_NE
S.xincurvum_NY2paternal_NY
S.xincurvum_sc35apaternal_Ontario
S.xincurvum_4bbpaternal_MI
S.xincurvum_sc33apaternal_Ontario
S.xincurvum_sm1apaternal_WI
S.xincurvum_NY5apaternal_NY
S.magnicamporum_sm18a_TA
S.magnicamporum_sm15a_Ontario
S.magnicamporum_sm26k_IL
S.magnicamporum_sm12a_GA
S.magnicamporum_sm17a_TN
S.magnicamporum_sm11a_VA
S.magnicamporum_sm8a_VA
S.magnicamporum_15e_TX
S.magnicamporum_sm13a_Ontario
S.magnicamporum_15c_MO
S.magnicamporum_sm25g_IL
S.magnicamporum_15b_TX
S.magnicamporum_15g_WI
S.magnicamporum_15f_WI
S.diluvialis_sd1paternal_ID
S.diluvialis_sd2paternal_WA
S.magnicamporum_sm7h_NM
S.diluvialis_0a1paternal_NE
S.magnicamporum_sm16a_MS
S.magnicamporum_sm19a_IL
S.magnicamporum_sm22c_WI
S.xincurvum_sm23bpaternal_IL
S.magnicamporum_sm23e_IL
S.xincurvum_soch10apaternal_VT
S.xincurvum_sm1bpaternal_WI
S.xincurvum_sm21fpaternal_WI
S.triloba_FL165
S.triloba_FL164
S.triloba_FL135
S.triloba_FLAvon
S.triloba_FL130
S.triloba_FL148
S.triloba_FL36
S.triloba_17h_FL
S.triloba_si2_FL
S.triloba_17n_FL
S.triloba_17m_FL
S.triloba_itch2_FL
S.ovalis_18b_FL
S.ovalisv.erostellata_19b_SC
S.ovalisv.erostellata_19e_FL
S.ovalisv.erostellata_19c_NC
S.ovalisv.erostellata_19a_VA
S.ovalisv.erostellata_sov2_WI
S.ovalisv.erostellata_sov3_NC
S.ovalisv.erostellata_19d_SC


S.lacerav.gracilis_11a_SC
S.lacerav.gracilis_11f_AR
S.lacerav.gracilis_11c_NC
S.lacerav.gracilis_11d_TX
S.lacerav.gracilis_11b_WV
S.lacerav.gracilis_11e_MO
S.lacera_f0c_MO
S.lacera_10d_WI
S.eatonii_7b_FL
S.eatonii_7a_FL
S.brevilabris_1g_FL
S.brevilabris_1e_TX
S.brevilabris_1a_FL
S.torta_28f_PuertoRico
S.torta_28g_PuertoRico
S.torta_28h_PuertoRico
S.torta_28c_FL
S.torta_28b_FL
S.torta_28a_FL
S.floridana_8b_FL
S.floridana_8f_FL
S.floridana_8c_FL
S.floridana_8a_FL
S.vernalis_27a_IA
S.vernalis_27n_TX
S.vernalis_27f_TX
S.vernalis_27e_WV
S.vernalis_27o_TX
S.vernalis_27d_NC
S.vernalis_27c_SC
S.vernalis_27b_FL
S.vernalis_27g_FL
S.vernalis_27h_SC
S.vernalis_27k_FL
S.vernalis_27m_FL
S.delitescens_5bpaternal_AZ
S.delitescens_5cpaternal_AZ
S.delitescens_5apaternal_AZ
S.laciniata_slt5a_LA
S.laciniata_slt1a_FL
S.laciniata_slt1c_FL
S.laciniata_26728A_FL
S.laciniata_12b_GA
S.laciniata_slt1b_FL
S.odorata_17e_NC
S.odorata_17d_NC
S.odorata_so1d_NC
S.odorata_17g_FL
S.odorata_17z_FL
S.odorata_so5h_NC
S.odorata_so13_VA
S.odorata_so10i_FL
S.odorata_17c_NC
S.odorata_17i_TX
S.odorata_so12_TN
S.odorata_so11_KY
S.odorata_17opq_GA
S.tuberosa_26b_SC
S.tuberosa_26f_SC
S.tuberosa_26c_WV
S.tuberosa_26g_FL
S.tuberosa_26e_FL
S.sinensis_ssi6_Australia
S.australis
S.sinensis_ssi4_Russia
S.sinensis_14317_Yunnan
S.sinensis_ssi8_Zhejiang
S.sinensis_ssi7_LaReunion
S.lancea_ssi5_Yunnan
S.sinensis_2184_Malaysia
S.nivea_Taiwan
S.sunii_Ganzu
S.spiralis_10451_UK
S.aestivalis_21058_UK
S.aestivalis_21059_Switzerland


S.praecox_22b_SC
S.praecox_22a_FL
S.praecox_albotabia_23a_FL
S.praecox_22e_NC
S.praecox_22d_FL
S.praecox_22f_SC
S.praecox_22i_FL
S.sylvatica_ssy2_AR
S.sylvatica_ssy3_AR
S.sylvatica_25c_NC
S.sylvatica_25b_SC
S.sylvatica_25d_FL
S.sylvatica_25a_FL
S.sylvatica_ssy1_LA
S.praecox_22c_TX
S.nebulorum
S.graminea
S.diluvialis_6a1maternal_NE
S.diluvialis_sd2maternal_WA
S.diluvialis_6a2maternal_NE
S.romanzoffiana_24e_WA
S.romanzoffiana_24t_OR
S.xstellata_ss8cmaternal_CA
S.romanzoffiana_24pq_CA
S.romanzoffiana_24i_MT
S.romanzoffiana_24w_AK
S.romanzoffiana_24u_OR
S.xstellata_ss2maternal_CA
S.xstellata_ss5maternal_CA
S.romanzoffiana_24f_OR
S.romanzoffiana_24b_MI
S.romanzoffiana_24a_WY
S.xstellata_ss7maternal_CA
S.romanzoffiana_24o_WI
S.xstellata_ss8amaternal_CA
S.romanzoffiana_24m_NY
S.romanzoffiana_24j_MT
S.delitescens_5amaternal_AZ
S.delitescens_5bmaternal_AZ
S.delitescens_5cmaternal_AZ
S.xstellata_ss8bmaternal_CA
S.romanzoffiana_24d_AK
S.xstellata_ss4maternal_CA
S.romanzoffiana_sp1_ID
S.diluvialis_sd1maternal_ID
S.romanzoffiana_24g_UT
S.romanzoffiana_24s_Scotland
S.romanzoffiana_24r_Scotland
S.romanzoffiana_sr2_Manitoba
S.romanzoffiana_sr4_AZ
S.romanzoffiana_sr3_NM
S.romanzoffiana_24n_CA
S.perexilis_31f_CA
S.perexilis_ss6_CA
S.xstellata_ss2paternal_CA
S.perexilis_ss3_OR
S.perexilis_31e_OR
S.perexilis_31a_OR
S.xstellata_ss4paternal_CA
S.xstellata_ss8bpaternal_CA
S.xstellata_ss7paternal_CA
S.stellata_ss1paternal_CA
S.xstellata_ss8cpaternal_CA
S.xstellata_ss8apaternal_CA
S.xstellata_ss5paternal_CA
S.porrifolia_21q_CA
S.porrifolia_21m_CA
S.porrifolia_21s_CA
S.porrifolia_21f_CA
S.porrifolia_21r_OR
S.porrifolia_21j_CA
S.porrifolia_21o_CA
S.porrifolia_21d_OR
S.porrifolia_21x_OR
S.porrifolia_21n_OR
S.infernalis_9b_NV
S.infernalis_9c_NV
S.infernalis_9a_NV
S.porrifolia_21u_CA
S.stellata_ss1maternal_CA
S.porrifolia_21t_OR



Millions of years

Fig. 9[following page]. Chronogram: BioGeoBEARS estimate of ancestral distribution regions. **Map:** Physiographic Divisions of The United States (USGS) used to code the BioGeoBEARS ARE analysis. Colors on the map represent the base colors used in the BioGeoBEARS analysis. Colors on the chronogram represent both individual regions, as well as combinations of regions.

In general, colors on the chronogram can be interpreted in the following way:

Red: Atlantic Geological Coastal Plain

Orange: Appalachian Highlands

Magenta/Pink: Mexico + the Caribbean

Purple/Plum: Old World (Eurasia and Australia)

Green: Interior Lowlands

Greenish-brown: Appalachian Highlands + Interior Lowlands + Ozark Plateau & Ouachita Mountains

Blues: primarily Western North America, including: Rocky Mountains + Pacific Mountain systems + Basin & Range + Boreal North

Yellow: Ozark Plateau & Ouachita Mountains

Bright-blue: Basin & Range, & Intermountain Region [BR]



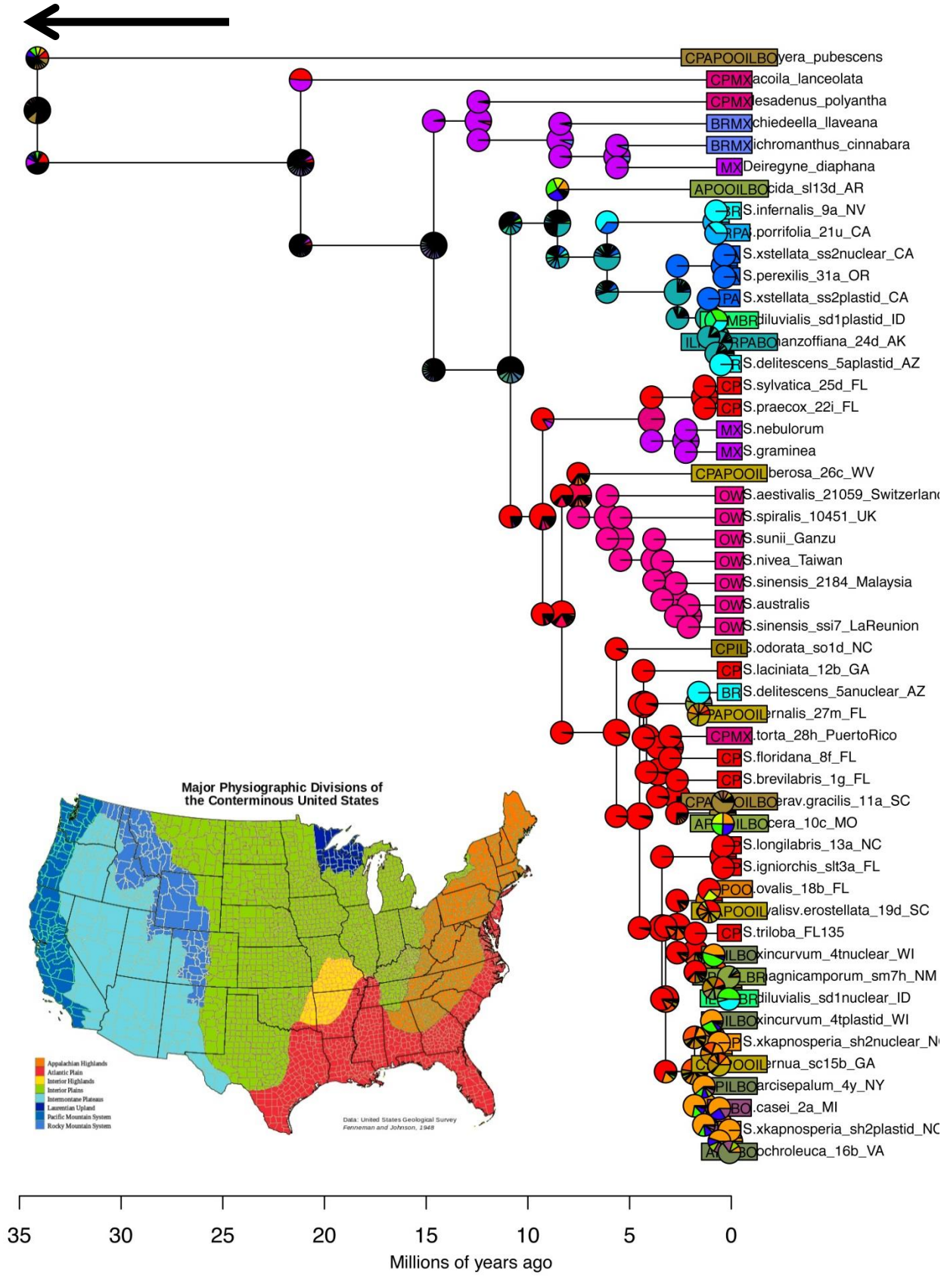

CPAPOOILBO vera_pubescens
CPMX acoila_lanceolata
CPMX esadenus_polyantha
BRMX chiedeella_llaveana
BRMX ichromanthus_cinnabara
MX Deiregyne_diaphana
APOOILBO cida_sl13d_AR
S.infernalis_9a_NV
.porrifolia_21u_CA
S.xstellata_ss2nuclear_CA
S.perexilis_31a_OR
S.xstellata_ss2plastid_CA
diluvialis_sd1plastid_ID
anzoffiana_24d_AK
S.delitescens_5aplastid_AZ
S.sylvatica_25d_FL
S.praecox_22i_FL
S.nebulorum
S.graminea
berosa_26c_WV
S.aestivalis_21059_Switzerlan
S.spiralis_10451_UK
S.sunii_Ganzu
S.nivea_Taiwan
S.sinensis_2184_Malaysia
S.australis
S.sinensis_ssi7_LaReunion
.odorata_so1d_NC
S.laciniata_12b_GA
S.delitescens_5anuclear_AZ
rnalis_27m_FL
.torta_28h_PuertoRico
S.floridana_8f_FL
S.brevilabris_1g_FL
erav.gracilis_11a_SC
cera_10c_MO
S.longilabris_13a_NC
S.igniorchis_slt3a_FL
.ovalis_18b_FL
valisv.erostellata_19d_SC
S.triloba_FL135
xincurvum_4tnuclear_WI
agnicamporum_sm7h_NM
diluvialis_sd1nuclear_ID
xincurvum_4tplastid_WI
S.xkapnosperia_sh2nuclear_N
ernua_sc15b_GA
arcisepalum_4y_NY
.casei_2a_MI
S.xkapnosperia_sh2plastid_NC
chroleuca_16b_VA
Major Physiographic Divisions of the Conterminous United States
Appalachian Highlands
Atlantic Plain
Interior Highlands
Interior Plains
Intermontane Plateaus
Laurentian Upland
Pacific Mountain System
Rocky Mountain System
Data: United States Geological Survey
Fenneman and Johnson, 1948
35
30
25
20
15
10
5
0
Millions of years ago

Fig. 10 [following page]. Chronogram: BioGeoBEARS estimate of ancestral distribution regions. **Map:** Geographic estimate of North American ca. 16-11 mybp. Colors on the chronogram represent both individual regions, as well as combinations of regions.

The "?" indicate areas BioGeoBEARS could not confidently estimate. Based on the distribution of the sister clade to *Spiranthes*, the clade likely evolved in Northern Mexico and the southwestern United States.

In general, colors on the chronogram can be interpreted in the following way:

Red: Atlantic Geological Coastal Plain

Orange: Appalachian Highlands

Magenta/Pink: Mexico + the Caribbean

Purple/Plum: Old World (Eurasia and Australia)

Green: Interior Lowlands

Greenish-brown: Appalachian Highlands + Interior Lowlands + Ozark Plateau & Ouachita Mountains

Blues: primarily Western North America, including: Rocky Mountains + Pacific Mountain systems + Basin & Range + Boreal North

Yellow: Ozark Plateau & Ouachita Mountains

Bright-blue: Basin & Range, & Intermountain Region



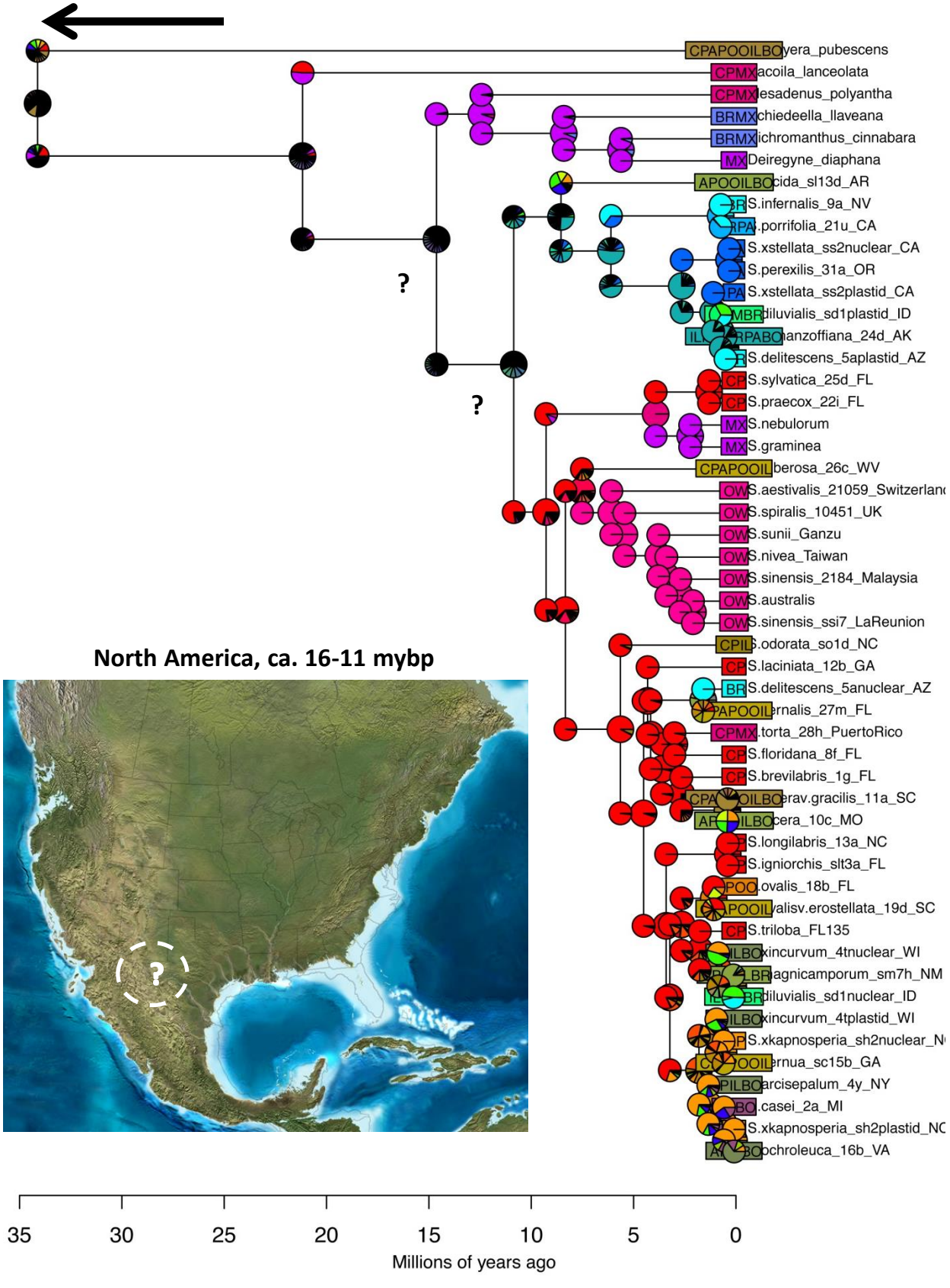

CPAPOOILBO yera_pubescens
CPMX acoila_lanceolata
CPMX esadenus_polyantha
BRMX chiedeella_llaveana
BRMX ichromanthus_cinnabara
MX Deiregyne_diaphana
APOOILBO cida_sl13d_AR
S.infernalis_9a_NV
.porrifolia_21u_CA
S.xstellata_ss2nuclear_CA
S.perexilis_31a_OR
S.xstellata_ss2plastid_CA
diluvialis_sd1plastid_ID
anzoffiana_24d_AK
S.delitescens_5aplastid_AZ
S.sylvatica_25d_FL
S.praecox_22i_FL
S.nebulorum
S.graminea
berosa_26c_WV
S.aestivalis_21059_Switzerlan
S.spiralis_10451_UK
S.sunii_Ganzu
S.nivea_Taiwan
S.sinensis_2184_Malaysia
S.australis
S.sinensis_ssi7_LaReunion
.odorata_so1d_NC
S.laciniata_12b_GA
S.delitescens_5anuclear_AZ
rnalis_27m_FL
.torta_28h_PuertoRico
S.floridana_8f_FL
S.brevilabris_1g_FL
erav.gracilis_11a_SC
cera_10c_MO
S.longilabris_13a_NC
S.igniorchis_slt3a_FL
.ovalis_18b_FL
valisv.erostellata_19d_SC
S.triloba_FL135
xincurvum_4tnuclear_WI
agnicamporum_sm7h_NM
diluvialis_sd1nuclear_ID
xincurvum_4tplastid_WI
S.xkapnosperia_sh2nuclear_N
rnua_sc15b_GA
arcisepalum_4y_NY
.casei_2a_MI
S.xkapnosperia_sh2plastid_NC
chroleuca_16b_VA
?
?
North America, ca. 16-11 mybp
?
35
30
25
20
15
10
5
0
Millions of years ago

Fig. 11 [following page]. Chronogram: BioGeoBEARS estimate of ancestral distribution regions. **Map:** Geographic estimate of North American ca. 16-11 mybp. Colors on the chronogram represent both individual regions, as well as combinations of regions.

The gradient arrow indicates the migration of *Spiranthes lucida* from an ancestor which may have occurred in the Intermountain West or the western Interior Lowlands, toward the Appalachian Highlands. [For clarity, and because the position of *S. lucida* within the tree is somewhat unusual, this arrow will not be displayed in subsequent maps. However, *S. lucida* does represent an independent lineage of *Spiranthes* within Eastern North America that does not share a MRCA with any other Eastern North American *Spiranthes*.]

In general, colors on the chronogram can be interpreted in the following way:

Red: Atlantic Geological Coastal Plain

Orange: Appalachian Highlands

Magenta/Pink: Mexico + the Caribbean

Purple/Plum: Old World (Eurasia and Australia)

Green: Interior Lowlands

Greenish-brown: Appalachian Highlands + Interior Lowlands + Ozark Plateau & Ouachita Mountains

Blues: primarily Western North America, including: Rocky Mountains + Pacific Mountain systems + Basin & Range + Boreal North

Yellow: Ozark Plateau & Ouachita Mountains

Bright-blue: Basin & Range, & Intermountain Region



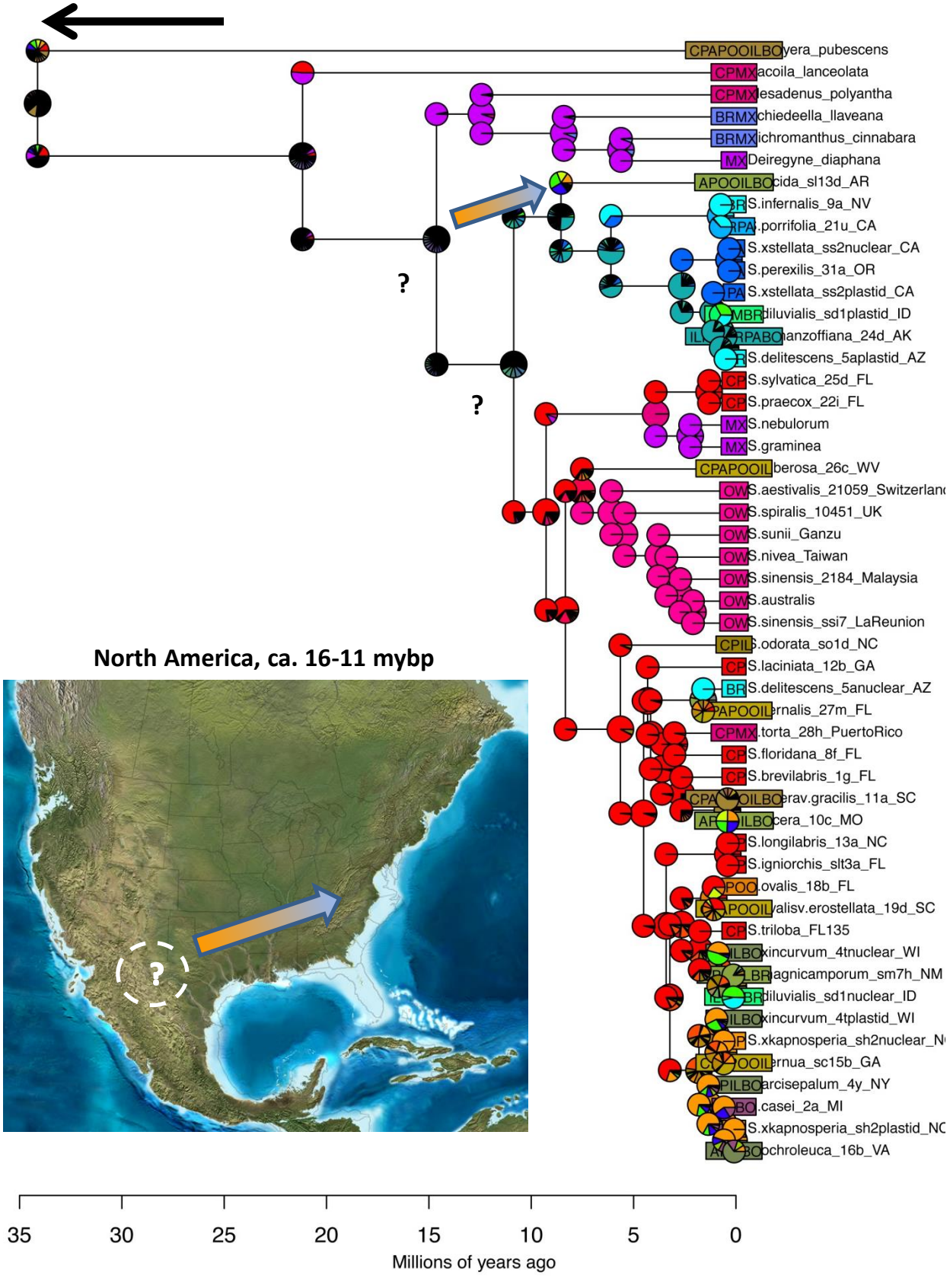

CPAPOOILBO yera_pubescens
CPMX acoila_lanceolata
CPMX esadenus_polyantha
BRMX chiedeella_llaveana
BRMX ichromanthus_cinnabara
MX Deiregyne_diaphana
APOOILBO cida_sl13d_AR
S.infernalis_9a_NV
.porrifolia_21u_CA
S.xstellata_ss2nuclear_CA
S.perexilis_31a_OR
S.xstellata_ss2plastid_CA
diluvialis_sd1plastid_ID
anzoffiana_24d_AK
S.delitescens_5aplastid_AZ
S.sylvatica_25d_FL
S.praecox_22i_FL
S.nebulorum
S.graminea
CPAPOOIL berosa_26c_WV
S.aestivalis_21059_Switzerlan
S.spiralis_10451_UK
S.sunii_Ganzu
S.nivea_Taiwan
S.sinensis_2184_Malaysia
S.australis
S.sinensis_ssi7_LaReunion
.odorata_so1d_NC
S.laciniata_12b_GA
S.delitescens_5anuclear_AZ
ernalis_27m_FL
.torta_28h_PuertoRico
S.floridana_8f_FL
S.brevilabris_1g_FL
erav.gracilis_11a_SC
cera_10c_MO
S.longilabris_13a_NC
S.igniorchis_slt3a_FL
.ovalis_18b_FL
valisv.erostellata_19d_SC
S.triloba_FL135
xincurvum_4tnuclear_WI
agnicamporum_sm7h_NM
diluvialis_sd1nuclear_ID
xincurvum_4tplastid_WI
S.xkapnosperia_sh2nuclear_N
ernua_sc15b_GA
arcisepalum_4y_NY
.casei_2a_MI
S.xkapnosperia_sh2plastid_NC
bchroleuca_16b_VA
?
?
North America, ca. 16-11 mybp
?
35
30
25
20
15
10
5
0
Millions of years ago

Fig. 12 [following page]. Chronogram: BioGeoBEARS estimate of ancestral distribution regions. **Map:** Geographic estimate of North American ca. 10-8 mybp. Colors on the chronogram represent both individual regions, as well as combinations of regions.

The blue and red on the map represent areas to which *Spiranthes* migrated: Western North America (blue), and the NAGCP (red).

In general, colors on the chronogram can be interpreted in the following way:

Red: Atlantic Geological Coastal Plain

Orange: Appalachian Highlands

Magenta/Pink: Mexico + the Caribbean

Purple/Plum: Old World (Eurasia and Australia)

Green: Interior Lowlands

Greenish-brown: Appalachian Highlands + Interior Lowlands + Ozark Plateau & Ouachita Mountains

Blues: primarily Western North America, including: Rocky Mountains + Pacific Mountain systems + Basin & Range + Boreal North

Yellow: Ozark Plateau & Ouachita Mountains

Bright-blue: Basin & Range, & Intermountain Region



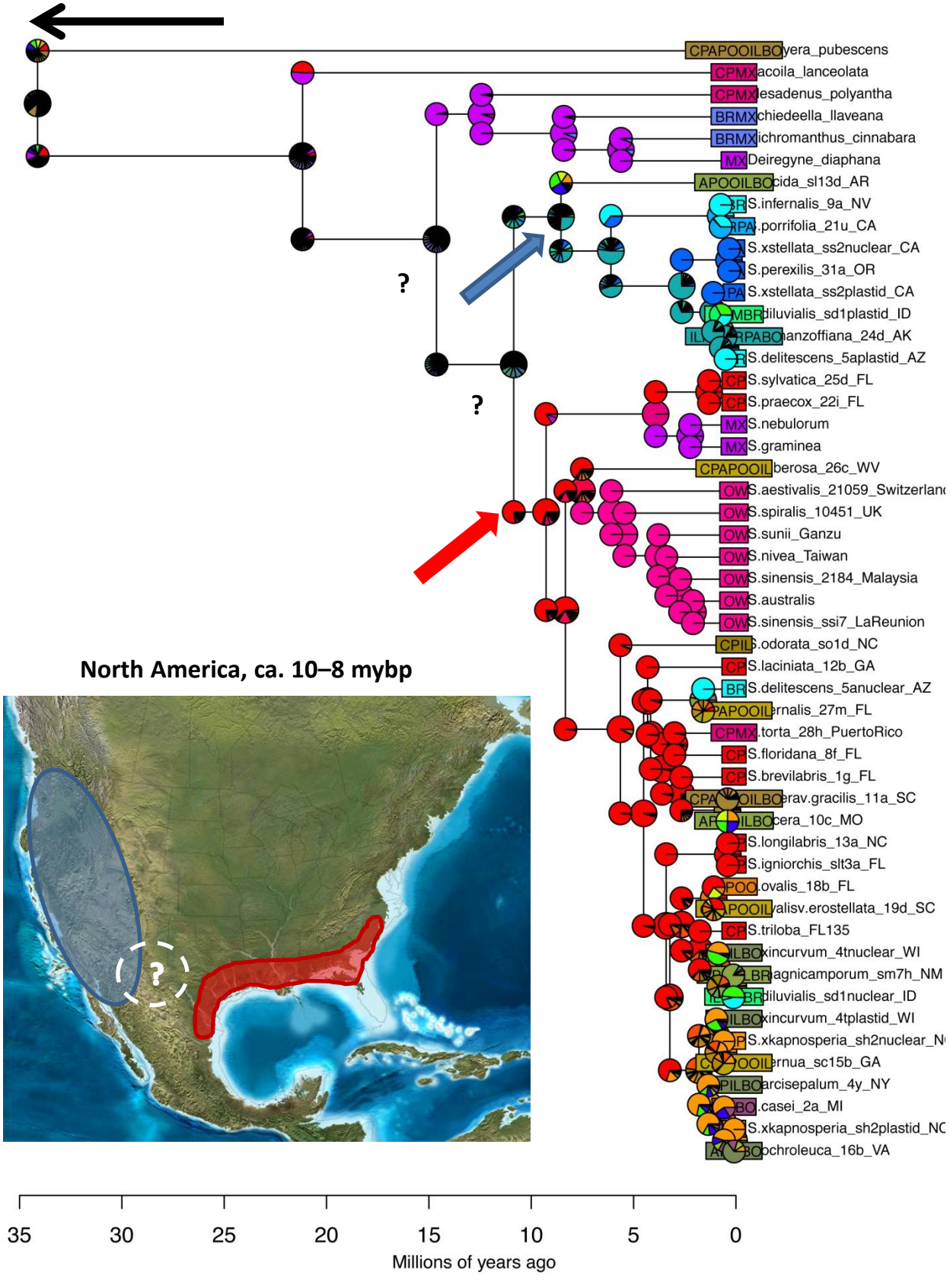

CPAPOOILBO vera_pubescens
CPMX acoila_lanceolata
CPMX esadenus_polyantha
BRMX chiedeella_llaveana
BRMX ichromanthus_cinnabara
MX Deiregyne_diaphana
APOOILBO cida_sl13d_AR
S.infernalis_9a_NV
.porrifolia_21u_CA
S.xstellata_ss2nuclear_CA
S.perexilis_31a_OR
S.xstellata_ss2plastid_CA
diluvialis_sd1plastid_ID
anzoffiana_24d_AK
S.delitescens_5aplastid_AZ
S.sylvatica_25d_FL
S.praecox_22i_FL
S.nebulorum
S.graminea
berosa_26c_WV
S.aestivalis_21059_Switzerlan
S.spiralis_10451_UK
S.sunii_Ganzu
S.nivea_Taiwan
S.sinensis_2184_Malaysia
S.australis
S.sinensis_ssi7_LaReunion
.odorata_so1d_NC
S.laciniata_12b_GA
S.delitescens_5anuclear_AZ
rnalis_27m_FL
.torta_28h_PuertoRico
S.floridana_8f_FL
S.brevilabris_1g_FL
erav.gracilis_11a_SC
cera_10c_MO
S.longilabris_13a_NC
S.igniorchis_slt3a_FL
.ovalis_18b_FL
valisv.erostellata_19d_SC
S.triloba_FL135
xincurvum_4tnuclear_WI
agnicamporum_sm7h_NM
diluvialis_sd1nuclear_ID
xincurvum_4tplastid_WI
S.xkapnosperia_sh2nuclear_N
rnua_sc15b_GA
arcisepalum_4y_NY
.casei_2a_MI
S.xkapnosperia_sh2plastid_NC
bchroleuca_16b_VA
?
?
North America, ca. 10–8 mybp
?
35
30
25
20
15
10
5
0
Millions of years ago

Fig. 13 [following page]. Chronogram: BioGeoBEARS estimate of ancestral distribution regions. **Map:** Geographic estimate of North American ca. 10-8 mybp. Colors on the chronogram represent both individual regions, as well as combinations of regions.

The pink/magenta arrow indicates a dispersal event to the Old World (most likely Europe, as the ancestors of the European species –*S*. *aestivalis* and *S*. *spiralis* – evolved before MRCAs of the Asian species.

In general, colors on the chronogram can be interpreted in the following way:

Red: Atlantic Geological Coastal Plain

Orange: Appalachian Highlands

Magenta/Pink: Mexico + the Caribbean

Purple/Plum: Old World (Eurasia and Australia)

Green: Interior Lowlands

Greenish-brown: Appalachian Highlands + Interior Lowlands + Ozark Plateau & Ouachita Mountains

Blues: primarily Western North America, including: Rocky Mountains + Pacific Mountain systems + Basin & Range + Boreal North

Yellow: Ozark Plateau & Ouachita Mountains

Bright-blue: Basin & Range, & Intermountain Region



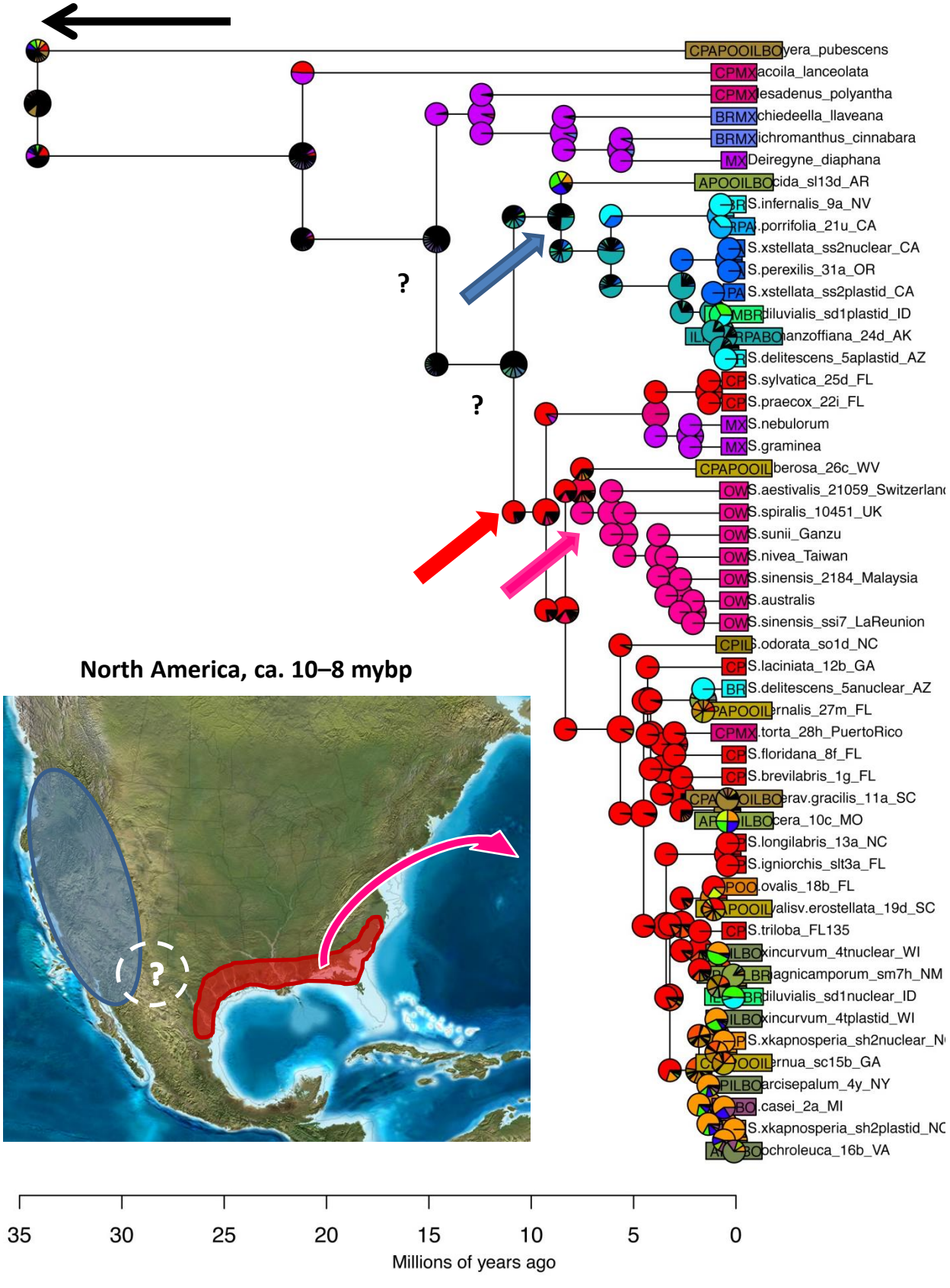

CPAPOOILBO vera_pubescens
CPMX acoila_lanceolata
CPMX esadenus_polyantha
BRMX chiedeella_llaveana
BRMX ichromanthus_cinnabara
MX Deiregyne_diaphana
APOOILBO cida_sl13d_AR
S.infernalis_9a_NV
.porrifolia_21u_CA
S.xstellata_ss2nuclear_CA
S.perexilis_31a_OR
S.xstellata_ss2plastid_CA
diluvialis_sd1plastid_ID
anzoffiana_24d_AK
S.delitescens_5aplastid_AZ
S.sylvatica_25d_FL
S.praecox_22i_FL
S.nebulorum
S.graminea
berosa_26c_WV
S.aestivalis_21059_Switzerlan
S.spiralis_10451_UK
S.sunii_Ganzu
S.nivea_Taiwan
S.sinensis_2184_Malaysia
S.australis
S.sinensis_ssi7_LaReunion
.odorata_so1d_NC
S.laciniata_12b_GA
S.delitescens_5anuclear_AZ
rnalis_27m_FL
.torta_28h_PuertoRico
S.floridana_8f_FL
S.brevilabris_1g_FL
erav.gracilis_11a_SC
cera_10c_MO
S.longilabris_13a_NC
S.igniorchis_slt3a_FL
.ovalis_18b_FL
valisv.erostellata_19d_SC
S.triloba_FL135
xincurvum_4tnuclear_WI
agnicamporum_sm7h_NM
diluvialis_sd1nuclear_ID
xincurvum_4tplastid_WI
S.xkapnosperia_sh2nuclear_N
rnua_sc15b_GA
arcisepalum_4y_NY
.casei_2a_MI
S.xkapnosperia_sh2plastid_NC
bchroleuca_16b_VA
?
?
North America, ca. 10–8 mybp
?
35
30
25
20
15
10
5
0
Millions of years ago

Fig. 14 [following page]. Chronogram: BioGeoBEARS estimate of ancestral distribution regions. **Map:** Geographic estimate of North American ca. 4 mybp. Colors on the chronogram represent both individual regions, as well as combinations of regions.

The purple arrow indicates a migration event for the MRCA of *S*. *graminea* and *S*. *nebulorum* from the NAGCP (as indicated by the red arrow and pie-charts) to the mountains of southern Mexico and Central America.

In general, colors on the chronogram can be interpreted in the following way:

Red: Atlantic Geological Coastal Plain

Orange: Appalachian Highlands

Magenta/Pink: Mexico + the Caribbean

Purple/Plum: Old World (Eurasia and Australia)

Green: Interior Lowlands

Greenish-brown: Appalachian Highlands + Interior Lowlands + Ozark Plateau & Ouachita Mountains

Blues: primarily Western North America, including: Rocky Mountains + Pacific Mountain systems + Basin & Range + Boreal North

Yellow: Ozark Plateau & Ouachita Mountains

Bright-blue: Basin & Range, & Intermountain Region



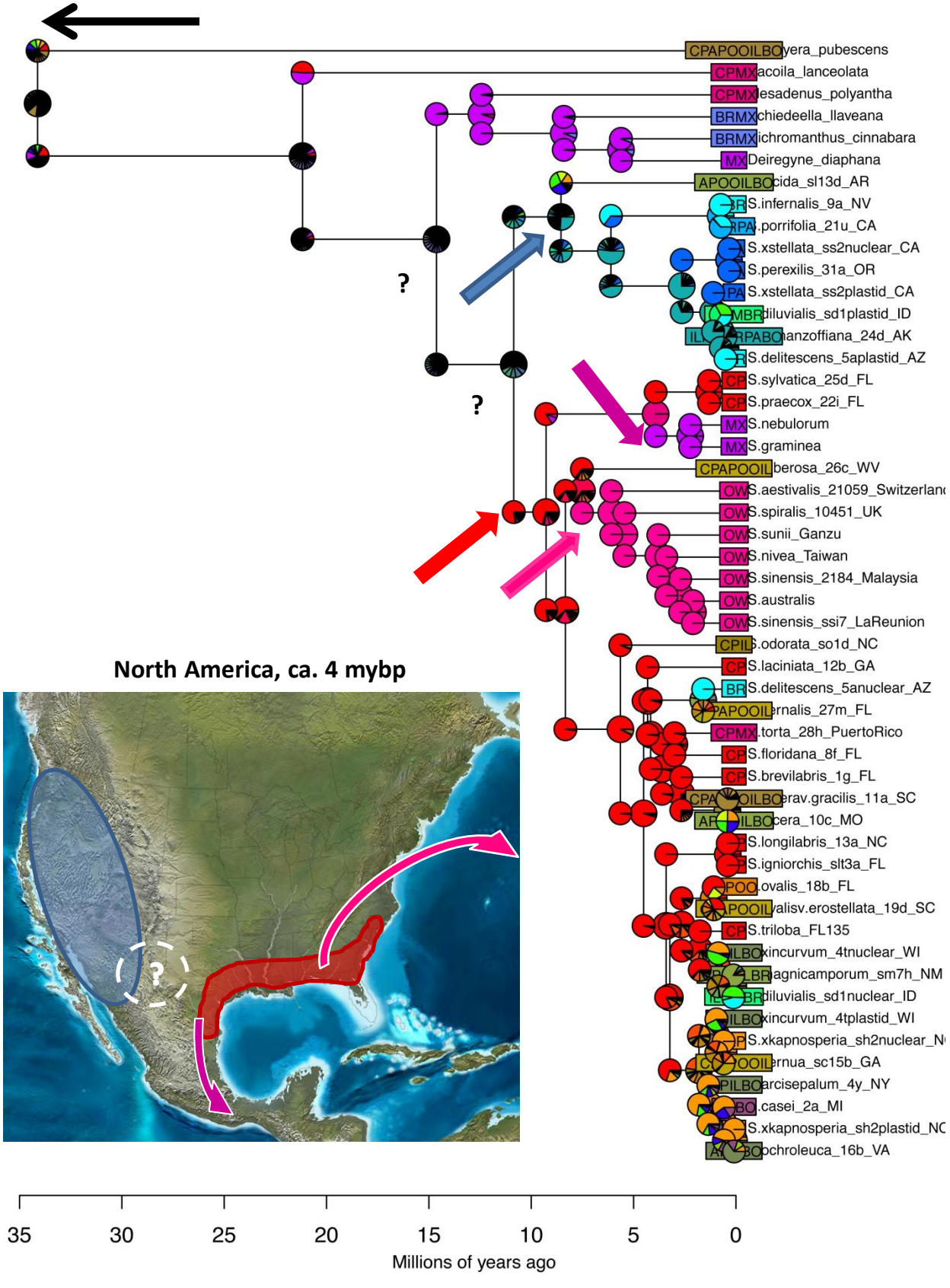

CPAPOOILBO yera_pubescens
CPMX acoila_lanceolata
CPMX esadenus_polyantha
BRMX chiedeella_llaveana
BRMX ichromanthus_cinnabara
MX Deiregyne_diaphana
APOOILBO cida_sl13d_AR
S.infernalis_9a_NV
.porrifolia_21u_CA
S.xstellata_ss2nuclear_CA
S.perexilis_31a_OR
S.xstellata_ss2plastid_CA
diluvialis_sd1plastid_ID
anzoffiana_24d_AK
S.delitescens_5aplastid_AZ
S.sylvatica_25d_FL
S.praecox_22i_FL
S.nebulorum
S.graminea
berosa_26c_WV
S.aestivalis_21059_Switzerlan
S.spiralis_10451_UK
S.sunii_Ganzu
S.nivea_Taiwan
S.sinensis_2184_Malaysia
S.australis
S.sinensis_ssi7_LaReunion
.odorata_so1d_NC
S.laciniata_12b_GA
S.delitescens_5anuclear_AZ
rnalis_27m_FL
.torta_28h_PuertoRico
S.floridana_8f_FL
S.brevilabris_1g_FL
erav.gracilis_11a_SC
cera_10c_MO
S.longilabris_13a_NC
S.igniorchis_slt3a_FL
.ovalis_18b_FL
valisv.erostellata_19d_SC
S.triloba_FL135
xincurvum_4tnuclear_WI
agnicamporum_sm7h_NM
diluvialis_sd1nuclear_ID
xincurvum_4tplastid_WI
S.xkapnosperia_sh2nuclear_N
rnua_sc15b_GA
arcisepalum_4y_NY
.casei_2a_MI
S.xkapnosperia_sh2plastid_NC
chroleuca_16b_VA
?
?
North America, ca. 4 mybp
?
35
30
25
20
15
10
5
0
Millions of years ago

Fig. 15 [following page]. Chronogram: BioGeoBEARS estimate of ancestral distribution regions. **Map:** Geographic estimate of North American ca. 3-2 mybp. Colors on the chronogram represent both individual regions, as well as combinations of regions.

The orange area and arrows denote the evolution and migration of ancestral taxa to the Appalachian Highlands. The green and greenish-brown area and pie-charts indicate the evolution and migration of groups into broad regions encompassing the Appalachian Highlands + Interior Lowlands + Ozark Plateau & Ouachita Mountains

In general, colors on the chronogram can be interpreted in the following way:

Red: Atlantic Geological Coastal Plain

Orange: Appalachian Highlands

Magenta/Pink: Mexico + the Caribbean

Purple/Plum: Old World (Eurasia and Australia)

Green: Interior Lowlands

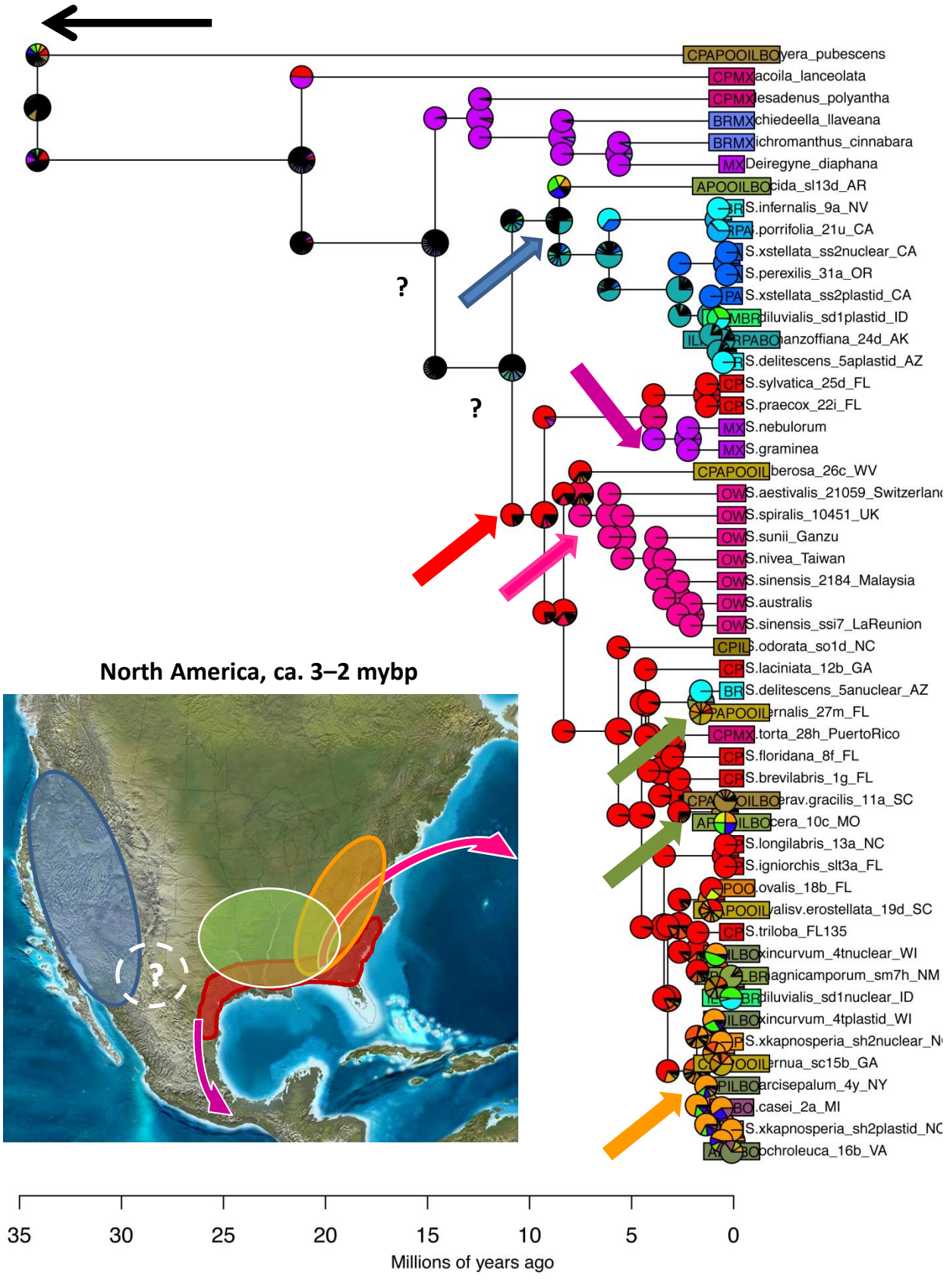
Greenish-brown: Appalachian Highlands + Interior Lowlands + Ozark Plateau & Ouachita Mountains

Blues: primarily Western North America, including: Rocky Mountains + Pacific Mountain systems + Basin & Range + Boreal North

Yellow: Ozark Plateau & Ouachita Mountains

Bright-blue: Basin & Range, & Intermountain Region




CPAPOOILBO vera_pubescens
CPMX acoila_lanceolata
CPMX esadenus_polyantha
BRMX chiedeella_llaveana
BRMX ichromanthus_cinnabara
MX Deiregyne_diaphana
APOOILBO cida_sl13d_AR
S.infernalis_9a_NV
.porrifolia_21u_CA
S.xstellata_ss2nuclear_CA
S.perexilis_31a_OR
S.xstellata_ss2plastid_CA
diluvialis_sd1plastid_ID
anzoffiana_24d_AK
S.delitescens_5aplastid_AZ
S.sylvatica_25d_FL
S.praecox_22i_FL
S.nebulorum
S.graminea
berosa_26c_WV
S.aestivalis_21059_Switzerlan
S.spiralis_10451_UK
S.sunii_Ganzu
S.nivea_Taiwan
S.sinensis_2184_Malaysia
S.australis
S.sinensis_ssi7_LaReunion
.odorata_so1d_NC
S.laciniata_12b_GA
S.delitescens_5anuclear_AZ
rnalis_27m_FL
.torta_28h_PuertoRico
S.floridana_8f_FL
S.brevilabris_1g_FL
erav.gracilis_11a_SC
cera_10c_MO
S.longilabris_13a_NC
S.igniorchis_slt3a_FL
.ovalis_18b_FL
valisv.erostellata_19d_SC
S.triloba_FL135
xincurvum_4tnuclear_WI
agnicamporum_sm7h_NM
diluvialis_sd1nuclear_ID
xincurvum_4tplastid_WI
S.xkapnosperia_sh2nuclear_N
rnua_sc15b_GA
arcisepalum_4y_NY
.casei_2a_MI
S.xkapnosperia_sh2plastid_NC
chroleuca_16b_VA
?
?
North America, ca. 3–2 mybp
?
35
30
25
20
15
10
5
0
Millions of years ago