Molecular systematics, biogeography, and mycorrhizal associations in the Acianthinae (Orchidaceae), with a focus on the genus *Corybas*.

By

Stephanie Pimm Lyon

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

Doctor of Philosophy
(Botany)

at the
UNIVERSITY OF WISCONSIN-MADISON
2014

Date of final oral examination: 4/24/2014

The dissertation is approved by the following members of the Final Oral Committee:
Thomas Givnish, Professor, Botany
Kenneth Sytsma, Professor, Botany
Kenneth Cameron, Professor, Botany
Cecile Ané, Associate Professor, Botany and Statistics
Daniel Young, Professor, Entomology
Mark Clements, CSIRO Australia

Dedicated to Ezra and Akiva, without whose love, patience, and support, I could not have completed it.

Acknowledgements

I gratefully acknowledge my graduate committee—Dr. Thomas Givnish, Dr. Mark Clements, Dr. Kenneth Cameron, Dr. Kenneth Sytsma, Dr. Cecile Ané, and Dr. Daniel Young—for invaluable assistance, guidance, and advice throughout my time as a graduate student. I would also like to thank past and present members of the Givnish lab—Dr. Phil Gonsiska, Dr. Bob Wernerehl, Dr. Emily Sessa, Kate Gerndt, Dr. Mercedes Ames Sevillano, Dr. Becky Shirk, and Dr. Jackson Moeller—as well as a number of fellow UW-Madison Botany graduate students, particularly Abigail Mazie, Alison Scott, Dr. Andy Gardener, Dr. Bryan Drew, Dr. Brian Walsh, Dr. Rafael Arevalo-Burbano, Brian Sidoti, Lauren Moscoe, and Dr. Liz Georgian.

This project relied heavily on assistance from many international scientists, botanists, and orchid enthusiasts, and I am much indebted to them. I would particularly like to thank the staff at CSIRO Black Mountain, especially Dr. Chris Howard, Dr. Joe Miller, and Dr. Judy West. Numerous samples were provided by collectors for the Orchid Research Group. In South Australia, Joe Quarmby, Bob Bates, and Cathy and Malcolm Houston collected numerous specimens and organized my field trips. In Western Australia, Andrew Brown and Dr. Kevin Thiele assisted with collecting locations, permits, and specimen transfer. The New Zealand samples were used with permission of Dr. Brian Molloy. In peninsular Malaysia, I had substantial assistance from Dr. Rusea Go and Muiching Tan (Universiti Putra Malaysia) and staff members of FRIM (Forest Research Institute of Malaysia), particularly Ong Poh Teck. In Sabah, Tony Lamb, and Joan Periera and Jamirus Jumian at the Sandakan herbarium organized my field trips and provided advice as well as field and herbarium assistance. In Sarawak, I had substantial help from Dr. Julia Sang and Ling Chea Yiing (Sarawak Forestry Corporation). The research in Papua New Guinea would not have been possible without substantial assistance from Dr. Ed de Vogel (Leiden), Nathalie and Foreting Juhonewe, and Drs. Shelley James and Allen Allison (Bishop Museum). Taiwanese orchid researchers Dr. Yung-I Lee and Wei-Min Lin organized my trip to Taiwan and provided specimens and field assistance. Dr. Iriwati and Richa Kusuma organized my brief trip to Java. I thank the

herbaria at Kew, the Smithsonian, Leiden, FRIM, Canberra, and Bogor for loans of specimens and allowing destructive sampling in a few cases. I am also grateful to the governments of South Australia and Western Australia, Malaysia, and Papua New Guinea for granting collection permits and research visas.

Ezra Lyon and Dr. Becky Shirk provided critical bioinformatics assistance. I thank the UW-Madison High Throughput Computing center for access to the HPC cluster, and Marie Adams at the Biotech Center for assistance and advice on GBS. Dr. Jane Bradbury and Peter Gregg collected the flow cytometry data, and the use of those facilities and instrumentation was supported by the University of Wisconsin Materials Research Science and Engineering Center (DMR-1121288).

I thank my parents, Karen Lindebrekke and Stuart Pimm, and husband Ezra Lyon for support and editing assistance in the final stages of thesis writing. My mother Karen Lindebrekke assisted with fieldwork in Taiwan, and Ezra Lyon, Akiva Lyon, and Tom Givnish assisted with fieldwork in Western Australia.

Financial support was provided by the National Science Foundation, the Australian Orchid Foundation, the American Orchid Society, the Botanical Society of America, Vilas travel fund (administered by UW-Madison Graduate School), and the following funds administered by the UW-Madison Department of Botany: the Davis Travel Fund, Eldon Newcomb Fellowship, the Owen and Edith Allen Fellowship, and the Judy Croxdale Fund.

Abstract

The orchid subtribe Acianthinae, containing roughly 170 species in five genera, consists of almost exclusively terrestrial, diminutive species, most of which seem to be pollinated by small flies. The group is poorly studied, and presents unique opportunities for evolutionary studies. While the vegetative features are fairly uniform within the group, floral diversity is quite substantial, especially in the large genus *Corybas*. Though part of the primarily Australian Diurideae, this particular subtribe has a remarkably wide range spanning much of Australasia and the Malay Archipelago, into mainland Asia and the Pacific. This is the first phylogenetic study to extensively sample the Acianthinae outside of Australia, New Zealand, and New Caledonia. By using multiple genes, we achieve good resolution and support, allowing us to propose a revised generic classification system than would both minimize taxonomic changes yet incorporate our new understanding of evolutionary relationships in the group. The phylogeny also reveals that some morphological traits that have been used to define taxonomic groups in the past are quite labile, and indicates a remarkable case of floral convergence. Biogeographical studies indicate a mid-Oligocene origin in Australia with extensive dispersal via West Wind Drift in the southern parts of its range. In the tropical parts of its range, dispersal appears more limited, mostly occurring on a smaller scale—with some dramatic exceptions. Much diversification appears to occur locally, especially within the rapidly uplifting New Guinea. We also employ Next Generation Sequencing genotyping techniques to address relationships within one very recently evolved clade endemic to Australia, the genus *Corysanthes* Jones et al. This results in unprecedented resolution within and among species, allowing us to evaluate phylogeographic structure and make recommendations on species delimitation. In addition, we present results of an extensive study of the mycorrhizal associations in the Corysanthes clade. All species appear to be strongly associated with one to several undescribed *Tulasnella* fungi, most of which have not been previously detected in other orchids. Species within the clade clearly differ in regards to their mycorrhizal preferences, though there is a strong environmental signal in their patterns of association.

Chapter 1: Phylogeny, morphological evolution, and biogeography of the orchid subtribe Acianthinae

Abstract

We present the most comprehensive phylogeny of the Acianthinae (Orchidaceae: Orchidoideae: Diurideae) to date, including all major lineages and over 80 taxa of *Corybas*, the largest genus in the group. With strong backbone resolution and support, we propose a revised generic classification scheme that minimizes taxonomic changes but accounts for the polyphyly of the genus *Acianthus*. *Corybas* is strongly supported as monophyletic, and several of the genera that have been proposed as segregates are clearly not natural groups. We examine several morphological characters that have been used to subdivide *Corybas*, and conclude that while some floral characters, such as the presence of spurs and some minor details of the lateral tepals, are highly conserved, other characters such as tepal length are more labile. Some remarkable cases of convergence in floral form are reported. Historical biogeographic reconstructions of the Acianthinae support an origin in Australia about 27 My. Much exchange between Australia and New Zealand and Australia and New Caledonia is apparent, primarily following the patterns expected from West Wind Drift, but only following the reemergence of these Zealandian fragments post drowning. Our reconstructions of the two genera (*Corybas* and *Stigmatodactylus*) that have dispersed beyond these southern landmasses support a fairly restrictive path of dispersal along the intervening islands to mainland Asia. Dispersal northwards began between 10-12 My, congruent with the timing of major collision between the Australian and Asian plates. *Corybas* dispersed directly from Australia to the Sunda shelf, at a time when New Guinea was primarily submerged, and only colonized New Guinea around 8 My coinciding with the onset of major uplift and mountain building. Despite the finding that many lineages are highly geographically conserved, and most dispersal seems to take place between adjacent landmasses, a few instances of remarkable long-distance dispersal are inferred.

Introduction

The orchid subtribe Acianthinae, with around 170 recognized species, was first recognized by Rudolf Schlechter (1926), and lies within the largely Australian and almost exclusively terrestrial tribe Diurideae. The group is primarily defined by vegetative features, in particular a single, thin, broadly heart-shaped leaf, and nearly spherical underground tuberoids. As treated by Chase et al. (2003) and the World Checklist of Selected Plant Families from Kew (WCSP, 2014), the subtribe Acianthinae contains five genera: *Acianthus* (~ 20 species), *Cyrtostylis* (~ 6 species), *Stigmatodactylus* (~ 10 species), *Townsonia* (~ 2 species), and *Corybas* (~ 135 species). Because of the widespread use of Kew's classification, we use these genera throughout the text, but refer to other classifications schemes as shown in Table 1.

Within the subtribe, floral morphology is more variable than vegetative morphology (Fig. 1). Most genera have multiple, open flowers, with narrow lateral petals and sepals and a labellum that is either broad and flat or somewhat keeled. The common names of several of these genera—e.g. "gnat orchid", "crane fly orchid", "mosquito orchid"—reflect not only their appearance (drab coloration and thin appendages), but also refer to their pollination by various small dipterans, especially in the families Sciaridae, Mycetophilidae, Empididae, and Anisopodidae (Pridgeon, 2001). Members of *Acianthus* and *Cyrtostylis* generally produce nectar. Members of *Stigmatodactylus* and *Townsonia* produce no nectar reward and rely at least partially on selfing, though many species of *Stigmatodactylus* have relatively large, strikingly colored flowers in pinks and blues, suggesting a generalized deceptive pollination system. *Corybas*, by far the largest genus, has only a single flower. The flowers of *Corybas* are complex and highly modified, with a trap-like structure formed by the greatly enlarged dorsal sepal and the labellum. They are frequently heavily marked with red or purple, and the labellum often shows

modifications such as a large, central mounded boss and/or a strongly fringed or toothed margin (Fig. 1). Like other non-selfing members of subtribe Acianthinae, *Corybas* is pollinated by small dipterans; unlike most other genera in the subtribe, however, *Corybas* lacks nectar-secreting tissues and is thought to be pollinated via their mimicry of mushroom brood sites of fungus gnats of family Mycetophilidae (Jones, 1971; Fuller, 1979; Pridgeon, 2001). We have observed *Exechia* females in *Corybas aconitiflorus*, unidentified mycetophilids in herbarium specimens of *C. longipedunculatus* and *C. aff. calophyllus*, as well as evidence of mycetophilid pollination in Bornean collections of *C. carinatus*. Pollination studies of the *Corybas trilobus* complex have yielded multiple collections of *Mycetophila* bearing pollinia (C. Lehnebach, pers. comm.). Studies of *Corybas cheesemanii* found no evidence for mycetophilid brood-site deception, but were unable to identify any pollinators whatsoever, coincident with high rates of selfing (Kelly et al., 2013), a common pattern in New Zealand orchids.

Gross floral form varies substantially within *Corybas,* and different shapes and forms have been used to characterize infrageneric groups as well as to define segregate genera (Clements et al., 2002; Jones et al., 2002). However, the consistency of the association of these morphological variants with phylogeny across a wider, more representative sample of *Corybas* species is yet to be demonstrated. Many species of *Corybas* have prominent filiform lateral sepals and petals. Elongated sepals, though not petals, are also found in *Acianthus caudatus* and *A. atepalus*. Such structures are frequently associated with sapromyophily (Faegri and van der Pijl 1979; Vogel and Martens 2000), and may serve as osmophores, tactile guides, or visual cues to potential pollinators. Scent is usually important in attracting pollinators in cases of brood-site deception (Urru et al., 2011; Jürgens et al., 2013); fungus-mimicking volatiles have been suspected but never documented in *Corybas.* In many taxa, however, filiform appendages are reduced to

vestigial structures. Another floral trait (unique to *Corybas*) possibly associated with sapromyophily is the possession of a pair of spurs or open auricles at the base of the labellum. The auricles might promote the release of fungus-like odors (Jones, 1971), or serve as "windows" that directly affect the movement of pollinators (Faegri and van der Piejl 1979). The spurs seen in many taxa are an enigma, given that *Corybas* secretes no nectar, but personal observations suggest that they may be important in positioning pollinators for effective removal of pollinia.

The great majority of diurid lineages are restricted to the southern landmasses of Australia, New Zealand, and New Caledonia. Occasionally a genus ranges into New Guinea, Java, or even mainland Asia, but this is usually due to recent dispersal of one or two widespread species (e.g. *Caladenia carnea*, *Microtis parviflora*, *Thelymitra javanica*). The Acianthinae is unusual in having two genera, *Stigmatodactylus* and *Corybas*, whose ranges not only extend well beyond the southern landmasses of Australia, New Zealand, and New Caledonia, but also have the majority of their diversity located outside of these areas. Like almost all orchids, members of the subtribe have tiny, wind-dispersed seeds. However, these plants are also very small in stature. *Corybas,* for instance—which has perhaps the widest distribution of any diurid—is typically only a few centimeters high and has to undergo dramatic peduncle elongation in order to even release its seeds above the forest floor boundary layer. *Corybas* and its relatives are often overlooked, and are rarely collected outside of Australia and New Zealand, but the existing collection records suggest high rates of endemism.

The aggregated distribution of the Acianthinae spans one of the most geologically complex and dynamic regions on Earth, including Australia, New Zealand, New Caledonia, New Guinea, and the Malay Archipelago. It ranges east to the Society Islands, south to several

Subantarctic islands, and north and west to Taiwan and Japan, mainland China, India, and the Himalayas (see inset in Fig. 10). The peculiar patterns of diversity evident on different islands in this region played an important role in shaping early ideas about evolution and biogeography, and it remains an area of particular interest for biogeographers (Lohman et al., 2011). This makes the *Acianthinae* a fascinating group in which to study historical biogeography.

Finally, the classification of taxa within the Acianthinae has been the subject of extensive debate within the botanical community. Table 1 presents a brief history of the changing concepts of the Acianthinae in the last century, and of various different generic and infrageneric delimitation schemes. Preliminary analyses of chloroplast sequences (Kores et al., 2001) and variation in morphology and nrDNA ITS sequences (Clements et al., 2002) showed that *Acianthus* was polyphyletic. *Cyrtostylis*, often considered part of *Acianthus*, was shown to be sister to *Corybas*, which had often been placed in its own subtribe. *Acianthus atepalus,* recently transferred to the monotypic genus *Spuracianthus* by Szlachetko and Margonska (2001), and *Townsonia* fell outside the main Acianthinae clade. Of the remaining *Acianthus* taxa, those with a single viscidium (*Acianthus* subgen. Univiscidiatus Kores) were found to be more closely related to *Stigmatodactylus* than to those taxa with two viscidia (*Acianthus* subgen. *Acianthus* Kores).

Jones et al. (2002) and Jones and Clements (2004) used these results to inform their revision of the Acianthinae, naming each strongly supported or highly divergent lineage as a distinct genus and ultimately dividing Acianthinae into 14 genera, while transferring *Townsonia* and *Spuracianthus* into newly erected subtribes. Jones et al. (2001) and Jones and Clements (2002a; b) took a similar approach to the hallmark Australian orchid genera *Caladenia* and *Pterostylis*, increasing the former from one to six genera, and the latter from one to 16 genera. These steps

are part of a broad trend in Australian orchid systematics in the last 15 years, in which the number of recognized species has risen by one-third, and the number of recognized genera has risen from 110 to 182. In fact, 45% of Australian orchid species have been placed in a new genus since 2000, raising questions about the desirability of these kinds of recent nomenclatural change (Hopper 2009).

Regardless of one's philosophy regarding "splitting" and "lumping" in taxonomy, the multiplication of genera in subtribe Acianthinae by Jones et al. (2002) was fraught for several reasons. First, their conclusions were based almost entirely on the nrDNA ITS sequence data of Clements et al. (2002). The ITS region is difficult to align across subtribe Acianthinae and especially tribe Diuridae, and has a number of peculiarities that can mislead phylogenetic inference (Álvarez and Wendel 2003). Second, few of the relationships within or among the major clades recognized were strongly supported. Third, while the study of Clements et al. (2002) was pioneering in many ways, it had important shortcomings in taxonomic sampling; only 27 of ca. 135 species of the large genus *Corybas* were included, and those that were included had a strong geographic bias toward Australia, New Zealand, and New Caledonia. Currently, New Guinea has 49 recognized taxa, more than one-third of all *Corybas* species, despite little collecting and taxonomic attention in recent years. Clements et al. (2002) were able to include only two New Guinea taxa, and none of the numerous species from west of Wallace's Line (Dransfield et al., 1986). Finally, even though *Corybas* in the broad sense was resolved as monophyletic by Clements et al. (2002), Jones et al. (2002) split it into eight genera. Two of these genera were monotypic: the New Zealand endemics *Corybas oblongus*, assigned to *Singularybas*, and *Corybas cryptanthus*, assigned to *Molloybas*. Two other larger genera were each represented by a single species in the Clements et al. (2002) phylogeny, and many other

species were assigned to these groups based solely on morphology, without confirmation that the supposed morphological synapomorphies were, in fact, phylogenetically informative.

Opponents of the new classification scheme by Jones et al. (2002) argued for the conservation of historically recognized, monophyletic genera (e.g., Hopper 2009), and eventually the consortium of major Australian herbaria rejected these taxonomic changes in favor of older, broader generic concepts (Entwisle and Weston, 2005). To make matters even more confusing, many native plant societies in Australia and New Zealand did accept the new genera. What was lost in the debate, however, were the taxonomic changes needed to create monophyletic taxa from polyphyletic *Acianthus* despite independent confirmation of this problem by Kores et al. (2001), and the need to determine broad-scale patterns of morphological evolution in a far better sampled *Corybas*.

To address these challenges, here we present an analysis of phylogenetic relationships in the Acianthinae based on an extensive sampling of taxa from throughout much of its range, using DNA sequences from five rapidly evolving loci in the chloroplast genome, as well as ITS, *PhyC*, and *Agt1* in the nuclear genome. Our objectives are to:

- Develop a robust molecular phylogeny for the Acianthinae, using nuclear and plastid data from a greatly expanded set of taxa representing several different regions and morphological groupings, and evaluate generic delimitation schemes within this framework;
- Evaluate broad patterns of relationships within *Corybas* and their association with aspects of floral morphology proposed as synapomorphies by Clements et al. (2002) and Jones et al. (2002); and

- Use this phylogeny to infer the pattern and timing of biogeographic diversification in the subtribe, in a region with a complex history of plate tectonics and island building, and notable biogeographic barriers for other groups.

Methods

Taxon sampling

Our collection efforts focused heavily on the large genus *Corybas*. We amassed tissue samples of roughly 80 *Corybas* taxa—roughly three times the number studied by Clements et al. (2002)—through extended expeditions to Australia, New Guinea, Borneo, peninsular Malaysia, and Taiwan by the senior author, and through additional archived collections from these areas as well as from mainland China, Java, New Caledonia, New Zealand, and several smaller islands in the southwest Pacific, spanning most of the range of *Corybas*. Our sampling excluded only the Himalayas, Sumatra, the Philippines, and the biogeographic region between the Sunda Shelf and New Guinea known as Wallacea (Lohman et al., 2011). These areas have not been sufficiently explored, but limited collection records suggest that *Corybas* is not particularly common or diverse there. Our sampling included all the genera recognized by Jones et al. (2002) and captures the vast majority of morphological variation in the group. Whenever possible, we initially sequenced two or more individuals per taxon, usually from multiple populations. This allowed us to confirm monophyly of species and to confirm field identification in the few cases where non-flowering material was used. For this paper, in most cases we have included only one accession per taxon in the figures; Table 2 lists the voucher specimens for all samples that were sequenced. Species delimitation of taxa and fine-scale relationships in particular clades are addressed elsewhere (Howard et al. 2014; Chapter 2). However, for two widespread Sundaland

species, *Corybas pictus* and *Corybas carinatus*, we include multiple samples from widely separated regions and different substrate types. We also include several undescribed taxa from New Guinea, which will be described in a separate publication.

We sampled all other major lineages in subtribe Acianthinae: *Townsonia*, *Stigmatodactylus*, the anomalous species *Acianthus atepalus*, the three major lineages of the primarily Australian *Acianthus* clade (*A. caudatus*, *A. exsertus*, *A. fornicatus*), both large-flowered and small-flowered forms of New Caledonian *Acianthus*, and both major lineages of *Cyrtostylis* (*C. robusta*, *C. reniformis*) (Kores et al., 2001; Clements et al., 2002). For outgroups, we included representatives from the four other species-rich diurid subtribes: *Eriochilus cucullatus* from Caladeniinae, *Microtis parviflora* from Prasophyllinae, *Diuris sulphurea* from Diurideae, and *Chiloglottis trapeziformis* from Thelymitrinae. Inclusion of these outgroups also permits us to use calibration points from previously published molecular dating analyses of the family Orchidaceae (Ramírez et al., 2007; Gustafsson et al., 2010).

DNA extraction and sequencing

We extracted genomic DNA from silica-dried tissue collected in the field or, less commonly, from cultivated plants or herbarium specimens. Whenever possible, we used tissue from flowering individuals or plants immediately adjacent to flowering individuals within the same clonal patch. Most members of Acianthinae spread vegetatively via stolonoid roots (Pridgeon and Chase, 1995) and many taxa (particularly in *Corybas*) can form large colonies of primarily sterile individuals. Tissues were ground using a TissueLyser (QIAGEN, Germantown, MD) at 30 Hz for 2 minutes. We extracted genomic DNA using either the EZNA Plant Mini Kit (Omega Bio-tek, Norcross, GA) or DNEasy Plant Mini Kit (QIAGEN, Germantown, MD). For

most herbarium specimens, we used the EZNA High Performance Plant Mini Kit (Omega Bio-tek, Norcross, GA), which employs a CTAB-based approach to obtain somewhat higher yields. DNA extracts of *Corybas mirabilis* from Vanuatu and *Corybas imperatorius* from Java were provided by the Kew DNA Bank.

To reconstruct phylogenetic relationships in Acianthinae, we used four highly variable, rapidly evolving intergenic spacers (*psbJ-petA*, *rps16-trnQ*, *psbD-trnT*, *trnL-trnF*) and one gene (*matK*) in the chloroplast genome, as well as nrDNA ITS, *PhyC* (phytochrome C), and *Agt1* (α-glucoside transporter 1) from the nuclear genome. We followed a supermatrix approach similar to that of Pirie et al. (2008), sequencing *matK* and *Agt1* for only a handful of accessions chosen to represent all major clades. This allowed us to reduce sequencing costs while improving backbone resolution and support though inclusion of the more conserved *matK* and *Agt1* regions. DNA amplification and sequencing followed Shaw et al. (2007) for the chloroplast spacers. For *matK*, we followed the protocol of Molvray et al. (2001) but developed our own internal primers. For ITS, we used the standard primers of White et al. (1990), but substituted the ITS5A primer developed by Downie and Katz-Downie (1996) to correct for the two base-pair substitutions specific to the angiosperms. For *PhyC*, we followed the PCR program of Russell et al. (2010), but pursued multiple rounds of iterative primer design to develop primers that would amplify across Acianthinae. For *Agt1*, we used the primer sequences and PCR programs of Li et al. (2008).

For herbarium specimens, amplification of *PhyC* and *Agt1* was generally impossible. Most specimens, however, yielded sufficiently high-quality DNA to permit sequencing of all other regions used in this study. In a few cases, however, one or more regions would not amplify. When necessary, *trnL-trnF* was amplified in two pieces using the primers TabC/TabD and

TabE/TabF (Taberlet et al., 1991). The ITS1 region was sometimes amplified separately when the entire ITS region failed to amplify, using the primers ITS5A and ITS2 (White et al., 1990, Downie and Katz-Downie, 1996); typically, the ITS2 region could not be amplified in these cases. The number of PCR cycles was increased from 30 to 35 when initial attempts at amplification resulted in very weak products. In two cases, *Corybas aristatus* and *Corybas aff. gastrosiphon* SLE583, we were only able to amplify a single fragment at the 5' end of the *trnL-trnF* region. Recognizing the potential for inadvertent amplification of contaminants in such cases, we checked these sequences carefully for uniqueness within the sequencing run and confirmed that their placements in the phylogeny was additionally supported by morphological characters. Prior to sequencing, PCR products were imaged on a 1.5% agarose gel. PCR reactions were cleaned using ExoSAP (Affymetrix, Santa Clara, CA), and sequencing reactions were cleaned using a Sephadex column in a Millipore plate (EMD Millipore, Billerica, MA). We sequenced all reactions using ABI BigDye technology (Life Technologies, Grand Island, NY) at the UW-Madison Biotech Center.

The *matK* and *trnL-trnF* sequences for *Corybas neocaledonicus*, *Cyrtostylis huegelli*, *Acianthus elegans*, *A. confusus*, *A. cymbalariifolius*, and *Stigmatodactylus sikokianus* were downloaded from NCBI GenBank, and the ITS sequence of *C. neocaledonicus* was obtained directly from Dr. Paul Kores.

Phylogenetic analyses

Sequences were assembled and edited using Geneious Pro v 5.4.3 (Biomatters Ltd., Auckland, New Zealand). Alignments were generated using various algorithms in Geneious,

typically ClustalX (Larkin et al., 2007) or the Consensus Align algorithm, and adjusted manually as necessary to account for the misplacement of indels using these algorithms. With ITS, *PhyC*, and *Agt1,* some sequences showed a small percentage of polymorphic bases. Polymorphisms were coded using the standard IUPAC nucleotide ambiguity codes.

The incongruence length difference (ILD) test (Farris et al., 1994), as implemented in PAUP 4.0d102 (Swofford, 2003), was used to test for congruence between nuclear and chloroplast data sets. However, given the tendency for this test to incorrectly reject the hypothesis of congruence in certain circumstances, including when different partitions follow different models of evolution (Darlu and Lecointre, 2002), we also relied on visual inspection of our gene trees for instances of hard incongruence (i.e., strongly supported, conflicting topologies). Despite several rounds of iteratively removing taxa whose placements conflicted, we were unable to identify a data set for which the ILD test did not indicate significantly different evolutionary histories for the separate partitions. On the other hand, there were very few lineages (a total of four) that showed strongly supported, conflicting placements in separate analyses of the different partitions. We thus analyzed our data sets both separately and combined. We focus on results from the combined analyses, pointing out these few areas of well-supported conflict.

We used maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) to reconstruct relationships in *Corybas* and subtribe Acianthinae. MP analyses were implemented in PAUP 4.0d102, using 100 repetitions of random sequence addition and TBR swapping to search for multiple islands of equally parsimonious trees. Parsimony bootstrap analyses used 100 repetitions each using TBR and 10 random starting trees. The monophyly of the genera *Corybas s.l.* and *Acianthus s.l.,* as well as various groups within *Corybas,* were evaluated using the Templeton (1983) test as implemented in PAUP.

Before beginning model-based ML and BI analyses, the optimal model for evolution was chosen using jModelTest (Posada, 2008). For both the concatenated chloroplast data and *Agt1*, a model of GTR+G was determined to be optimal using the Akaike Information Criterion (AIC). For the ITS and *PhyC* data sets, the GTR+G+I model was selected as optimal. Maximum likelihood and Bayesian analyses were run via the CIPRES science gateway V3.3 (Miller et al., 2010), using RAxML 7.2.7 (Stamatakis, 2014) and MrBayes 3.2.2 (Ronquist et al., 2012), respectively. RAxML ran 100 ML bootstrap replicates for each analysis. For the combined data, we ran our ML analysis using GARLI 2.01 (Zwickl, 2006; also implemented on the CIPRES gateway) allowing for separate models for each partition but used RAxML for bootstrap analyses. Two simultaneous runs were implemented for each MrBayes analysis, using four chains each, standard prior settings, and up to one million generations (sampled every 1000 generations and discarding the first 25% as burn-in). Analyses were automatically stopped when the two runs had sufficiently converged. Posterior probabilities for clades were estimated as the frequency of trees containing a particular clade in the post-burn-in fraction of sampled trees.

Molecular dating and ancestral areas reconstruction

Based on the evident variation in branch lengths in our phylogenies, we conducted a dating analysis using the Bayesian relaxed-clock approach implemented in BEAST v1.7.5. (Drummond et al., 2012) For calibration, we used dates estimated for major splits in tribe Diurideae: 32 My for the split of *Diuris* from *Chiloglottis*, 34 My for the split of *Microtis* from *Eriochilus*, and 39 My as the crown age of the Diurideae. There is no fossil record for Diurideae, so these values were estimates from a family-wide analysis by Gustaffson et al. (2010). We used only chloroplast data for this reconstruction. Dates were modeled as normally distributed parameters

with a mean as listed above and standard deviations of 3 My each (roughly 10% of the mean, in accordance with the confidence intervals shown in Gustaffson et al. (2010)). We used a Yule process (speciation only) tree prior, a random starting tree, an uncorrelated log normal relaxed clock, and a GTR+G model of evolution as selected in previous analyses. The analysis was run for 100 million generations (logged every 1000), the first 30 million of which were discarded as burn-in. Following the run, we confirmed that the estimated sample size (ESS) for all estimated parameters was over 200.

Ancestral biogeographic distributions were reconstructed using Lagrange (Ree and Smith, 2008). Table 3 shows the main dispersal probability matrix used. Probabilities were based on the proximity, connectivity, and emergence of landmasses as inferred from the geological and climatological history for Southeast Asia, Australasia, and the Pacific (Sanmartin and Ronquist, 2004; Hall, 2009; Lohman et al., 2011). Within each time slice, dispersal between adjacent (though not usually contiguous) landmasses was set at 0.9 and dispersal between non-adjacent landmasses was set at 0.1. Dispersal between individual continental areas and the smaller Pacific islands was set at 0.5, given that all neighboring continents might contribute to the Pacific flora, but that the individual island targets are small and distant from putative source areas. Because of the high rates of endemism in the Acianthinae, the highly fragmented range it occupies, and our relatively dense taxon sampling, the maximum number of areas that could be occupied by a single ancestral lineage was set to two. The results of this analysis were also compared to the results obtained using a matrix allowing equal probability of dispersal between all regions at all times. Ancestral distributions at nodes were reconstructed as the state with the highest probability. Partially shaded squares (Fig. 10) indicate that the most likely reconstruction had less than twice the probability of the next most likely reconstruction.

A few terminal taxa were coded as having distributions covering two areas. These codings represent taxa known to be closely related to the sampled taxa based on ITS sequences (Clements et al., 2002; Howard et al., 2014) but with disjunct distributions, typically representing recent dispersals between Australia and New Zealand or Australia and New Caledonia. Despite attempts to sample as much of the subtribe as possible and across as broad a geographical range as possible, there are several unsampled Acianthinae taxa that likely represent additional dispersal events—but because we have no molecular data regarding their placement, we could not include their distributions in the analyses. However, we feel that our reconstructions capture the major dispersal and radiation events in the subtribe.

Morphological character scoring and ancestral trait reconstruction

Five morphological characters were scored for ancestral state reconstruction on the concatenated chloroplast tree. Because the major clades in the rest of subtribe Acianthinae have been well characterized in regards to anatomy and morphology (Kores, 1995; Clements et al., 2002), we focused on the traits that have been used to delimit groups within *Corybas s.l.* The presence of spurs, open auricles, or neither, was determined from the literature and from direct observations of specimens. All observations agreed with the literature except in the case of *Corybas oblongus*, thought by van Royen (1983) to have spurs when in fact it has auricles—this trait was correctly scored by Clements et al. (2002).

The presence or absence of an elongated column, a prominently inflated labellum, and a large, shielding dorsal sepal—all somewhat subjective traits, though distinctive at their morphological extremes—were scored following Clements et al. (2002) and Jones et al. (2002),

except in cases where observations of field or herbarium specimens contradicted these, such as in the case of *Corybas ponapensis.*

Because flower size within a species often varies, but component parts remain proportional to each other, we scored the length of the lateral sepals relative to the dorsal sepals. While this is technically a continuous character, we scored it as discrete with 3 states: the lateral sepal less than half the length of the dorsal sepal, between half the length of the dorsal sepal and equal in length with the dorsal sepal, and longer than the dorsal sepal. Measurements were derived from the literature (using the midpoint value when a range was given), or assessed directly from specimen images in cases where the taxa are undescribed or where there was doubt about the representativeness of measurements provided in the literature.

Ancestral character states were reconstructed using Mesquite v2.75 (Maddison and Maddison, 2011), using the "trace character history" option and parsimony ancestral state reconstruction. The states for all characters were treated as unordered.

Results

Relationships in the Acianthinae

We present the combined, supermatrix-derived phylogeny for all eight regions sequenced in this study in Fig. 2, with alternate reconstructions for two cases of hard incongruence shown in Fig. 3.

Our analyses strongly support the monophyly of the Acianthinae (Fig. 2). A well-supported clade consisting of *Townsonia* and *Acianthus atepalus* (*Spuracianthus* Szlachetko) is shown to be sister to the rest of the subtribe. The primarily Australian clade of *Acianthus* (*Acianthus s.s.*) diverges subsequently, and a well-supported clade consisting of the two major

groups of primarily New Caledonian *Acianthus*, together with a monophyletic *Stigmatodactylus*, is then recovered as sister to a clade consisting of *Cyrtostylis* and *Corybas s.l.* Within the New Caledonian *Acianthus* (excluding *A. atepalus*), there are both large-flowered taxa with wide, multi-veined lateral petals (*Acianthopsis sens.* Jones et al.) and smaller flowered taxa with narrow, single veined lateral petals (*Acianthella* Jones and Clements). While our sampling of these groups was limited, we also recovered each of these groups as monophyletic, though not always with strong support. The genus *Acianthus* is clearly polyphyletic in our analyses, consisting of at least three different lineages. Templeton tests indicated that monophyly of the genus could be rejected as significantly less parsimonious ($p<0.001$ for both chloroplast and ITS data).

Corybas s.l. was recovered as monophyletic and sister to *Cyrtostylis* in all cases except for the *PhyC* analysis, though the sister relationship of *Cyrtostylis* and *Corybas* was not always strongly supported. Analyses of *PhyC* data placed one clade (*Anzybas* Jones et al.) as sister to *Cyrtostylis*. A Templeton test, however, indicated that the monophyly of *Corybas* could not be rejected ($p=0.3359$), and the combined data set strongly supported monophyly of *Corybas.*

Relationships within *Corybas*

The Anzybas clade (*Anzybas* Jones et al.) is strongly supported as monophyletic in all analyses. Its exact placement varies among the different analyses, though it is almost always among the first clades to diverge. In the combined data, it is strongly supported as sister to the rest of *Corybas*. The other early-diverging taxon showing variable placement is the anomalous New Zealand species *Corybas oblongus* (*Singularybas* Jones et al.). *Corybas oblongus* represents one of the only instances of hard incongruence in our analyses. In analyses of the ITS

data set, it is strongly supported as sister to the rest of the genus, while in analyses of the chloroplast data, it is strongly supported as sister to the spurred clade (Fig. 3).

Our analyses support the monophyly of both *Corysanthes* Jones et al. and *Nematoceras* Jones et al. The Corysanthes clade contains the least amount of genetic variation of any non-monotypic clade identified by Clement et al. (2002). The recent radiation of this group makes reconstructing evolutionary history difficult; this clade will be examined in greater detail in Chapter 2. The achlorophyllous *C. cryptanthus* (*Molloybas* Jones et al.) is clearly related to the Nematoceras and Corysanthes clades. Relationships among these three lineages, however, are not resolved by our analyses; most often the three lineages appeared as a trichotomy. Even in analyses of ITS and *Agt1*, neither of which is specifically related to photosynthesis, the exact relationships of *C. cryptanthus* to the Nematoceras and Corysanthes clades are unclear.

The remaining *Corybas* taxa fall into one large, well-supported clade. All of these taxa have a pair of enclosed spurs at the base of the labellum (discussed below). The taxa within this clade were variously assigned to *Corybas s.s.*, *Gastrosiphon*, and *Calcearia* by Jones et al. The monophyly of all three of these taxa was generally rejected with Templeton tests (*Corybas s.s.*: chloroplast $p<0.0001$, ITS $p<0.0001$, *PhyC* $p=0.0075$; *Gastrosiphon*: chloroplast $p=0.0029$, ITS $p=0.0018$, *PhyC* $p=0.4328$, *Calcearia*: chloroplast $p<0.0001$, ITS $p=0.0039$, *PhyC* $p=1.0$). While the *PhyC* results do not directly contradict the monophyly of *Gastrosiphon* and *Calcearia*, they also fail to provide support for their monophyly. In most analyses, *Calcearia* Jones et al. appears paraphyletic with respect to *Gastrosiphon* and some purported members of *Corybas s.s.* Most taxa assigned to *Corybas s.s.* do, in fact, share a recent common ancestor. However, there appear to have been at least two separate evolutions of a very similar morphology within the clade consisting primarily of taxa with *Gastrosiphon*-type morphology (see Figs. 8, 9).

Within the spurred clade as a whole, the first clade to diverge is a primarily Australian group, here referred to as the *C. aconitiflorus* clade. This clade is the subject of a separate paper (Howard et al. 2014). Our results largely echo their findings, though here we find the New Zealand taxon *C. cheesemanii* to be relatively genetically distinct, and confirm that *C. imperatorius* from Java is indeed a member of this group.

All the taxa sampled from New Guinea fall in a single clade, except for *Corybas epiphyticus,* which appears as sister to the large New Guinea clade plus the *C. pictus* clade. The remaining sampled New Guinea taxa fall into three distinct clades. One is a fairly distinctive morphological group not recognized by any previous authors, with lateral sepals that emerge on the dorsal side of the spurs, largely tubular flowers without an expanded labellum blade, a narrow, acute dorsal sepal, and often an elongated ovary and/or peduncle (Fig.1 T). This clade, containing *C. naviculisepalus*, also includes at least two taxa from outside of New Guinea, *C. solomonensis* from the nearby Solomon Islands and the newly described *C. puniceus* from Taiwan. A second large subclade consists primarily of taxa with *Gastrosiphon*-type morphology. Within this group, there appear to be at least two separate evolutions of *Corybas s.s.*-like morphologies. The clade also includes *Corybas ponapensis* from Micronesia, previously assigned to *Calcearia* Jones et al., though examination of better-preserved specimens reveals that it is actually similar to *C. gastrosiphon*. The third major group consists of a species complex, all related to *Corybas boridiensis*.

The large New Guinea clade is sister to the *Corybas pictus* clade. *C. pictus* itself, thought to be widespread on the Sunda shelf, does not appear monophyletic. Populations identified as *C. pictus* from different areas (Java, Sarawak, Sabah) are deeply divergent and are intermixed with other, morphologically distinct, taxa. The *C. pictus* accessions from Java are sister to the rest of

the group. Within Sabah, all accessions form a well-supported but highly diverse clade. Interestingly, there seems to be some phylogenetic signal of edaphic conditions. *Corybas serpentinus* is variously embedded in a grade of serpentine-associated *Corybas pictus* accessions, with the crown taxa consisting of non-serpentine collections from Mt. Kinabalu and Mt. Alab. Populations from Sarawak limestone (variously called *C. pictus* or *C. crenulatus*) are more closely related to the peninsular Malaysian *C. geminigibbus*, *C. calcicola*, and *C. selangorensis*—the first two of which are also limestone-affiliated.

Despite its strikingly similar coloration, *C. comptus* was not found to be part of the *C. pictus* group. Instead it was found to be part of a well-supported clade consisting of those Sundaland taxa with partially fused lateral petals (see Dransfield et al. 1986). This clade includes *C. carinatus*, the other "widespread" taxon on the Sunda shelf, which also appears to be non-monophyletic. *C. carinatus* from Java is more closely related to the peninsular Malaysian *C. calopeplos* than it is to *C. carinatus* from Sarawak, Sabah, and peninsular Malaysia, which together form a clade. ITS data strongly support the sister relationship of this clade to a mainland Asian clade, here represented by *C. himalaicus*, *C. sinii*, and *C. taliensis/shanlinshiensis*—all of these except *C. sinii* also have fused lateral petals. Chloroplast data, however, strongly support a clade consisting of the Sundaland taxa with fused petals and *C. ridleyanus* and *C. villosus*, both from peninsular Malaysia. This is the other main instance of hard incongruence in our data, and may represent a case of chloroplast capture—though additional nuclear data would be needed to test that hypothesis.

Corybas morphological evolution

The *Corybas* taxa with spurs (Corybas *s.s.*, *Gastrosiphon* Jones et al., and *Calcearia* Jones et al., together corresponding to van Royen's subgenus Corybas) form a clade, but those with open auricles do not (Templeton tests: p= 0.023 for chloroplast data, p=0.174 for ITS, p=0.001 for *PhyC*). This suggests a single origin of labellum spurs from an ancestor with open auricles (Fig. 4).

There appear to have been multiple transitions among short, medium, and long lateral sepals in the Acianthinae (Fig. 5). The genera forming a grade relative to *Corybas* all have lateral sepals that are roughly the same length as, or slightly shorter than, the dorsal sepal. However, in *Corybas,* both extremes for lateral sepal length can be found: very short (less than half the length of the dorsal sepal) and long (longer than the dorsal sepal, often substantially). Most transitions occur between adjacent categories. In groups such as the *C. naviculisepalus* clade, these transitions appear particularly frequent. There are also three fairly dramatic transitions within the genus, from long to very short lateral sepals—these correspond to the evolution of the Corysanthes clade, the *C. aconitiflorus* clade, and the primarily Gastrosiphon-type clade.

A curved, elongated column lacking a ventral pad is characteristic of most genera in the Acianthinae, but is present only in the Singularybas and Anzybas lineages of *Corybas*. These two lineages are, in all reconstructions, among the first to diverge—though never sister to one another—and this is likely a shared, ancestral character. A shorter column with a ventral pad appears to have evolved twice, based on the chloroplast analyses (Fig. 6), but because of the significant conflict regarding placement of *C. oblongus* (Singularybas) in the ITS data, we cannot be certain of this.

The markedly inflated "pouch" on the dorsal side of the labellum characteristic of *Gastrosiphon* Jones et al. does appear to have evolved only once, though it has been lost twice since then (Fig. 7). The strongly hooded dorsal sepal, the supposed distinguishing characteristic of *Corybas s.s.*, appears to have evolved at least three times (Fig. 8). The convergence in this trait is particularly striking between the *C. aconitiflorus* clade and the New Guinea taxa *C. aff. calophyllus* and *C. aff. simbuensis* (Fig. 9).

Biogeography and molecular dating

The BEAST analysis yielded an estimated crown age for the Acianthinae of around 27 My, in the mid-Oligocene, and for *Corybas* about 15 My, in the mid-Miocene (Fig. 10). Because of the time frame and geology of the region (see Discussion), we interpret essentially all range disjunctions in terms of dispersal. This makes it is difficult to interpret the few nodes that were reconstructed as occupying two regions simultaneously. Fortunately, most ancestral area reconstructions were more straightforward.

There were few differences between the differently parameterized Lagrange models. The model in which all dispersal events, at all times, had equal probability differed primarily in that *Stigmatodactylus* had similar likelihood of dispersal from New Guinea directly to mainland Asia as compared to having first dispersed from New Guinea to Sundaland. Ancestral areas at several nodes were somewhat ambiguous (state with highest likelihood less than twice the next highest likelihood), as indicated in Fig. 10 by half-colored boxes. Almost all of these ambiguities involve uncertainty about the exact placement reconstruction of nodes involving dispersal between Australia and New Zealand, and Australia and New Caledonia. The one major exception is the node leading to essentially all of the New Guinea, Sundaland, and mainland Asian *Corybas* taxa.

While the most likely reconstruction identifies this node as being present only in Sundaland, the second most likely reconstruction shows a split between Sundaland and Sundaland plus New Guinea.

Lineages are remarkable geographically constrained, though there are numerous inferred dispersals between Australia and New Zealand, and Australia and New Caledonia. The earliest dispersal to New Zealand occurred around 12.5 My, with subsequent dispersals around 9 My and 8 My, and four additional dispersals in the last one million years. If the root of the Acianthinae is reconstructed as being restricted to Australia, the most likely reconstruction in our analysis, the earliest dispersal to New Caledonia occurred around 19 My. Additional dispersal events between Australia and New Caledonia appear at around 4 My and 3 My, with one additional lineage recently shared between them.

Corybas does not appear in New Guinea until 8.5 My at the earliest. However, dispersal to New Guinea is reconstructed at around 12 My for *Stigmatodactylus*. There are at least five dispersals across Wallace's line, located between Sundaland and New Guinea. The earliest appears to have occurred around 11 My, from Australia to Sundaland, with no apparent involvement of New Guinea. Subsequently, around 7.5 My at the latest, *Corybas* appears in New Guinea. The most likely reconstruction shows one lineage present in *both* New Guinea and Sundaland until around 7 My, possibly suggesting a role for Wallacea as a bridge. The *C. pictus* clade, apparently present on both sides of Wallace's line based on collection records, represents at least one additional dispersal event. *Stigmatodactylus* likely dispersed from New Guinea to Sundaland sometime around 7 My, and a final dispersal event (not shown) appears to have occurred in the *C. aconitiflorus* clade, giving rise to *C. imperatorius* on Java.

There are also very recent dispersals from New Guinea to Vanuatu (~ 2 My), New Guinea to Pohnpei (~ 0.5 My), and, rather surprisingly, from New Guinea to Taiwan (around 1 My).

Discussion

Implications for taxonomy

The Acianthinae, as defined by Chase et al. (2003) and others, is strongly supported as monophyletic, and we find no indication that the Spuracianthinae and Townsoniinae should be recognized as separate subtribes. As suggested by previous analyses (Kores et al., 2001; Clements et al., 2002), the genus *Acianthus* is clearly polyphyletic. The simplest classification scheme for the Acianthinae recognizing morphologically distinct, monophyletic genera would consist of only five genera: *Corybas*, *Cyrtostylis*, *Stigmatodactylus*, *Acianthus*, and *Townsonia*. These are the same five genera currently recognized by the Kew checklist (WCSP, 2014). We propose that the myriad genera used to classify members of Acianthinae in Jones et al. (2002) be largely reduced to infrageneric status, and the genera *Acianthus*, *Stigmatodactylus*, and *Townsonia* re-circumscribed to incorporate closely related lineages. These proposed changes are summarized in the last column of Table 1.

While our analyses recover the monophyly of the *Acianthus* segregates proposed by Jones et al. 2002 and Jones and Clements 2004, support for *Acianthella* is weak. On the other hand, both *Acianthella* and *Acianthopsis* form a strongly supported clade with *Stigmatodactylus*. The most straightforward solution is to include these primarily New Caledonian species in *Stigmatodactylus.* All three groups have a single viscidium and appendages on the posterior margin of the stigma. Additional similarities can be found between *Stigmatodactylus* and the

large-flowered, New Caledonian taxa (*Acianthopsis sens.* Jones et al.), particularly the presence of a single, basal callus on the labellum and appendages on the ventral surface of the column.

The Jones et al. genus *Nemacianthus* is monotypic, restricted to *Acianthus caudatus.* While its morphology is distinctive and other authors have ascribed it special status (Kores, 1995), it is clearly part of the *Acianthus s.s.* clade. To avoid proliferation of monotypic genera (Humphreys and Linder, 2009), we recommend including *Acianthus caudatus* in the genus *Acianthus*.

Acianthus atepalus is strongly supported as sister to *Townsonia* in our analyses, in contrast to the molecular findings of Clements et al. (2002). These two lineages are separated by long branches, and *Townsonia* is distinctive in having a laxly branching rhizome with very small tubers in the axils. However, we propose sinking *Acianthus atepalus* into *Townsonia*, as they share several morphological features, including a winged column, reduced or absent lateral petals, a simple lip, and leaves with veins that do not anastomose at the tip.

Our analyses, in agreement with the previously published molecular analyses of Kores et al. (2001) and Clements et al. (2002), continue to support *Cyrtostylis* as a distinct, monophyletic genus, more closely related to *Corybas* than to any lineages of *Acianthus*. Its distinctive morphology is described in detail elsewhere (Jones and Clements, 1987).

Many taxonomists (e.g. Entwisle and Weston, 2005; Hopper, 2009; Humphreys and Linder, 2009) have argued for the need to maintain nomenclatural stability whenever possible, while still recognizing monophyletic, diagnosable groups. Based on those criteria, we recommend treating *Corybas s.l.* as a single genus. The genus is distinguished from the rest of the subtribe by a number of characters: 1) a single, large flower (except in rare cases); 2) a labellum that is folded inwards along the lateral margins—together with the enlarged dorsal

sepal, this forms a tube in most taxa; 3) a pair of either spurs or auricles at the base of the labellum; and 4) lateral petals that are rotated to the ventral surface of the flower during development, emerging in the small space between the lateral sepals. Unlike its sister clade *Cyrtostylis*, *Corybas* has only a single viscidium and pollinarium. It is also well supported as monophyletic.

Branch lengths might argue for the recognition of the Anzybas clade as a distinct genus. However, while the Anzybas clade is morphologically distinctive in many ways (Fig. 1 I), particularly within the context of Australia and New Zealand, many of its distinguishing characters are shared with the monotypic and variably placed Singularybas (see below). These differences seem minor in comparison to the features uniting the genus *Corybas s.l.* as a whole.

Morphological evolution in the genus *Corybas*

Most of the characters that distinguish the Anzybas and Singularybas lineages are likely symplesiomorphic, including a longer, curved column. Despite their similarities, these lineages are not closely related, though both appear to have been among the first lineages to diverge. Two additional traits associated with these lineages are also likely pleisiomorphic: a slight tendency to have a second flower (very rare), and the presence of a second, smaller bract subtending the flower (the only one in the rest of the genus). Because the exact reconstruction of the root of *Corybas* is somewhat ambiguous, it is not clear whether a shorter column evolved once or twice. Regardless, we hypothesize that a shorter column, in conjunction with a narrow, tubular base to the labellum, may facilitate more precise placement of pollinia on the thorax of pollinators.

Trait reconstruction shows that the ancestral *Corybas* had open auricles, and that spurs have arisen just once. As previously mentioned, the functions of both spurs and auricles are

unclear, though both are usually thought to be related to pollination. The sister group to *Corybas*, *Cyrtostylis*, has backwards-projecting labellum auricles that clasp the base of the column, and it may be that open auricles in *Corybas* were a natural consequence of in-folding of the labellum margins. While the transition to fully enclosed spurs occurred only once, closed spur taxa account for approximately 85% of the species diversity in *Corybas*. This suggests that spurs may have proven a more successful strategy for interacting with pollinators, though it may not be possible to disentangle morphology from environment in this case, as most of the spurred lineages are found in the tropics. Occasional "stuck" pollinators are often found with each of their forelegs in a spur (personal observation), and these structures could be important for positioning of pollinators in order to increase the success of pollinarium removal and deposition. Many *Corybas* have downward-pointing calli in the labellum tube, presumably to force pollinators down towards the column, and spurs may provide a surface that gnats can use for leverage when exiting the flowers. There is significant variation in the length of these structures, and, together with varying column and labellum tube lengths, this variation might allow for more precise adaptation to the size variation found in Mycetophilid pollinators.

While other Acianthinae genera have lateral sepals roughly equal in length to the prominent dorsal sepal, in *Corybas* we see two different, opposing trends in lateral sepal length (often associated with changes in the length of lateral petals as well): in some clades, the lateral tepals have become dramatically elongated, while in other lineages they are essentially vestigial. Because filiform appendages are strongly associated with fly pollination (Faegri and van der Pijl, 1979), it is tempting to suppose that the exaggeration and loss of these features is tied to changes in pollination syndrome. Studies of the genus *Tacca* (Zhang et al., 2005, 2011) have suggested that long, filiform structures may actually be unrelated to pollination, since several species with

them seem to be predominantly selfing. However, the presence of facultative selfing does not necessarily mean that selection by pollinators does not influence floral form (Fenster and Marten-Rodriguez, 2007).

To the extent that there are data on pollination, *Corybas* with very short tepals appear to have similar pollination systems to those with very long tepals. The most striking difference between the two extremes is found between the species in Australia and those in New Zealand, particularly in the closely related Corysanthes and Nematoceras clade. All Australian taxa have very short lateral tepals, except in the relatively rare Anzybas clade (generally found on wetter soils, sometimes in swamps)—whereas all taxa in New Zealand, except the most recent dispersals, have long tepals. Because of the development and expansion of aridity in Australia, particularly since the onset of the Pleistocene (Crisp and Cook, 2007), and the maintenance of cooler, wet conditions in at least some parts of New Zealand, it is possible that reduction of tepals could, in some cases, be tied to changes in climate. Long, thin tepals with a lot of evaporative surface area would be unlikely to persist in drier climates, as they may put species with them at a disadvantage due to increased water loss.

On the other hand, in New Guinea, species in the Gastrosiphon clade and *Corybas "sp1"* from the *C. naviculisepalus* clade, which have very short lateral tepals, are found in wet, cool, relatively high elevation areas. If anything, in New Guinea, taxa with longer lateral tepals tend to be found at lower elevations in habitats that are perhaps slightly warmer, drier, and/or more open (personal observation). However, the higher elevation taxa in New Guinea are found at much higher elevations (up to 3700 m) than anywhere else in the range of *Corybas*. This presents an interesting possibility—that long tepals are optimal only in an intermediate level of humidity. Certainly very cold, damp air would be less optimal for the dispersal of volatiles (Peñuelas,

2008)—if indeed the filiform structures are related to scent production—perhaps rendering them useless under these conditions. Again, these hypotheses require more explicit testing.

The reduction of lateral tepals seems to be associated with the development of other features, such as a prominent, broad dorsal sepal—especially in those with a *Corybas aconitiflorus*-like form. This suggests that there may exist distinct strategies for attracting pollinators—one strategy involving filiform appendages, and another involving more obvious visual mimicry. In species lacking long filiform appendages, is not uncommon for the dorsal sepal to be distinctly ridged, lined, or bright white; from above, the dorsal sepal may strongly resemble a basidiocarp even to the human eye. There also appears to be a third strategy, the formation of kettle-like flowers with a ventrally inflated labellum that superficially resemble other fungus gnat brood-site deceptive flowers such as *Arisaema* (Vogel and Martens, 2000). This strategy appears to have arisen only once, with two reversals to the prominent dorsal sepal strategy. A few taxa do not seem to fit neatly into any of these groups (*Corybas "sp1"*), or in some cases fit multiple groups—for instance, *C. koresii* has an inflated labellum, a prominent dorsal sepal, and elongated tepals.

Biogeography

Our results suggest a crown date for the Acianthinae around 27 My (mid-Oligocene), with an origin in Australia. At that time, the ancient continent of Gondwana had long since broken apart, Africa having split off at around 135 My, Zealandia at around 80 My, and Australia having separated from Antarctica and South America between 35-52 My (Sanmartin and Ronquist, 2004) . Both New Zealand and New Caledonia, as well as many of the landmasses

in the Indo Australian archipelago, were either submerged below the ocean, or had only recently emerged (Hall, 2001, 2009; Sanmartin and Ronquist, 2004; Trewick et al., 2007; Grandcolas et al., 2008). Because of this history it is unlikely that vicariance events, especially at the continental level, play a substantial role in the evolutionary history of the Acianthinae. Indeed, this is the story that is emerging for much of the southern hemisphere flora (Swenson et al., 2001; Sanmartin and Ronquist, 2004; Bartish et al., 2005; Cook and Crisp, 2005; Perrie and Brownsey, 2007; Trewick et al., 2007; Keppel et al., 2009).

While dispersal is undoubtedly the primary force driving distributional patterns in the Acianthinae, additional biogeographic questions remain regarding: the relative importance of wind currents versus proximity in determining dispersal patterns, the predictability of dispersal trajectories, and the relative importance of dispersal versus *in situ* diversification for generating observed patterns of morphological diversity.

While wind patterns are known to be important determinants of distributions in wind-dispersed plants, Muñoz et al. (2004) also demonstrated the importance of proximity *per se* in determining dispersal patterns in ferns. As orchid seeds are about an order of magnitude larger than most fern spores and have similar problems maintaining long-term viability, we might expect proximity to be an important factor.

The opening of the Drake passage between Antarctica and South America around 30 My resulted in the establishment of modern wind patterns, especially the strong Westerlies (Sanmartin and Ronquist, 2004) that are thought to be responsible for a great deal of long-distance dispersal (LDD) in the southern hemisphere (Muñoz et al., 2004; Sanmartin and Ronquist, 2004; Cook and Crisp, 2005; Queiroz, 2005; Perrie and Brownsey, 2007; Sanmartín et al., 2007), a phenomenon known as West Wind Drift. This wind pattern has played an obvious

role in dispersing several Acianthinae lineages: there appear to have been eight dispersals from Australia to New Zealand and four from Australia to New Caledonia. Several of these events appear to have happened in the last million years, indicating that this process is still very much ongoing. Importantly, none of these inferred dispersal events predate the estimated re-emergence times of New Caledonia (37 Ma) and New Zealand (23 Ma) following their presumed submergences.

Given the relatively large expanse of ocean between Australia and New Zealand (~ 4000 km) compared to the narrow Torres Straight separating Australia and New Guinea (which was exposed land during glacial maxima), it is remarkable that there are no clear cases of Australia-New Guinea dispersal in the Acianthinae. This is certainly suggestive of the greater importance of wind connectivity as opposed to physical proximity.

The Westerlies are particularly strong, directional winds, and the cold air may help preserve seed viability during transfer. However, within the Malesian region (the Malay peninsula, the Malay and Philippine archipelagos, Wallacea and New Guinea), wind patterns are complicated, vary seasonally, and generally do not strongly contradict patterns of adjacency. While the lack of dispersal between Australia and New Guinea remains an enigma, this does not rule out the importance of proximity as a factor determining range expansion. Researchers have reconstructed the biogeographical history of numerous animal and plant (including some wind-dispersed) groups in the Malesian region (reviewed in Turner et al., 2001; Van Welzen et al., 2003, 2011), and most taxa do seem to follow a fairly predictable dispersal trajectory through the archipelago, with adjacent areas showing similar flora and fauna. Van Welzen (2003) suggested this was likely due to the more or less linear series of landmasses, restricting dispersal to very few possible routes.

Only two lineages in the Acianthinae have dispersed through this region. The earliest dispersal into Malesia appears to be *Stigmatodactylus,* as early as 12 My. It is not clear from our reconstruction whether this occurred via Australia or New Caledonia. At 12 My most of New Guinea was largely still below water, and much of the mountain building on the island has likely occurred in the last 5 My (Hall, 2009). It is possible that that *Stigmatodactylus* persisted in northeastern Australia or New Caledonia for some time before dispersing to New Guinea, and subsequently went extinct in one of these regions. If so, the colonization of New Guinea may have occurred closer to 7 My. The Australian mesic biome in particular has faced substantial contraction and extinction (Byrne et al., 2011), and no doubt this hampers our ability to accurately reconstruct dispersal history in this group. Additional inferred dispersal events in *Stigmatodactylus*, from New Guinea to the Sunda shelf to eastern Asia, follow the typical patterns discussed by Van Welzen et al. (2003, 2011).

For *Corybas,* the analyses presented here suggest *direct* dispersal from Australia to the Sunda shelf around 11 My. Our findings suggest a later colonization of New Guinea, around 7 My, from the Sunda shelf (likely via Wallacea). This result was surprising, and runs contrary to expectations that New Guinea that served as a gateway to SE Asia. New Guinea was still largely submerged around 11 My, and there are seasonal wind currents that run from the western coast of Australia towards Java. The *Corybas* flora of Java has a few interesting taxa which suggest that it might have served as a gateway to the Sunda shelf. We have evidence for a much more recent dispersal from Australia to Java, in the form of Java's *C. imperatorius,* which forms part of the mostly Australian *C. aconitiflorus* complex. However, at 11 My there were only isolated volcanic islands in the area that is now Java; the only extensive area of uplands was found in northern Borneo (Hall, 2009).

Corybas is not the only diurid genus that seems to have bypassed New Guinea. *Microtis unifolia,* found in eastern Australia and New Zealand, has a range that also extends via Java through Southeast Asia and into eastern continental Asia, but has never been reported in New Guinea. There is a possibility for "stepping-stone" dispersal between Australia and Java in this case, as *Microtis unifolia* has been found on the island of Timor (Silveira et al., 2008), along with several other diurid genera—though not, to our knowledge, *Corybas*. Timor was uplifted within the last 5 My and other members of the lesser Sundas are relatively young (Hall, 2009)—so this cannot account for the earlier inferred dispersal. More problematic is the lack of appropriate climate for most terrestrial orchids in northwestern Australia—only a few species of *Calochilus* and *Arthrochilus* can be found there today—and the *Corybas* and *Microtis* taxa present in southwestern Australia are completely unrelated to those lineages that have colonized the Sunda shelf. One possibility is that, during wetter periods of Australia's history, there may have been additional diurid lineages—including spurred *Corybas*—in the Kimberley region or Arnhem Land, that have since gone extinct.

Once in Sundaland, Corybas seems to have rapidly dispersed to mainland Asia, but we have no evidence of subsequent exchange between those regions, reinforcing the idea that the Kangar-Pattani line between Thailand and Malaysia is a major biogeographic barrier in plants (Woodruff, 2010). Within mainland Asia, however, individual taxa can span wide geographic regions; *C. taliensis* and *C. sinii* range from all the way from western China to Taiwan. Dispersal from mainland China to Taiwan is well documented in other plant groups and is in accordance with prevailing wind patterns (Wei et al., 2010). Cooler winds may promote seed viability during long distance dispersal events in this region, as in the south.

Within the tropics, long distance dispersal in *Corybas* is substantially less common, and lineages are highly geographically constrained. The case of New Guinea is particularly striking. New Guinea has the highest *Corybas* diversity in the region with a large number of morphological forms—yet all but one New Guinean species sampled resulted from a single colonization event dated to roughly 5 My. This rapid speciation and morphological diversification has likely been facilitated by extensive mountain building in New Guinea over the last 5 million years. A similar process of rapid speciation driven by mountain building has been inferred in the Andes (Young et al., 2002).

Local diversification also appears to be an important process on the relatively more stable Sunda shelf. Though we treat the Sundaland as a single biogeographical area, *Corybas* and *Stigmatodactylus* are restricted to patches of montane forest surrounded by unsuitable lowland habitat. Dispersal among these areas may have increased during glacial maxima due to cooling and expansion of montane forests (Wang et al., 2009). However, it is telling that even the supposed “widespread" Sundaland taxa show deep genetic divergences among disjunct areas. *C. pictus* populations from Java, for instance, are very genetically distinct from *C. pictus* populations in Borneo and peninsular Malaysia.

The presence of *Corybas* on young volcanic archipelagos, such those found in Vanuatu, Micronesia, and the Society Islands, indicates that long distance dispersal certainly does take place in the tropics. New Guinea, despite its high rates of local endemism, appears to have been a major source for these areas, in accordance with the literature (Keppel et al., 2009). The apparent direct dispersal of the undescribed *Corybas “sp2”* from New Guinea to Taiwan, however, is quite surprising, and serves as a reminder of the importance of occasional stochastic events.

Literature Cited

BARTISH, I., U. SWENSON, J. MUNZINGER, AND A.A. ANDERBERG. 2005. Phylogenetic relationships among New Caledonian Sapotaceae (Ericales): molecular evidence for generic polyphyly and repeated dispersal. *American Journal of Botany* 92: 667–673.

BYRNE, M., D.A. STEANE, L. JOSEPH, D.K. YEATES, G.J. JORDAN, D. CRAYN, K. APLIN, ET AL. 2011. Decline of a biome: evolution, contraction, fragmentation, extinction and invasion of the Australian mesic zone biota. *Journal of Biogeography* 38: 1635–1656.

CLEMENTS, M.A., D.L. JONES, I.K. SHARMA, M.E. NIGHTINGALE, M.J. GARRATT, K.. FITZGERALD, A.M. MACKENZIE, AND B.P.J. MOLLOY. 2002. Phylogenetics of Diurideae (Orchidaceae) based on the internal transcribed spacer (ITS) regions of nuclear ribosomal DNA. *Lindleyana* 17: 135–171.

COOK, L.G., AND M.D. CRISP. 2005. Not so ancient: the extant crown group of Nothofagus represents a post-Gondwanan radiation. *Proceedings of the Royal Society B: Biological Sciences* 272: 2535–2544.

CRISP, M.D., AND L.G. COOK. 2007. A congruent molecular signature of vicariance across multiple plant lineages. *Molecular Phylogenetics and Evolution* 43: 1106–1117.

DARLU, P., AND G. LECOINTRE. 2002. When does the incongruence length difference test fail? *Molecular Biology and Evolution* 19: 432–437.

DOWNIE, S.R., AND D.S. KATZ-DOWNIE. 1996. A molecular phylogeny of Apiaceae subfamily Apioideae: evidence from nuclear ribosomal DNA internal transcribed spacer sequences. *American Journal of Botany* 83: 234–251.

DRANSFIELD, J., J.B. COMBER, AND G. SMITH. 1986. A Synopsis of Corybas (Orchidaceae) in West Malesia and Asia. *Kew Bulletin* 41: 575–613.

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.

ENTWISLE, T.J., AND P.H. WESTON. 2005. Majority rules, when systematists disagree. *Australian Systematic Botany* 18: 1–6.

FAEGRI, K., AND L. VAN DER PIJL. 1979. The principles of pollination ecology. Pergamon Press.

FARRIS, J.S., M. KÄLLERSJÖ, A.G. KLUGE, AND C. BULT. 1994. Testing Significance of Incongruence. *Cladistics* 10: 315–319.

FENSTER, C.B., AND S. MARTEN-RODRIGUEZ. 2007. Reproductive assurance and the evolution of pollination specialization. *International Journal of Plant Sciences* 168: 215–228.

FULLER, G. 1979. Pollination of Corybas macranthus (Hook. f.) Rechb. f. *Orchids in New Zealand*37–39.

GRANDCOLAS, P., J. MURIENNE, T. ROBILLARD, L. DESUTTER-GRANDCOLAS, H. JOURDAN, E. GUILBERT, AND L. DEHARVENG. 2008. New Caledonia: a very old Darwinian island? *Philosophical Transactions of the Royal Society B: Biological Sciences* 363: 3309–3317.

GUSTAFSSON, A., C. 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.

HALL, R. 2001. Cenozoic reconstructions of SE Asia and the SW Pacific: changing patterns of land and sea. *In* Faunal and Floral Migration and Evolution in SE Asia-Australasia, CRC Press.

HALL, R. 2009. Southeast Asia's changing palaeogeography. *Blumea - Biodiversity, Evolution and Biogeography of Plants* 54: 148–161.

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

HOWARD, C., S.P. LYON, A.K. VARAN, J.T. MILLER, AND M.A. CLEMENTS. 2014. Lack of genetic variation in the Australian Helmet Orchids questions the validity of the threatened species, Corybas dowlingii. *Australian Systematic Botany* in review: .

HUMPHREYS, A.M., AND H.P. LINDER. 2009. Concept versus data in delimitation of plant genera. *Taxon* 58: 1054–1074.

JONES, D.L. 1971. The pollination of Corybas diemenicus (Lindl.) Rupp et Nicholls ex Rupp. f. Jones. *Orchadian* 87: 372–374.

JONES, D.L., AND M.A. CLEMENTS. 2002a. A review of Pterostylis (Orchidaceae): 1. A reassessment of Pterostylis R. Br. (Orchidaceae). *Australian Orchid Research* 4: 3–63.

JONES, D.L., AND M.A. CLEMENTS. 2002b. A review of Pterostylis (Orchidaceae): 2. A new classification of Pterostylis R. Br. (Orchidaceae). *Australian Orchid Research* 4: 64–124.

JONES, D.L., AND M.A. CLEMENTS. 2004. Acianthella. *The Orchadian* 14: 331.

JONES, D.L., AND M.A. CLEMENTS. 1987. Reinstatement of the genus Cyrtostylis R.Br. and its relationship with Acianthus R.Br. (Orchidaceae). *Lindleyana* 2: 156–160.

JONES, D.L., M.A. CLEMENTS, I.K. SHARMA, AND A.M. MACKENZIE. 2001. A new classification of Caladenia R. Br. (Orchidaceae). *The Orchadian* 13: 388–419.

JONES, D.L., M.A. CLEMENTS, I.K. SHARMA, A.M. MACKENZIE, AND B.P.J. MOLLOY. 2002. Nomenclature notes arising from studies intro the Tribe Diurideae (Orchidaceae). *Orchadian* 13: 437–468, 502–503.

JÜRGENS, A., S.-L. WEE, A. SHUTTLEWORTH, AND S.D. JOHNSON. 2013. Chemical mimicry of insect oviposition sites: a global analysis of convergence in angiosperms. *Ecology Letters* 16: 1157–1167.

KELLY, M., R. TOFT, AND A. GASKETT. 2013. Pollination and insect visitors to the putatively brood-site deceptive endemic spurred helmet orchid, Corybas cheesemanii. *New Zealand Journal of Botany* 51: 155–167.

KEPPEL, G., A.J. LOWE, AND H.P. POSSINGHAM. 2009. Changing perspectives on the biogeography of the tropical South Pacific: influences of dispersal, vicariance and extinction. *Journal of Biogeography* 36: 1035–1054.

KORES, P., M. MOLVRAY, P.H. WESTON, S.D. HOPPER, A.P. BROWN, K.M. CAMERON, AND M.W. CHASE. 2001. A phylogenetic analysis of Diurideae (Orchidaceae) based on plastid DNA sequence data. *American Journal of Botany* 88: 1903–1914.

KORES, P.J. 1995. A systematic study of the genus Acianthus (Orchidaceae: Diurideae). *Allertonia* 7: 87–220.

LARKIN, M.A., G. BLACKSHIELDS, N.P. BROWN, R. CHENNA, P.A. MCGETTIGAN, H. MCWILLIAM, F. VALENTIN, ET AL. 2007. Clustal W and Clustal X version 2.0. *Bioinformatics* 23: 2947–2948.

LI, M., J. WUNDER, G. BISSOLI, E. SCARPONI, S. GAZZANI, E. BARBARO, H. SAEDLER, AND C. VAROTTO. 2008. Development of COS genes as universally amplifiable markers for phylogenetic reconstructions of closely related plant species. *Cladistics* 24: 727–745.

LOHMAN, D.J., M. DE BRUYN, T. PAGE, K. VON RINTELEN, R. HALL, P.K.L. NG, H.-T. SHIH, ET AL. 2011. Biogeography of the Indo-Australian Archipelago. *Annual Review of Ecology, Evolution, and Systematics* 42: 205–226.

MADDISON, W.P., AND D.R. MADDISON. 2011. Mesquite: a modular system for evolutionary analysis. Available at: http://mesquiteproject.org.

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 Oleans, LA, 1–8.

MOLVRAY, M., P. KORES, AND M.W. CHASE. 2001. Polyphyly of myco-heterotrophic orchids and functional influences of floral and molecular characters. *In* Monocots: systematics and evolution, CSIRO Publishing, Collingwood, Victoria, Australia.

MUÑOZ, J., Á.M. FELICÍSIMO, F. CABEZAS, A.R. BURGAZ, AND I. MARTÍNEZ. 2004. Wind as a long-distance dispersal vehicle in the southern hemisphere. *Science* 304: 1144–1147.

PEÑUELAS, J. 2008. An increasingly scented world. *New Phytologist* 180: 735–738.

PERRIE, L., AND P. BROWNSEY. 2007. Molecular evidence for long-distance dispersal in the New Zealand pteridophyte flora. *Journal of Biogeography* 34: 2028–2038.

PIRIE, M.D., A.M. HUMPHREYS, C. GALLEY, N.P. BARKER, G.A. VERBOOM, D. ORLOVICH, S.J. DRAFFIN, ET AL. 2008. A novel supermatrix approach improves resolution of phylogenetic relationships in a comprehensive sample of danthonioid grasses. *Molecular Phylogenetics and Evolution* 48: 1106–1119.

POSADA, D. 2008. jModelTest: Phylogenetic Model Averaging. *Molecular Biology and Evolution* 25: 1253–1256.

PRIDGEON, A.M. 2001. Acianthinae. *Genera orchidacearum* 2: 64–83.

PRIDGEON, A.M., AND M.W. CHASE. 1995. Subterranean axes in tribe Diurideae (Orchidaceae): Morphology, anatomy, and systematic significance. *American Journal of Botany* 82: 1473–1495.

QUEIROZ, A. 2005. The resurrection of oceanic dispersal in historical biogeography. *Trends in Ecology & Evolution* 20: 68–73.

RAMÍREZ, S., 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.

REE, R., AND S.A. SMITH. 2008. Maximum likelihood inference of geographic range evolution by dispersal, local extinction, and cladogenesis. *Systematic Biology* 57: 4–14.

RONQUIST, F., M. TESLENKO, P. VAN DER MARK, D.L. AYRES, A. DARLING, S. HÖHNA, B. LARGET, ET AL. 2012. MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* 61: 539–542.

VAN ROYEN, P. 1983. Corybas (Orchidaceae) in its eastern areas. *Phanerogamarum monographiae* 16: 1–175.

RUSSELL, A., R. SAMUEL, 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.

SANMARTIN, I., AND F. RONQUIST. 2004. Southern hemisphere biogeography inferred by event-based models: plant versus animal patterns. *Systematic Biology* 53: 216.

SANMARTÍN, I., L. WANNTORP, AND R.C. WINKWORTH. 2007. West Wind Drift revisited: testing for directional dispersal in the Southern Hemisphere using event-based tree fitting. *Journal of Biogeography* 34: 398–416.

SILVEIRA, P., A. SCHUITEMAN, J.J. VERMEULEN, A.J. SOUSA, H. SILVA, J. PAIVA, AND E.D. DE VOGEL. 2008. The orchids of Timor: checklist and conservation status. *Botanical Journal of the Linnean Society* 157: 197–215.

STAMATAKIS, A. 2014. RAxML Version 8: A tool for Phylogenetic Analysis and Post-Analysis of Large Phylogenies. *Bioinformatics*btu033.

SWENSON, U., A. BACKLUND, S. MCLOUGHLIN, AND R. HILL. 2001. Nothofagus biogeography revisited with special emphasis on the enigmatic distribution of subgenus Brassospora in New Caledonia. *Cladistics* 17: 28–47.

SWOFFORD, D. 2003. PAUP*: phylogenetic analysis using parsimony, version 4.0b10.

SZLACHETKO, D.L., AND H.B. MARGONSKA. 2001. Genera et species Orchidalium. 2. *Polish Botanical Journal* 46: 27–29.

TABERLET, P., L. GIELLY, G. PAUTOU, AND J. BOUVET. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology* 17: 1105–1109.

TEMPLETON, A.R. 1983. Phylogenetic inference from restriction endonuclease cleavage site maps with particular reference to the evolution of humans and the apes. *Evolution* 37: 221–244.

TREWICK, S.A., A.M. PATERSON, AND H.J. CAMPBELL. 2007. Hello New Zealand. *Journal of Biogeography* 34: 1–6.

TURNER, H., P. HOVENKAMP, AND P.V. WELZEN. 2001. Biogeography of Southeast Asia and the west Pacific. *Journal of Biogeography* 28: 217–230.

URRU, I., M.C. STENSMYR, AND B.S. HANSSON. 2011. Pollination by brood-site deception. *Phytochemistry* 72: 1655–1666.

VOGEL, S., AND J. MARTENS. 2000. A survey of the function of the lethal kettle traps of Arisaema (Araceae), with records of pollinating fungus gnats from Nepal. *Botanical Journal of the Linnean Society* 133: 61–100.

WANG, X., X. SUN, P. WANG, AND K. STATTEGGER. 2009. Vegetation on the Sunda Shelf, South China Sea, during the Last Glacial Maximum. *Palaeogeography, Palaeoclimatology, Palaeoecology* 278: 88–97.

WCSP. 2014. World Checklist of Selected Plant Families. Facilitated by the Royal Botanic Gardens, Kew. Available at: http://apps.kew.org/wcsp/ [Accessed April 5, 2014].

WEI, X.-X., Z.-Y. YANG, Y. LI, AND X.-Q. WANG. 2010. Molecular phylogeny and biogeography of Pseudotsuga (Pinaceae): Insights into the floristic relationship between Taiwan and its adjacent areas. *Molecular Phylogenetics and Evolution* 55: 776–785.

VAN WELZEN, P.C., J.A. PARNELL, AND J.F. SLIK. 2011. Wallace's Line and plant distributions: two or three phytogeographical areas and where to group Java? *Biological Journal of the Linnean Society* 103: 531–545.

VAN WELZEN, P.C., H. TURNER, AND P. HOVENKAMP. 2003. Historical biogeography of Southeast Asia and the West Pacific, or the generality of unrooted area networks as historical biogeographic hypotheses. *Journal of Biogeography* 30: 181–192.

WHITE, T.J., T.D. BRUNS, S. LEE, AND J.W. TAYLOR. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR-Protocols : A guide to methods and applications*.

WOODRUFF, D.S. 2010. Biogeography and conservation in Southeast Asia: how 2.7 million years of repeated environmental fluctuations affect today's patterns and the future of the remaining refugial-phase biodiversity. *Biodiversity and Conservation* 19: 919–941.

YOUNG, K.R., C.U. ULLOA, J.L. LUTEYN, AND S. KNAPP. 2002. Plant evolution and endemism in Andean South America: An introduction. *The Botanical Review* 68: 4–21.

ZHANG, L., S.C. BARRETT, J.Y. GAO, L.J. CHEN, W.W. COLE, Y. LIU, Z.L. BAI, AND Q.J. LI. 2005. Predicting mating patterns from pollination syndromes: the case of "sapromyophily" in Tacca chantrieri (Taccaceae). *American Journal of Botany* 92: 517–524.

ZHANG, L., H.-T. LI, L.-M. GAO, J.-B. YANG, D.-Z. LI, C.H. CANNON, J. CHEN, AND Q.-J. LI. 2011. Phylogeny and evolution of bracts and bracteoles in Tacca (Dioscoreaceae). *Journal of Integrative Plant Biology* 53: 901–911.

ZWICKL, D.J. 2006. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. Ph.D. dissertation. The University of Texas at Austin.

Figures and Tables

Figure 1. Major lineages in the subtribe Acianthinae. **A.** *Acianthus (Spuracianthus) atepalus* (photo by H. Vandrot); **B.** *Townsonia viridis* (photo by M. Clements); **C.** *Acianthus (Nemacianthus) caudatus*; **D.** *Acianthus fornicatus*; **E.** *Stigmatodactylus croftianus*; **F.** *Acianthus (Acianthopsis) cymbalariifolius* (photo by M. Clements); **G.** *Acianthus (Acianthella) confusus* (photo by M. Clements); **H.** *Cyrtostylis robusta*; **I.** *Corybas (Anzybas) unguiculatus*; **J.** *Corybas (Singularybas) oblongus* (photo by M. Pratt); **K.** *Corybas (Nematoceras) acuminatus* (photo by M. Pratt); **L.** *Corybas (Corysanthes) recurvus*; **M.** *Corybas (Molloybas) cryptanthus* (photo by A. Brochart, https://orchid.unibas.ch); **N.** *Corybas dowlingii*; **O.** *Corybas gastrosiphon (Gastrosiphon schlechteri)*; **P.** *Corybas (Calcearia) carinatus* (Sabah); **Q.** *Corybas (Calcearia) shanlinshiensis/taliensis*; **R.** *Corybas (Calcearia) ridleyanus*; **S**. *Corybas (Calcearia) pictus* (Mt. Kinabalu, Sabah); **T.** *Corybas (Calcearia) amungwiwensis.* Unless otherwise noted, photos by S. Lyon. All other images used with permission.

Table 1. Classification history of the Acianthinae. Heavy black lines show varying concepts of the Acianthinae. Recommendations for revised genera shown in last column.

Table 2. Collection, voucher, and sequence information for all accessions included in this study. An "x" indicates sequence successfully obtained for this region and included in this study, but not yet submitted to GenBank.

Table 3. Primary dispersal matrix used for Lagrange analysis. Dispersal probabilities among major biogeographical regions in two different time slices, 0-15 My and 15-40 My. Biogeographical regions defined as shown in Figure 10.

Figure 2. Maximum likelihood tree for partitioned analysis of concatenated supermatrix data set (chloroplast, nrITS, *PhyC* and *Agt1*). Nodes with poor support due to significant, conflicting placement among partitions are indicated, and corresponding nodes can be seen in separate analyses presented in Figure 3. Colored boxes show the generic placements of taxa within the Jones et al. (2002) classification scheme.

Figure 3. Maximum likelihood trees for A) chloroplast data and B) ITS data analyzed separately.

Figures 4-8. Parsimony-based ancestral character state reconstructions for 5 morphological characters used in delimiting taxa by Jones et al. (2002) and others. Values above the branch indicate maximum parsimony/maximum likelihood bootstrap support values for each clade, below the branch show posterior probabilities from Bayesian analysis. Only the backbone and clades showing significant conflict in placement between these two regions are shown.

Figure 9. A remarkable case of convergence in floral morphology in the genus *Corybas*. **A.** *Corybas undulatus*; **B.** *C. imperatorius* (photo by J.B. Comber, https://orchid.unibas.ch); **C.** *C. dowlingii*; **D.** *C. aff. subalpinus*; **E.** *C. aff. simbuensis* (photo by N. Juhonewe); **F.** *C. aff. calophyllus*. While species depicted in in **B**, **C**, **E**, and **F** show very similar gross floral

morphology, phylogenetic analyses show that **B** and **C** are more closely related to **A**, whereas **E** and **F** are more closely related to **D**. Unless otherwise noted, photos by S. Lyon. All other images used with permission.

Figure 10. BEAST tree of concatenated chloroplast data, calibrated using time points from Gustaffson et al. (2010). Error bars indicate 95% confidence intervals. Lagrange ancestral area reconstruction, based on dispersal matrix shown in Table 3, is overlain. Boxes at nodes indicate most likely reconstruction. In cases where second most likely reconstruction was greater than 50% of the most likely reconstruction, boxes are half-shaded. Inset shows range of the Acianthinae, biogeographical areas as defined in this study, and direction and number of inferred dispersal events between areas.

Figure 1.

Table 1.

Schlechter 1926	van Royen 1983	Kores 1995	Szlachetko 2001, Szlachetko and Margonska 2001	Clements et al. 2002, Jones et al. 2002, Jones and Clements 2004	Chase et al. 2003, Kew 2014	Present
Corybas Salisb. sects Calcearia and Geosiphon (*C. saprophyticus*)	*Corybas* sect Corybas subsect Corybas		*Corybas* subgen Corybas sect Corybas	*Corybas*	*Corybas*	*Corybas* subgen Corybas
				Calcearia		
Corybas sect Gastrosiphon	*Corybas* sect Corybas subsect Gastrosiphon		*Corybas* subgen Corybas sect Gastrosiphon	*Gastrosiphon*		
	Corybas sect Steleocorys		*Corybas* subgen Steleocorys	*Singularybas (C. oblongus)*		*Corybas* subgen Singularybas
				Molloybas (C. cryptanthus)		*Corybas* subgen Corysanthes sect Molloybas
				Nematoceras		*Corybas* subgen Corysanthes sect Nematoceras
				Corysanthes		*Corybas* subgen Corysanthes sect Corysanthes
				Anzybas		*Corybas* subgen Anzybas
Acianthus Br, including *Cyrtostylis* Br, *Acianthus atepalus* Reichb.		*Acianthus* subgen Acianthus sect Cyrtostylis	*Acianthus*	*Cyrtostylis*	*Cyrtostylis*	*Cyrtostylis*
		Acianthus subgen Acianthus sect Acianthus		*Acianthus*	*Acianthus,* incl. *A. atepalus*	*Acianthus* sect Acianthus
		Acianthus subgen Acianthus sect Caudatus		*Nemacianthus*		*Acianthus* sect Nemacianthus
		Acianthus subgen Univiscidiatus sect Macropetalus	*Univiscidiatus* section Macropetalus	*Acianthopsis*		*Stigmatodactylus* subgen Acianthopsis
		Acianthus subgen Univiscidiatus sect Macropetalus	*Univiscidiatus* section Univiscidiatus	*Acianthella*		*Stigmatodactylus* subgen Acianthella
Stigmatodactylus Maxim.		*Stigmatodactylus*	*Stigmatodactylus*	*Stigmatodactylus*	*Stigmatodactylus*	*Stigmatodactylus* subgen Stigmatodactylus
Townsonia Cheesem.		*Townsonia*	*Townsonia*	*Townsonia*	*Townsonia*	*Townsonia* subgen Townsonia
			Spuracianthus (A. atepalus)	*Spuracianthus (A. atepalus)*		*Townsonia* subgen Spuracianthus

Taxon	Extraction	Locality	Voucher ID (HERB)	*matK*	*trnL-trnF*	*psbJ-petA*	*psbD-trnT*	*rps16-trnQ*	nrITS	*PhyC*	*Agt1*
Acianthus atepalus	SLE593	New Caledonia, Mt Mou	MAC9388 (CANB)	-	x	x	x	x	x	-	x
Acianthus bracteatus	SLE596	New Caledonia, Mt Mou	MAC9389 (CANB)	-	x	x	x	x	x	x	-
Acianthus caudatus	SLE066	Australia, NSW, Alum Mt	SPL043 (WIS)	-	x	x	x	x	x	x	x
Acianthus cf elegans		New Caledonia	KM322 (OKL)	AJ309999	AJ409370	-	-	-	-	-	-
Acianthus confusus		New Caledonia	KM330 (OKL)	AJ309992	AJ409371	-	-	-	-	-	-
Acianthus cymbalariifolius		New Caledonia	KM329 (OKL)	AJ309991	AJ409372	-	-	-	-	-	-
Acianthus exsertus	SLE061	Australia, NSW, Jervis Bay	SPL025 (WIS)	-	x	x	x	x	x	x	-
Acianthus fornicatus	SLE063	Australia, NSW, Batemans Bay	SPL003 (WIS)	x	x	x	x	x	x	x	-
Acianthus macroglossa	SLE597	New Caledonia, Mt Koghi	MAC9322 (CANB)	-	x	x	x	x	x	-	-
Acianthus pusillus	SLE075	Australia, Tas, Narawntapu NP (cult MAC)	MAC10739 (CANB)	-	x	x	x	x	x	x	-
Chiloglottis trapeziformis	SLE535	Australia	MAC11858 (CANB)	x	x	x	x	x	x	x	x
Corybas "pseudocalophyllus"	SLE302	Papua New Guinea, Simbu, Mt Wilhelm	SPL390 (L)	x	x	x	x	x	x	x	x
Corybas abditus	SLE564	Australia	DLJ3355 (CANB)	-	x	-	-	-	x	-	-
Corybas abellianus	SLE366	Australia, Qld, Davies Creek	KS176 (CANB)	-	x	x	x	x	x	x	-
Corybas aconitiflorus	SLE007	Australia, Tas, Douglas Apsley NP (cult MAC)	MAC10772 (CANB)	x	x	x	x	x	x	x	x
Corybas acuminatus	SLE330	New Zealand, W Coast, Mangatini River	Kyle 275/02 (CANB)	x	x	x	x	x	x	x	x
Corybas aff albipurpureus	SLE298	Papua New Guinea, Madang, Finisterre Mts	SPL447 (L)	-	x	x	x	x	x	x	-
Corybas aff aristatus	SLE287	Papua New Guinea, Morobe, Huon peninsula	SPL419 (L)	-	x	x	x	x	x	x	-
Corybas aff boridiensis	SLE304	Papua New Guinea, Morobe, Huon peninsula	SPL423 (L)	x	x	x	x	x	x	x	-
Corybas aff calophyllus	SLE569	Indonesia, W Papua, Walabu	Rose 106 (CANB)	-	x	-	-	-	x	-	-
Corybas aff gastrosiphon	SLE580	Indonesia, W Papua, Walabu	Rose 115 (CANB)	-	x	-	-	-	x	-	-
Corybas aff royenii	SLE282	Papua New Guinea, Simbu, nr Mt Wilhelm	SPL399 (L)	-	x	x	x	x	x	x	-
Corybas aff simbuensis	SLE012	Papua New Guinea, EHP, nr Kainantu	Cruttwell 1890 (CANB)	-	x	-	-	-	x	-	-
Corybas aff subalpinus	SLE314	Papua New Guinea, Simbu, Mt Wilhelm	SPL405 (L)	-	x	x	x	x	x	x	-
Corybas aff umbonatus	SLE296	Papua New Guinea, Madang, Finisterre Mts	SPL448 (L)	-	x	x	x	x	x	x	-
Corybas aff urikensis	SLE583	Papua New Guinea, W Sepik, Toricelli Mts	MAC9612 (CANB)	-	x	-	-	-	-	-	-
Corybas aff urikensis	SLE293	Papua New Guinea, SHP, Tari gap	SPL459 (L)	-	x	x	x	x	x	x	-
Corybas amungwiwensis	SLE280	Papua New Guinea, Morobe, Huon peninsula	SPL420 (L)	x	x	x	x	x	x	x	x
Corybas aristatus	SLE576	Papua New Guinea, WHP, nr Mt. Hagen	Hooglan & Pullen 5930 (CANB)	-	x	-	-	-	-	-	-
Corybas barbarae	SLE043	Australia, NSW, Alum Mt	BB0028 (CANB)	-	x	x	x	x	x	x	-
Corybas betsyae	SLE315	Papua New Guinea, Morobe, Bubuu Valley	SPL473 (L)	-	x	x	x	x	x	x	-
Corybas boridiensis	SLE316	Papua New Guinea, Morobe, Bubuu Valley	SPL479 (L)	-	x	x	x	x	x	x	x
Corybas calcicolus	SLE505	Malaysia, Perak, Mt Mesah	FRI77616 (FRIM)	-	x	x	x	x	x	x	-
Corybas calopeplos	SLE126	Malaysia, Kelantan	FRI71286 (FRIM)	-	x	x	x	x	x	x	x
Corybas carinatus	SLE122	Malaysia, Pahang, Cameron Highlands	FRI67654 (FRIM)	-	x	x	x	x	x	x	x
Corybas carinatus	SLE257	Indonesia, W Java, Mt Salak Peak II	SN (BO)	x	x	x	x	x	x	x	-
Corybas carinatus	SLE134	Malaysia, Sabah, Mt Alab	SPL096 (WIS)	-	x	x	x	x	x	x	-

Taxon	Extraction	Locality	Voucher ID (HERB)	*matK*	*trnL-trnF*	*psbJ-petA*	*psbD-trnT*	*rps16-trnQ*	nrITS	*PhyC*	*Agt1*
Corybas carinatus	SLE259	Malaysia, Sarawak, Mt Santubong	SPL365 (SAR)	-	x	x	x	x	x	x	-
Corybas carsei	SLE114	New Zealand, Waikato, Whangamarino wetlands	de Lange 047/98 (CANB)	-	x	x	x	x	x	x	x
Corybas cerasinus	SLE009	Australia, Qld, Mt Walker	LJ Roberts CHB517 (CANB)	-	x	x	x	x	x	-	-
Corybas cheesemanii	SLE335	New Zealand, Northland	Syddal 084/99 (CANB)	-	x	x	x	x	x	x	-
Corybas comptus	SLE137	Malaysia, Kelantan	FRI71271 (FRIM)	-	x	x	x	x	x	x	x
Corybas cryptanthus	SLE110	New Zealand, Northland	Molloy 115/99 (CANB)	x	x	x	-	x	x	x	x
Corybas dentatus	SLE490	Australia, SA, Frome Rd	ORG6324 (CANB)	-	x	x	x	x	x	x	-
Corybas despectans	SLE502	Australia, SA, Coonalpyn	ORG6312 (CANB)	-	x	x	x	x	x	x	-
Corybas despectans	SLE016	Australia, SA, Newland Head	SPL059 (WIS, CANB)	x	x	x	x	x	x	x	x
Corybas diemenicus	SLE038	Australia, Tas, Arthur R	ORG5513 (CANB)	x	x	x	x	x	x	x	x
Corybas dowlingii	SLE026	Australia, NSW, L Macquarie CA	Dowling 513 (CANB)	-	x	x	x	x	x	x	-
Corybas ekuamensis	SLE288	Papua New Guinea, EHP, Daulo Pass	SPL389 (L)	-	x	x	x	x	x	x	-
Corybas epiphyticus	SLE587	Papua New Guinea, New Ireland, Hans Meyer Rng	Sands 2420 (L)	-	x	x	-	-	x	-	-
Corybas expansus	SLE463	Australia, SA, Innes NP	ORG6310 (CANB)	-	x	x	x	x	x	x	-
Corybas fimbriatus	SLE035	Australia, Tas, Mt William (cult MAC)	MAC10757 (CANB)	x	x	x	x	x	x	x	x
Corybas fordhamii	SLE515	Australia	ORG4930 (CANB)	-	x	x	x	x	x	x	-
Corybas gastrosiphon	SLE292	Papua New Guinea, Madang, Finisterre Mts	SPL452 (L)	-	x	x	x	x	x	x	-
Corybas geminigibbus	SLE093	Malaysia, Kedah, Mt Jerai	Go & Tan SN (UPM)	-	x	x	x	x	x	x	-
Corybas gibbifer	SLE277	Papua New Guinea, SHP, nr Tari gap	SPL457 (L)	x	x	x	x	x	x	x	x
Corybas himalaicus?	SLE591	China, Yunan, Yong De Mt	SN (TAICH)	-	x	x	x	x	x	x	-
Corybas hispidus	SLE020	Australia, NSW, Mt Duval (cult MAC)	ORG5394 (CANB)	-	x	x	x	x	x	x	x
Corybas holttumii	SLE089	Malaysia, Pahang, Genting Highlands	SPL070 (WIS)	x	x	x	x	x	x	x	-
Corybas imperatorius			Comber 1589 (K)	-	x	-	-	-	x	-	-
Corybas incurvus	SLE034	Australia, ACT, Black Mt	SPL049 (WIS)	-	x	x	x	x	x	x	x
Corybas iridescens	SLE343	New Zealand, cult Canterbury	Molloy 094/99 (CANB)	-	x	x	x	x	x	x	-
Corybas limpidus	SLE406	Australia, WA, Munglinup Beach	SPL251 (PERTH, WIS)	-	x	x	x	x	x	x	-
Corybas macranthus	SLE115	New Zealand, Canterbury, View Hill	Molloy 018/98 (CANB)	-	x	x	x	x	x	x	-
Corybas mirabilis		Vanuatu, Aneityum, Mt. Inrerow	B. Lewis & J. McDonagh 186 (K)	-	x	x	x	x	x	-	-
Corybas montanus	SLE008	Australia, Qld, Mt Maroon	Jones 1826 (CANB)	-	x	-	-	-	x	-	-
Corybas muluensis	SLE132	Malaysia, Sabah, Mt Alab	SPL088 (SAN)	-	x	x	x	x	x	x	-
Corybas naviculisepalus	SLE581	Indonesia, W Papua, Kembu	Rose 135 (CANB)	-	x	x	x	x	x	-	-
Corybas neocaledonicus		New Caledonia, Mt Mou	KM316 (OKL)	AJ3110011	AJ409390	-	-	-	x	-	-
Corybas oblongus	SLE354	New Zealand, Northland	Molloy 091/99 (CANB)	x	x	x	x	x	x	x	x
Corybas orbiculatus	SLE112	New Zealand, Canterbury, Ashley River	Molloy 014/98 (CANB)	-	x	x	x	x	x	x	-
Corybas papa	SLE357	New Zealand, Taranaki	Dodunski 096/99 (CANB)	-	x	x	x	x	x	x	-
Corybas pictus	SLE258	Indonesia, W Java, Mt Salak Peak II	SN (BO)	x	x	x	x	x	x	x	-
Corybas pictus	SLE120	Malaysia, Sabah, Kinabalu NP, Liwagu Trail	SPL085 (SNP)	-	x	x	x	x	x	x	x

Taxon	Extraction	Locality	Voucher ID (HERB)	*matK*	*trnL-trnF*	*psbJ-petA*	*psbD-trnT*	*rps16-trnQ*	nrITS	*PhyC*	*Agt1*
Corybas pictus	SLE267	Malaysia, Sarawak, Padawan area	SPL350 (SAR, WIS)	x	x	x	x	x	x	x	-
Corybas pictus (serpentine)	SLE131	Malaysia, Sabah, Bukit Hempuen	SPL111 (WIS)	-	x	x	x	x	x	x	-
Corybas piliferus	SLE133	Malaysia, Sabah, Mt Alab	SPL093 (WIS)	-	x	x	x	x	x	x	x
Corybas ponapensis	SLE590	Micronesia, Pohnpei	A Rinehart 1433 (US)	-	x	-	-	-	x	-	-
Corybas pruinosus	SLE025	Australia, NSW, Alum Mt	BB0033 (WIS)	-	x	x	x	x	x	x	x
Corybas puniceus	SLE518	Taiwan, Shyrpishan	Lin SN (TAI)	-	x	x	x	x	x	x	-
Corybas recurvus	SLE005	Australia, WA, Two People's Bay (cult MAC)	MAC10956 (CANB)	x	x	x	x	x	x	x	x
Corybas ridleyanus	SLE099	Malaysia, Pahang, Cameron Highlands	SPL076 (WIS)	x	x	x	x	x	x	x	x
Corybas rivularis	SLE117	New Zealand, Taranaki	Dodinski 003/98 (CANB)	x	x	x	x	x	x	x	x
Corybas rotundifolius	SLE111	New Zealand, Northland	Forester 101/99	-	x	x	x	x	x	x	-
Corybas royenii	SLE297	Papua New Guinea, Simbu, Mt Wilhelm	SPL397 (L)	-	x	x	x	x	x	x	-
Corybas selangorensis	SLE092	Malaysia, Pahang, Genting Highlands	Go & Tan SN (UPM)	-	x	x	x	x	x	x	-
Corybas serpentinus	SLE124	Malaysia, Sabah, Maliau R	SPL104 (WIS)	-	x	x	x	x	x	x	-
Corybas shanlinshiensis	SLE516	Taiwan, Shanlinshi	SPL489 (WIS, TAICH)	-	x	x	x	x	x	x	-
Corybas sinii	SLE511	Taiwan, Yuan Zui Shan	SPL491 (TAICH)	x	x	x	x	x	x	x	-
Corybas sp. "crenulatus"	SLE125	Malaysia, Sabah, Kinabalu NP, Mesilau	SPL082 (SNP)	-	x	x	x	x	x	x	-
Corybas sp1	SLE307	Papua New Guinea, WHP, nr Minj	SPL408 (L)	-	x	x	x	x	x	x	-
Corybas sp2	SLE310	Papua New Guinea, Morobe, Huon peninsula	SPL425 (L)	-	x	x	x	x	x	x	-
Corybas striatus	SLE510	Papua New Guinea, Morobe, Bubuu Valley	SAJ0604 (BISH, WIS)	-	x	x	x	x	x	x	-
Corybas sulcatus	SLE342	Australia, Macquarie Is	K633 (CANB)	-	x	x	x	x	x	x	-
Corybas taliensis		China, Yunan, Malipo county	SN (TAICH)	-	x	x	-	-	x	-	-
Corybas trilobus	SLE320	New Zealand, Manawatu-Wanganui, Erua	Molloy 242/00 (CANB)	x	x	x	x	x	x	x	x
Corybas undulatus	SLE014	Australia, NSW, Jervis Bay	SPL019 (WIS)	x	x	x	x	x	x	x	x
Corybas unguiculatus	SLE055	Australia, SA, Nixon-Skinner CP	SPL064 (WIS)	x	x	x	x	x	x	x	x
Corybas villosus	SLE090	Malaysia, Pahang, Genting Highlands	SPL072 (WIS)	-	x	x	x	x	x	x	-
Cyrtostylis huegelii		Australia, WA	KM247 (OKL)	AJ310019	AJ409399	-	-	-	-	-	-
Cyrtostylis reniformis	SLE001	Australia, Tas, Mt George (cult MAC)	MAC10742 (CANB)	x	x	x	x	x	x	x	x
Cyrtostylis robusta	SLE068	Australia, SA	SPL060 (WIS)	-	x	x	x	x	x	x	-
Diuris sulphurea	SLE536	Australia	ORG3473 (CANB)	x	x	x	x	x	x	x	-
Eriochilus cucullatus	SLE537	Australia, ACT, Black Mt	MAC11855 (CANB)	x	x	x	x	x	x	x	x
Microtis parviflora	SLE538	Australia	MAC11119 (CANB)	x	x	x	x	x	x	x	x
Stigmatodactylus croftianus	SLE273	Papua New Guinea, Morobe, Bubuu Valley	SPL478 (L)	-	x	-	x	x	x	x	-
Stigmatodactylus lamrii	SLE136	Malaysia, Sabah, Kinabalu NP, Botanic Garden	SPL084 (WIS)	-	x	x	x	x	x	x	x
Stigmatodactylus sikokianus		Japan	KM328 (OKL)	AJ310075	AJ409453	-	-	-	-	-	-
Stigmatodactylus variegatus	SLE285	Papua New Guinea, Simbu, Mt Wilhelm	SPL407 (L)	-	x	x	x	x	x	x	-
Townsonia viridis	SLE595	Australia, Tas, Mother Cummings Peak	Campbell 94183 (CANB)	-	x	x	x	x	-	x	-

Table 3.

0-15 My

	Asia	Australia	New Caledonia	New Guinea	New Zealand	Pacific	Sundaland
Asia	1	0.1	0.1	0.1	0.1	0.5	0.9
Australia	0.1	1	0.9	0.9	0.9	0.1	0.9
New Caledonia	0.1	0.9	1	0.9	0.9	0.5	0.1
New Guinea	0.1	0.9	0.9	1	0.1	0.5	0.9
New Zealand	0.1	0.9	0.9	0.1	1	0.5	0.1
Pacific	0.5	0.1	0.5	0.5	0.5	1	0.5
Sundaland	0.9	0.9	0.1	0.9	0.1	0.5	1

15-40 My

	Asia	Australia	New Caledonia	New Guinea	New Zealand	Pacific	Sundaland
Asia	1	0.1	0.1	0.1	0.1	0.5	0.9
Australia	0.1	1	0.9	0.9	0.9	0.1	0.1
New Caledonia	0.1	0.9	1	0.9	0.9	0.5	0.1
New Guinea	0.1	0.9	0.9	1	0.1	0.5	0.5
New Zealand	0.1	0.9	0.9	0.1	1	0.5	0.1
Pacific	0.5	0.1	0.5	0.5	0.5	1	0.5
Sundaland	0.9	0.1	0.1	0.5	0.1	0.5	1

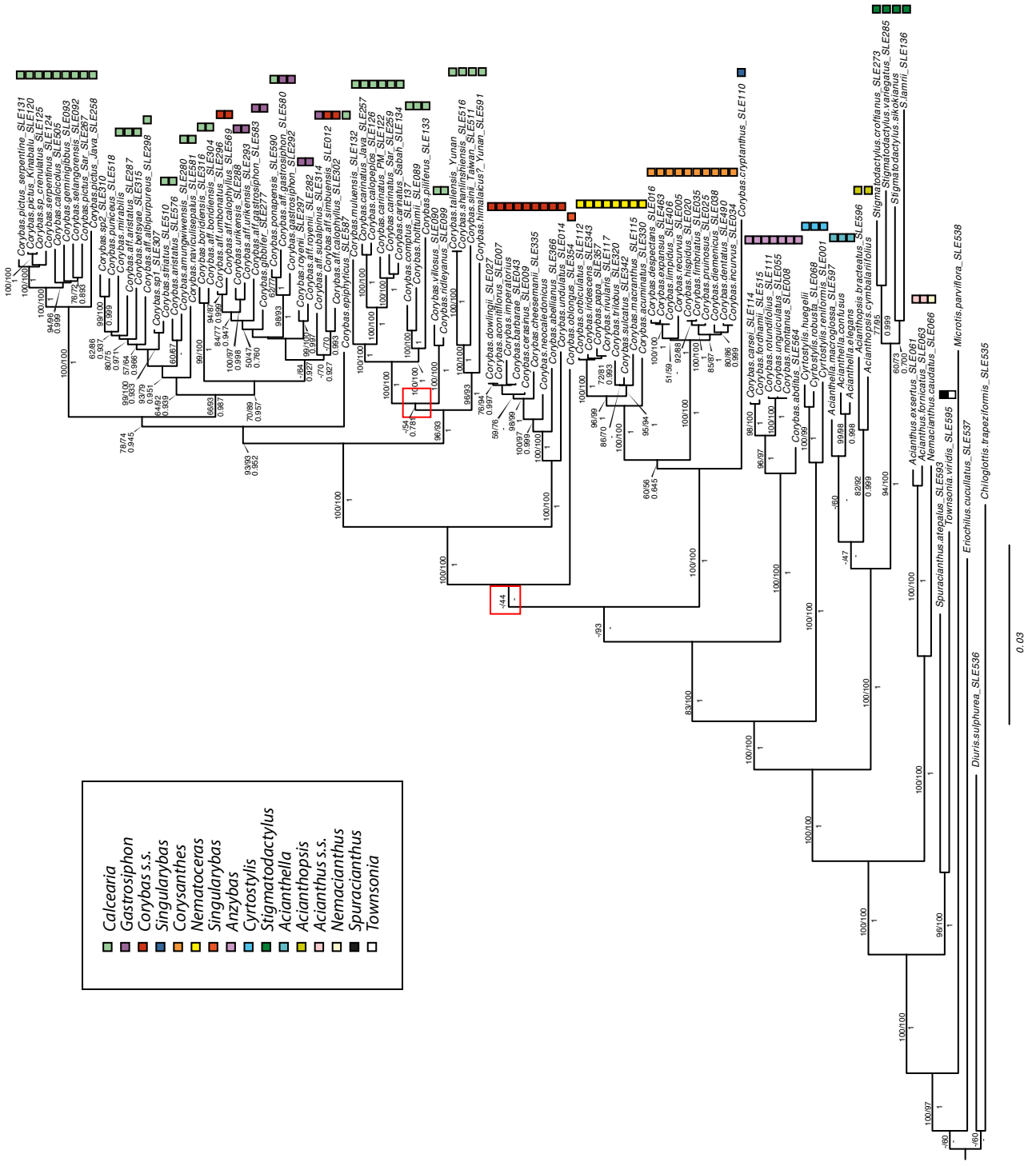
Calcearia
Gastrosiphon
Corybas s.s.
Singularybas
Corysanthes
Nematoceras
Singularybas
Anzybas
Cyrtostylis
Stigmatodactylus
Acianthella
Acianthopsis
Acianthus s.s.
Nemacianthus
Spuracianthus
Townsonia
0.03

Figure 2.

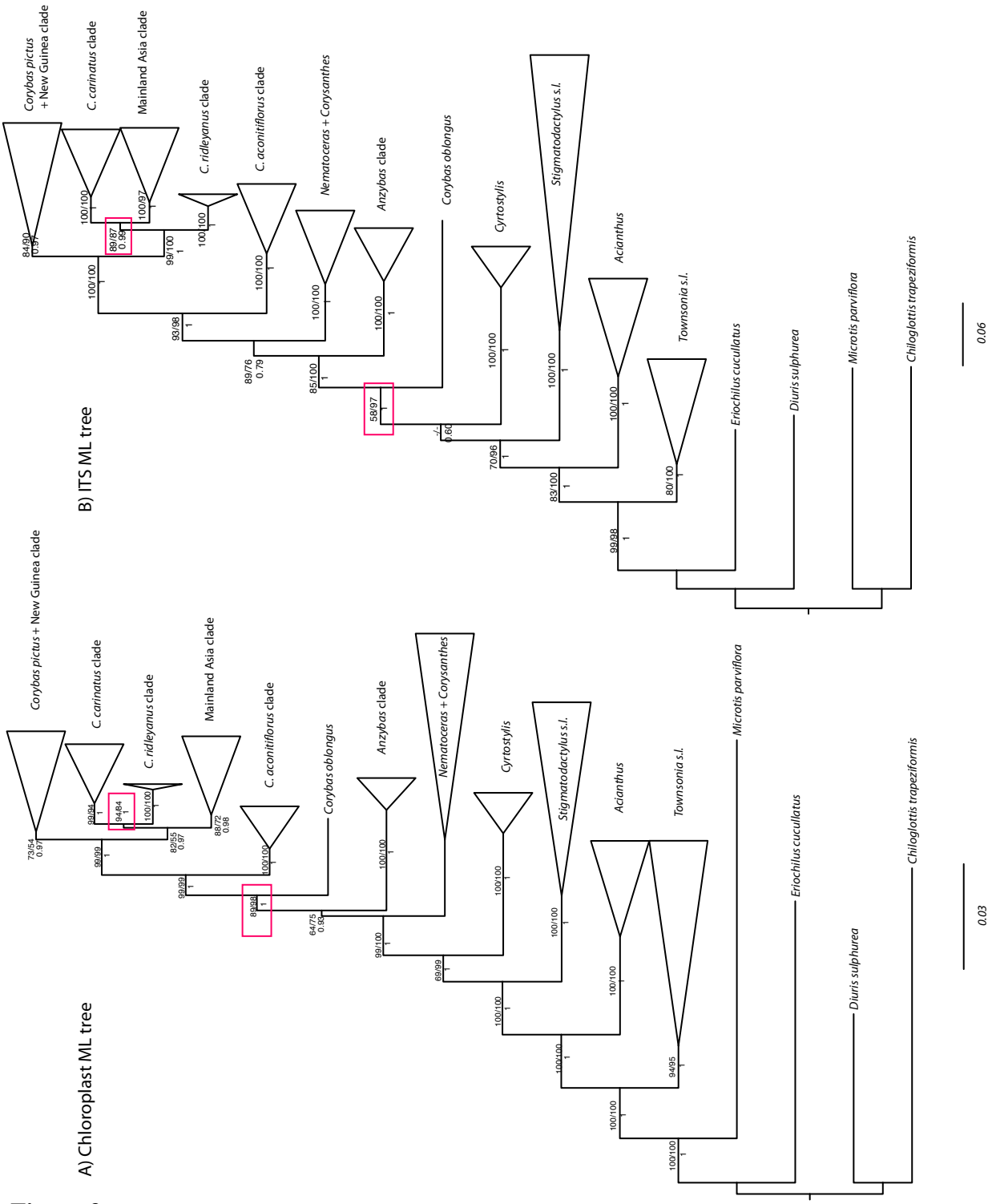
A) Chloroplast ML tree
B) ITS ML tree
Corybas pictus + New Guinea clade
C. carinatus clade
C. ridleyanus clade
Mainland Asia clade
C. aconitiflorus clade
Corybas oblongus
Anzybas clade
Nematoceras + Corysanthes
Cyrtostylis
Stigmatodactylus s.l.
Acianthus
Townsonia s.l.
Micrortis parviflora
Eriochilus cucullatus
Diuris sulphurea
Chiloglottis trapeziformis
0.03
0.06

Figure 3.

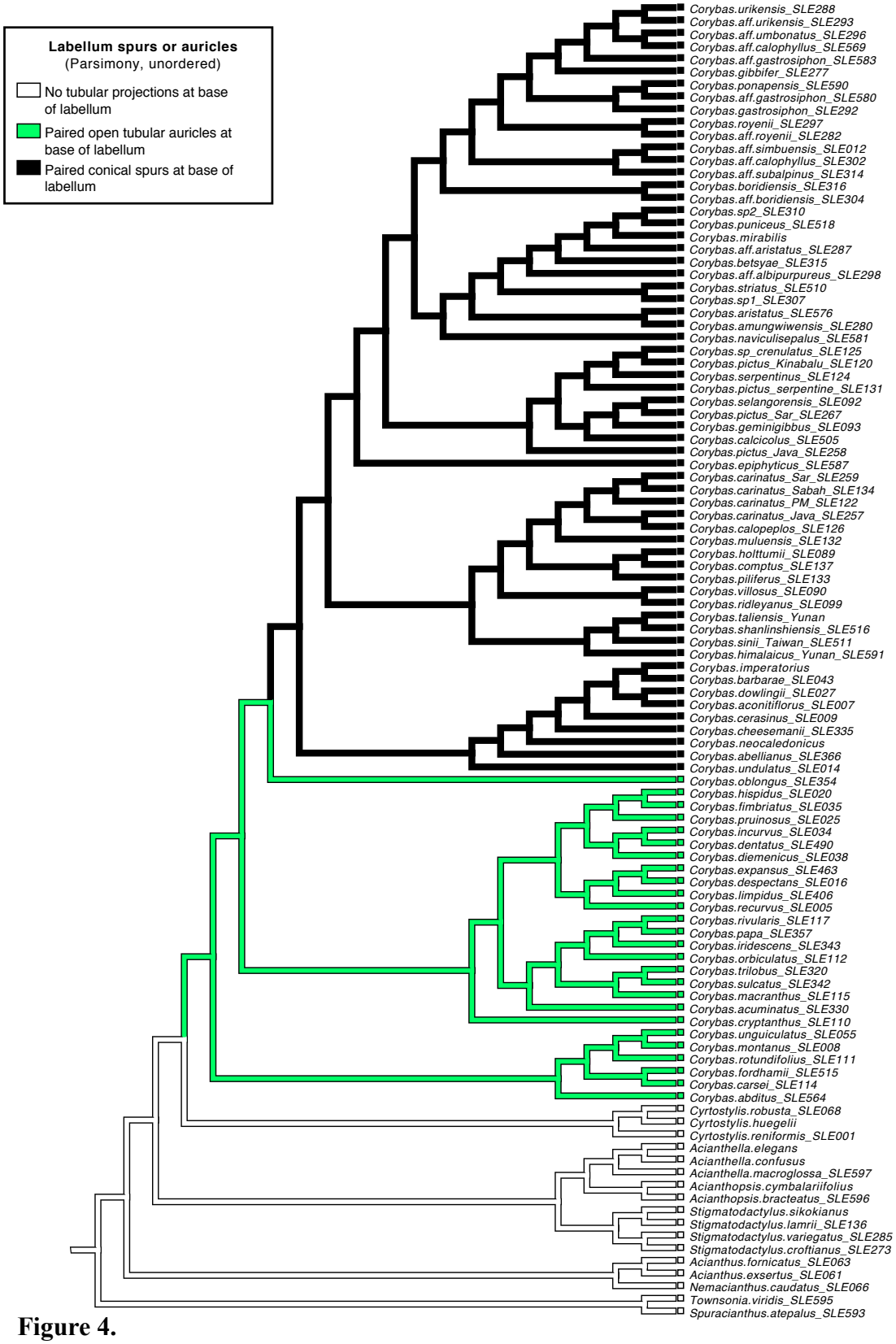
Labellum spurs or auricles
(Parsimony, unordered)
No tubular projections at base of labellum
Paired open tubular auricles at base of labellum
Paired conical spurs at base of labellum
Corybas.urikensis_SLE288
Corybas.aff.urikensis_SLE293
Corybas.aff.umbonatus_SLE296
Corybas.aff.calophyllus_SLE569
Corybas.aff.gastrosiphon_SLE583
Corybas.gibbifer_SLE277
Corybas.ponapensis_SLE590
Corybas.aff.gastrosiphon_SLE580
Corybas.gastrosiphon_SLE292
Corybas.royenii_SLE297
Corybas.aff.royenii_SLE282
Corybas.aff.simbuensis_SLE012
Corybas.aff.calophyllus_SLE302
Corybas.aff.subalpinus_SLE314
Corybas.boridiensis_SLE316
Corybas.aff.boridiensis_SLE304
Corybas.sp2_SLE310
Corybas.puniceus_SLE518
Corybas.mirabilis
Corybas.aff.aristatus_SLE287
Corybas.betsyae_SLE315
Corybas.aff.albipurpureus_SLE298
Corybas.striatus_SLE510
Corybas.sp1_SLE307
Corybas.aristatus_SLE576
Corybas.amungwiwensis_SLE280
Corybas.naviculisepalus_SLE581
Corybas.sp_crenulatus_SLE125
Corybas.pictus_Kinabalu_SLE120
Corybas.serpentinus_SLE124
Corybas.pictus_serpentine_SLE131
Corybas.selangorensis_SLE092
Corybas.pictus_Sar_SLE267
Corybas.geminigibbus_SLE093
Corybas.calcicolus_SLE505
Corybas.pictus_Java_SLE258
Corybas.epiphyticus_SLE587
Corybas.carinatus_Sar_SLE259
Corybas.carinatus_Sabah_SLE134
Corybas.carinatus_PM_SLE122
Corybas.carinatus_Java_SLE257
Corybas.calopeplos_SLE126
Corybas.muluensis_SLE132
Corybas.holttumii_SLE089
Corybas.comptus_SLE137
Corybas.piliferus_SLE133
Corybas.villosus_SLE090
Corybas.ridleyanus_SLE099
Corybas.taliensis_Yunan
Corybas.shanlinshiensis_SLE516
Corybas.sinii_Taiwan_SLE511
Corybas.himalaicus_Yunan_SLE591
Corybas.imperatorius
Corybas.barbarae_SLE043
Corybas.dowlingii_SLE027
Corybas.aconitiflorus_SLE007
Corybas.cerasinus_SLE009
Corybas.cheesemanii_SLE335
Corybas.neocaledonicus
Corybas.abellianus_SLE366
Corybas.undulatus_SLE014
Corybas.oblongus_SLE354
Corybas.hispidus_SLE020
Corybas.fimbriatus_SLE035
Corybas.pruinosus_SLE025
Corybas.incurvus_SLE034
Corybas.dentatus_SLE490
Corybas.diemenicus_SLE038
Corybas.expansus_SLE463
Corybas.despectans_SLE016
Corybas.limpidus_SLE406
Corybas.recurvus_SLE005
Corybas.rivularis_SLE117
Corybas.papa_SLE357
Corybas.iridescens_SLE343
Corybas.orbiculatus_SLE112
Corybas.trilobus_SLE320
Corybas.sulcatus_SLE342
Corybas.macranthus_SLE115
Corybas.acuminatus_SLE330
Corybas.cryptanthus_SLE110
Corybas.unguiculatus_SLE055
Corybas.montanus_SLE008
Corybas.rotundifolius_SLE111
Corybas.fordhamii_SLE515
Corybas.carsei_SLE114
Corybas.abditus_SLE564
Cyrtostylis.robusta_SLE068
Cyrtostylis.huegelii
Cyrtostylis.reniformis_SLE001
Acianthella.elegans
Acianthella.confusus
Acianthella.macroglossa_SLE597
Acianthopsis.cymbalariifolius
Acianthopsis.bracteatus_SLE596
Stigmatodactylus.sikokianus
Stigmatodactylus.lamrii_SLE136
Stigmatodactylus.variegatus_SLE285
Stigmatodactylus.croftianus_SLE273
Acianthus.fornicatus_SLE063
Acianthus.exsertus_SLE061
Nemacianthus.caudatus_SLE066
Townsonia.viridis_SLE595
Spuracianthus.atepalus_SLE593

Figure 4.

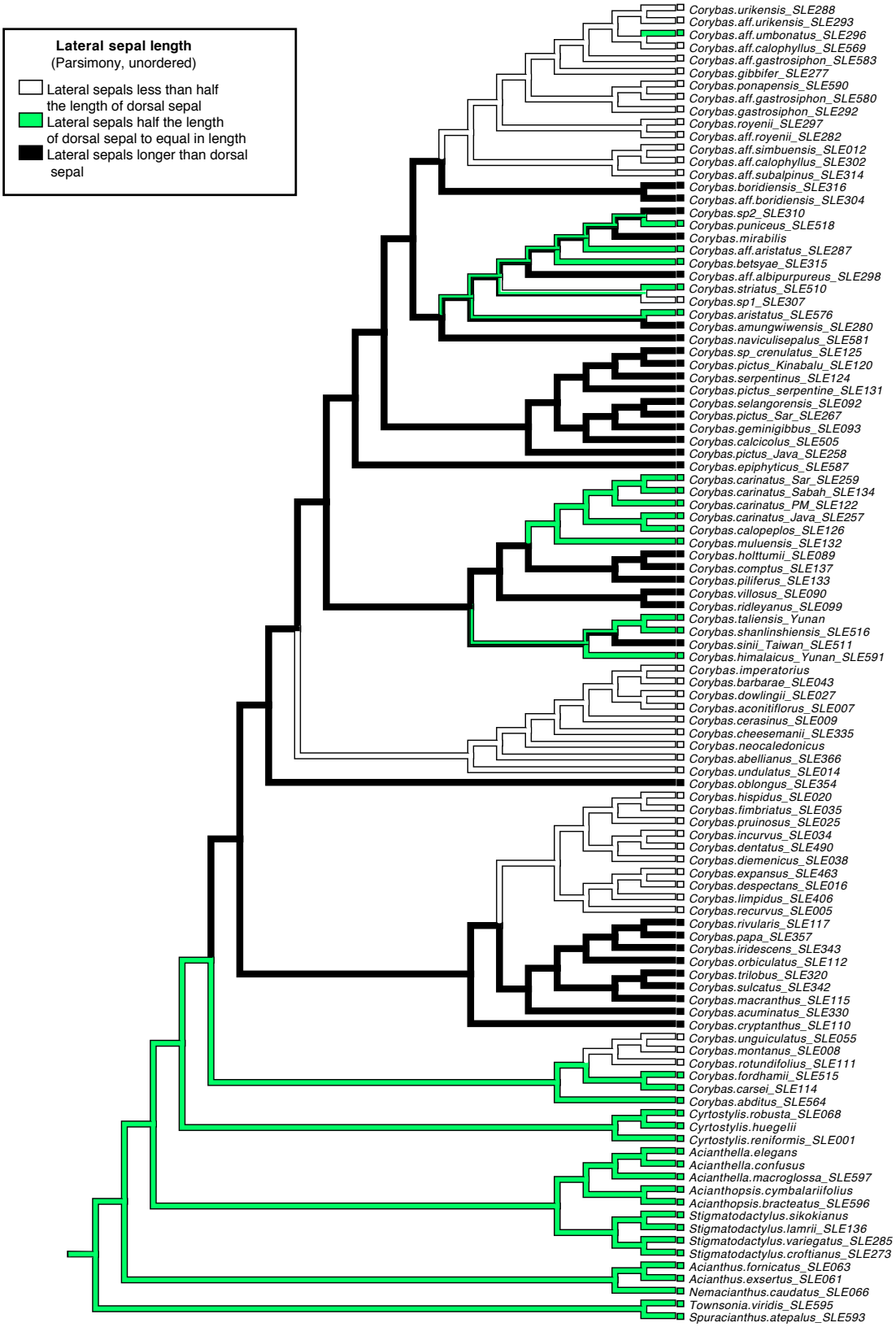
Lateral sepal length
(Parsimony, unordered)
Lateral sepals less than half the length of dorsal sepal
Lateral sepals half the length of dorsal sepal to equal in length
Lateral sepals longer than dorsal sepal
Corybas.urikensis_SLE288
Corybas.aff.urikensis_SLE293
Corybas.aff.umbonatus_SLE296
Corybas.aff.calophyllus_SLE569
Corybas.aff.gastrosiphon_SLE583
Corybas.gibbifer_SLE277
Corybas.ponapensis_SLE590
Corybas.aff.gastrosiphon_SLE580
Corybas.gastrosiphon_SLE292
Corybas.royenii_SLE297
Corybas.aff.royenii_SLE282
Corybas.aff.simbuensis_SLE012
Corybas.aff.calophyllus_SLE302
Corybas.aff.subalpinus_SLE314
Corybas.boridiensis_SLE316
Corybas.aff.boridiensis_SLE304
Corybas.sp2_SLE310
Corybas.puniceus_SLE518
Corybas.mirabilis
Corybas.aff.aristatus_SLE287
Corybas.betsyae_SLE315
Corybas.aff.albipurpureus_SLE298
Corybas.striatus_SLE510
Corybas.sp1_SLE307
Corybas.aristatus_SLE576
Corybas.amungwiwensis_SLE280
Corybas.naviculisepalus_SLE581
Corybas.sp_crenulatus_SLE125
Corybas.pictus_Kinabalu_SLE120
Corybas.serpentinus_SLE124
Corybas.pictus_serpentine_SLE131
Corybas.selangorensis_SLE092
Corybas.pictus_Sar_SLE267
Corybas.geminigibbus_SLE093
Corybas.calcicolus_SLE505
Corybas.pictus_Java_SLE258
Corybas.epiphyticus_SLE587
Corybas.carinatus_Sar_SLE259
Corybas.carinatus_Sabah_SLE134
Corybas.carinatus_PM_SLE122
Corybas.carinatus_Java_SLE257
Corybas.calopeplos_SLE126
Corybas.muluensis_SLE132
Corybas.holttumii_SLE089
Corybas.comptus_SLE137
Corybas.piliferus_SLE133
Corybas.villosus_SLE090
Corybas.ridleyanus_SLE099
Corybas.taliensis_Yunan
Corybas.shanlinshiensis_SLE516
Corybas.sinii_Taiwan_SLE511
Corybas.himalaicus_Yunan_SLE591
Corybas.imperatorius
Corybas.barbarae_SLE043
Corybas.dowlingii_SLE027
Corybas.aconitiflorus_SLE007
Corybas.cerasinus_SLE009
Corybas.cheesemanii_SLE335
Corybas.neocaledonicus
Corybas.abellianus_SLE366
Corybas.undulatus_SLE014
Corybas.oblongus_SLE354
Corybas.hispidus_SLE020
Corybas.fimbriatus_SLE035
Corybas.pruinosus_SLE025
Corybas.incurvus_SLE034
Corybas.dentatus_SLE490
Corybas.diemenicus_SLE038
Corybas.expansus_SLE463
Corybas.despectans_SLE016
Corybas.limpidus_SLE406
Corybas.recurvus_SLE005
Corybas.rivularis_SLE117
Corybas.papa_SLE357
Corybas.iridescens_SLE343
Corybas.orbiculatus_SLE112
Corybas.trilobus_SLE320
Corybas.sulcatus_SLE342
Corybas.macranthus_SLE115
Corybas.acuminatus_SLE330
Corybas.cryptanthus_SLE110
Corybas.unguiculatus_SLE055
Corybas.montanus_SLE008
Corybas.rotundifolius_SLE111
Corybas.fordhamii_SLE515
Corybas.carsei_SLE114
Corybas.abditus_SLE564
Cyrtostylis.robusta_SLE068
Cyrtostylis.huegelii
Cyrtostylis.reniformis_SLE001
Acianthella.elegans
Acianthella.confusus
Acianthella.macroglossa_SLE597
Acianthopsis.cymbalariifolius
Acianthopsis.bracteatus_SLE596
Stigmatodactylus.sikokianus
Stigmatodactylus.lamrii_SLE136
Stigmatodactylus.variegatus_SLE285
Stigmatodactylus.croftianus_SLE273
Acianthus.fornicatus_SLE063
Acianthus.exsertus_SLE061
Nemacianthus.caudatus_SLE066
Townsonia.viridis_SLE595
Spuracianthus.atepalus_SLE593

Figure 5.

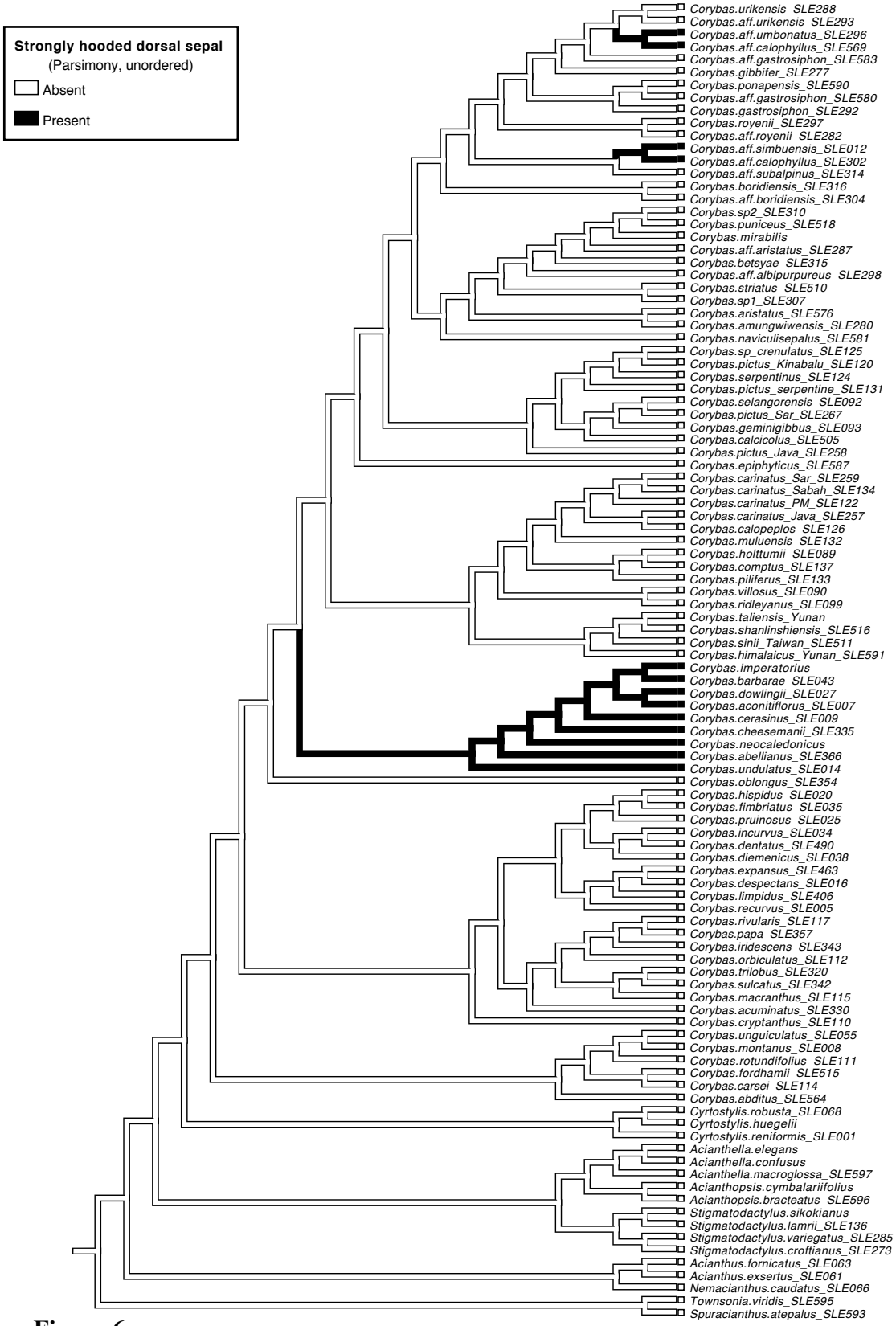
Strongly hooded dorsal sepal
(Parsimony, unordered)
Absent
Present
Corybas.urikensis_SLE288
Corybas.aff.urikensis_SLE293
Corybas.aff.umbonatus_SLE296
Corybas.aff.calophyllus_SLE569
Corybas.aff.gastrosiphon_SLE583
Corybas.gibbifer_SLE277
Corybas.ponapensis_SLE590
Corybas.aff.gastrosiphon_SLE580
Corybas.gastrosiphon_SLE292
Corybas.royenii_SLE297
Corybas.aff.royenii_SLE282
Corybas.aff.simbuensis_SLE012
Corybas.aff.calophyllus_SLE302
Corybas.aff.subalpinus_SLE314
Corybas.boridiensis_SLE316
Corybas.aff.boridiensis_SLE304
Corybas.sp2_SLE310
Corybas.puniceus_SLE518
Corybas.mirabilis
Corybas.aff.aristatus_SLE287
Corybas.betsyae_SLE315
Corybas.aff.albipurpureus_SLE298
Corybas.striatus_SLE510
Corybas.sp1_SLE307
Corybas.aristatus_SLE576
Corybas.amungwiwensis_SLE280
Corybas.naviculisepalus_SLE581
Corybas.sp_crenulatus_SLE125
Corybas.pictus_Kinabalu_SLE120
Corybas.serpentinus_SLE124
Corybas.pictus_serpentine_SLE131
Corybas.selangorensis_SLE092
Corybas.pictus_Sar_SLE267
Corybas.geminigibbus_SLE093
Corybas.calcicolus_SLE505
Corybas.pictus_Java_SLE258
Corybas.epiphyticus_SLE587
Corybas.carinatus_Sar_SLE259
Corybas.carinatus_Sabah_SLE134
Corybas.carinatus_PM_SLE122
Corybas.carinatus_Java_SLE257
Corybas.calopeplos_SLE126
Corybas.muluensis_SLE132
Corybas.holttumii_SLE089
Corybas.comptus_SLE137
Corybas.piliferus_SLE133
Corybas.villosus_SLE090
Corybas.ridleyanus_SLE099
Corybas.taliensis_Yunan
Corybas.shanlinshiensis_SLE516
Corybas.sinii_Taiwan_SLE511
Corybas.himalaicus_Yunan_SLE591
Corybas.imperatorius
Corybas.barbarae_SLE043
Corybas.dowlingii_SLE027
Corybas.aconitiflorus_SLE007
Corybas.cerasinus_SLE009
Corybas.cheesemanii_SLE335
Corybas.neocaledonicus
Corybas.abellianus_SLE366
Corybas.undulatus_SLE014
Corybas.oblongus_SLE354
Corybas.hispidus_SLE020
Corybas.fimbriatus_SLE035
Corybas.pruinosus_SLE025
Corybas.incurvus_SLE034
Corybas.dentatus_SLE490
Corybas.diemenicus_SLE038
Corybas.expansus_SLE463
Corybas.despectans_SLE016
Corybas.limpidus_SLE406
Corybas.recurvus_SLE005
Corybas.rivularis_SLE117
Corybas.papa_SLE357
Corybas.iridescens_SLE343
Corybas.orbiculatus_SLE112
Corybas.trilobus_SLE320
Corybas.sulcatus_SLE342
Corybas.macranthus_SLE115
Corybas.acuminatus_SLE330
Corybas.cryptanthus_SLE110
Corybas.unguiculatus_SLE055
Corybas.montanus_SLE008
Corybas.rotundifolius_SLE111
Corybas.fordhamii_SLE515
Corybas.carsei_SLE114
Corybas.abditus_SLE564
Cyrtostylis.robusta_SLE068
Cyrtostylis.huegelii
Cyrtostylis.reniformis_SLE001
Acianthella.elegans
Acianthella.confusus
Acianthella.macroglossa_SLE597
Acianthopsis.cymbalariifolius
Acianthopsis.bracteatus_SLE596
Stigmatodactylus.sikokianus
Stigmatodactylus.lamrii_SLE136
Stigmatodactylus.variegatus_SLE285
Stigmatodactylus.croftianus_SLE273
Acianthus.fornicatus_SLE063
Acianthus.exsertus_SLE061
Nemacianthus.caudatus_SLE066
Townsonia.viridis_SLE595
Spuracianthus.atepalus_SLE593

Figure 6.

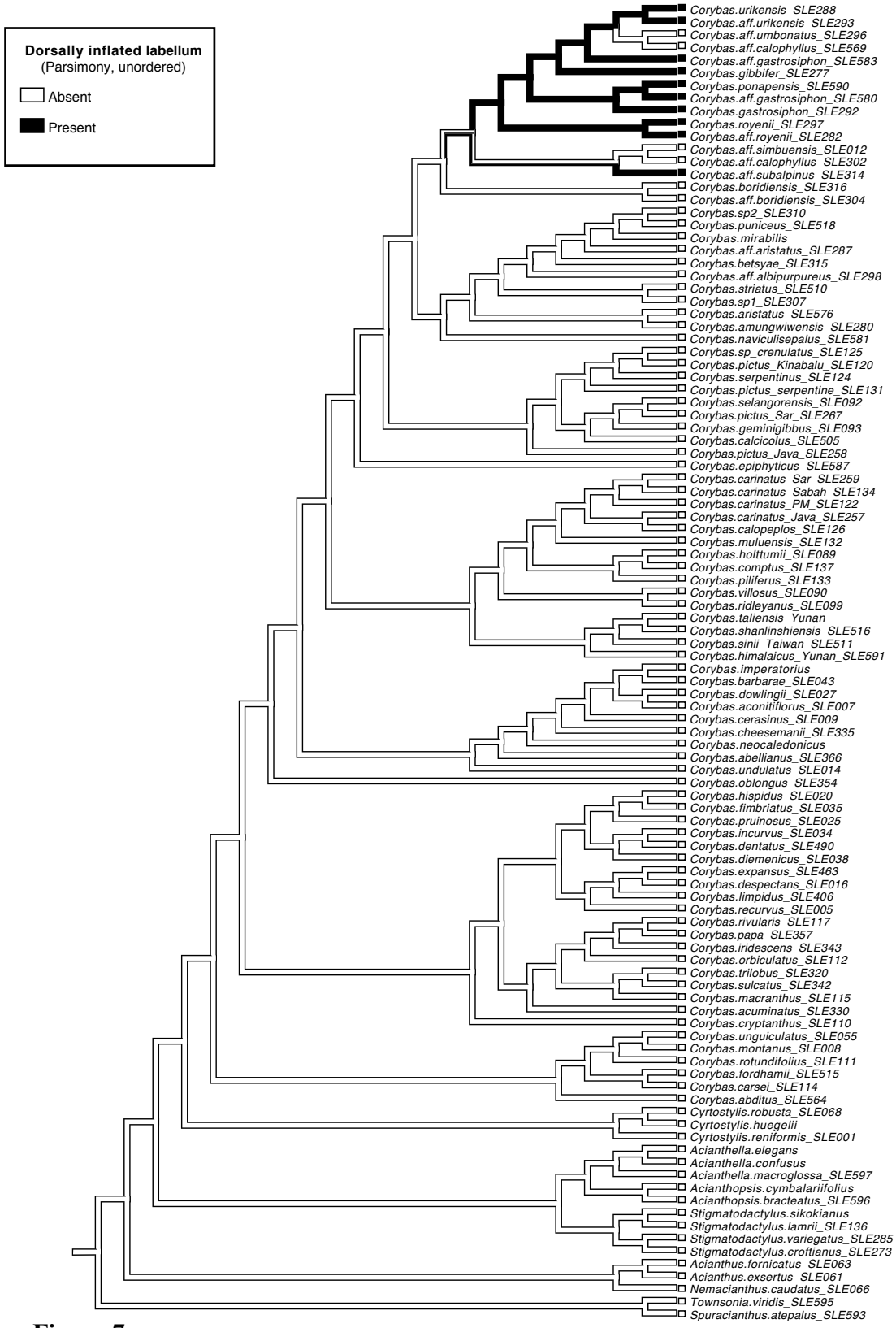
Dorsally inflated labellum
(Parsimony, unordered)
Absent
Present
Corybas.urikensis_SLE288
Corybas.aff.urikensis_SLE293
Corybas.aff.umbonatus_SLE296
Corybas.aff.calophyllus_SLE569
Corybas.aff.gastrosiphon_SLE583
Corybas.gibbifer_SLE277
Corybas.ponapensis_SLE590
Corybas.aff.gastrosiphon_SLE580
Corybas.gastrosiphon_SLE292
Corybas.royenii_SLE297
Corybas.aff.royenii_SLE282
Corybas.aff.simbuensis_SLE012
Corybas.aff.calophyllus_SLE302
Corybas.aff.subalpinus_SLE314
Corybas.boridiensis_SLE316
Corybas.aff.boridiensis_SLE304
Corybas.sp2_SLE310
Corybas.puniceus_SLE518
Corybas.mirabilis
Corybas.aff.aristatus_SLE287
Corybas.betsyae_SLE315
Corybas.aff.albipurpureus_SLE298
Corybas.striatus_SLE510
Corybas.sp1_SLE307
Corybas.aristatus_SLE576
Corybas.amungwiwensis_SLE280
Corybas.naviculisepalus_SLE581
Corybas.sp_crenulatus_SLE125
Corybas.pictus_Kinabalu_SLE120
Corybas.serpentinus_SLE124
Corybas.pictus_serpentine_SLE131
Corybas.selangorensis_SLE092
Corybas.pictus_Sar_SLE267
Corybas.geminigibbus_SLE093
Corybas.calcicolus_SLE505
Corybas.pictus_Java_SLE258
Corybas.epiphyticus_SLE587
Corybas.carinatus_Sar_SLE259
Corybas.carinatus_Sabah_SLE134
Corybas.carinatus_PM_SLE122
Corybas.carinatus_Java_SLE257
Corybas.calopeplos_SLE126
Corybas.muluensis_SLE132
Corybas.holttumii_SLE089
Corybas.comptus_SLE137
Corybas.piliferus_SLE133
Corybas.villosus_SLE090
Corybas.ridleyanus_SLE099
Corybas.taliensis_Yunan
Corybas.shanlinshiensis_SLE516
Corybas.sinii_Taiwan_SLE511
Corybas.himalaicus_Yunan_SLE591
Corybas.imperatorius
Corybas.barbarae_SLE043
Corybas.dowlingii_SLE027
Corybas.aconitiflorus_SLE007
Corybas.cerasinus_SLE009
Corybas.cheesemanii_SLE335
Corybas.neocaledonicus
Corybas.abellianus_SLE366
Corybas.undulatus_SLE014
Corybas.oblongus_SLE354
Corybas.hispidus_SLE020
Corybas.fimbriatus_SLE035
Corybas.pruinosus_SLE025
Corybas.incurvus_SLE034
Corybas.dentatus_SLE490
Corybas.diemenicus_SLE038
Corybas.expansus_SLE463
Corybas.despectans_SLE016
Corybas.limpidus_SLE406
Corybas.recurvus_SLE005
Corybas.rivularis_SLE117
Corybas.papa_SLE357
Corybas.iridescens_SLE343
Corybas.orbiculatus_SLE112
Corybas.trilobus_SLE320
Corybas.sulcatus_SLE342
Corybas.macranthus_SLE115
Corybas.acuminatus_SLE330
Corybas.cryptanthus_SLE110
Corybas.unguiculatus_SLE055
Corybas.montanus_SLE008
Corybas.rotundifolius_SLE111
Corybas.fordhamii_SLE515
Corybas.carsei_SLE114
Corybas.abditus_SLE564
Cyrtostylis.robusta_SLE068
Cyrtostylis.huegelii
Cyrtostylis.reniformis_SLE001
Acianthella.elegans
Acianthella.confusus
Acianthella.macroglossa_SLE597
Acianthopsis.cymbalariifolius
Acianthopsis.bracteatus_SLE596
Stigmatodactylus.sikokianus
Stigmatodactylus.lamrii_SLE136
Stigmatodactylus.variegatus_SLE285
Stigmatodactylus.croftianus_SLE273
Acianthus.fornicatus_SLE063
Acianthus.exsertus_SLE061
Nemacianthus.caudatus_SLE066
Townsonia.viridis_SLE595
Spuracianthus.atepalus_SLE593

Figure 7.

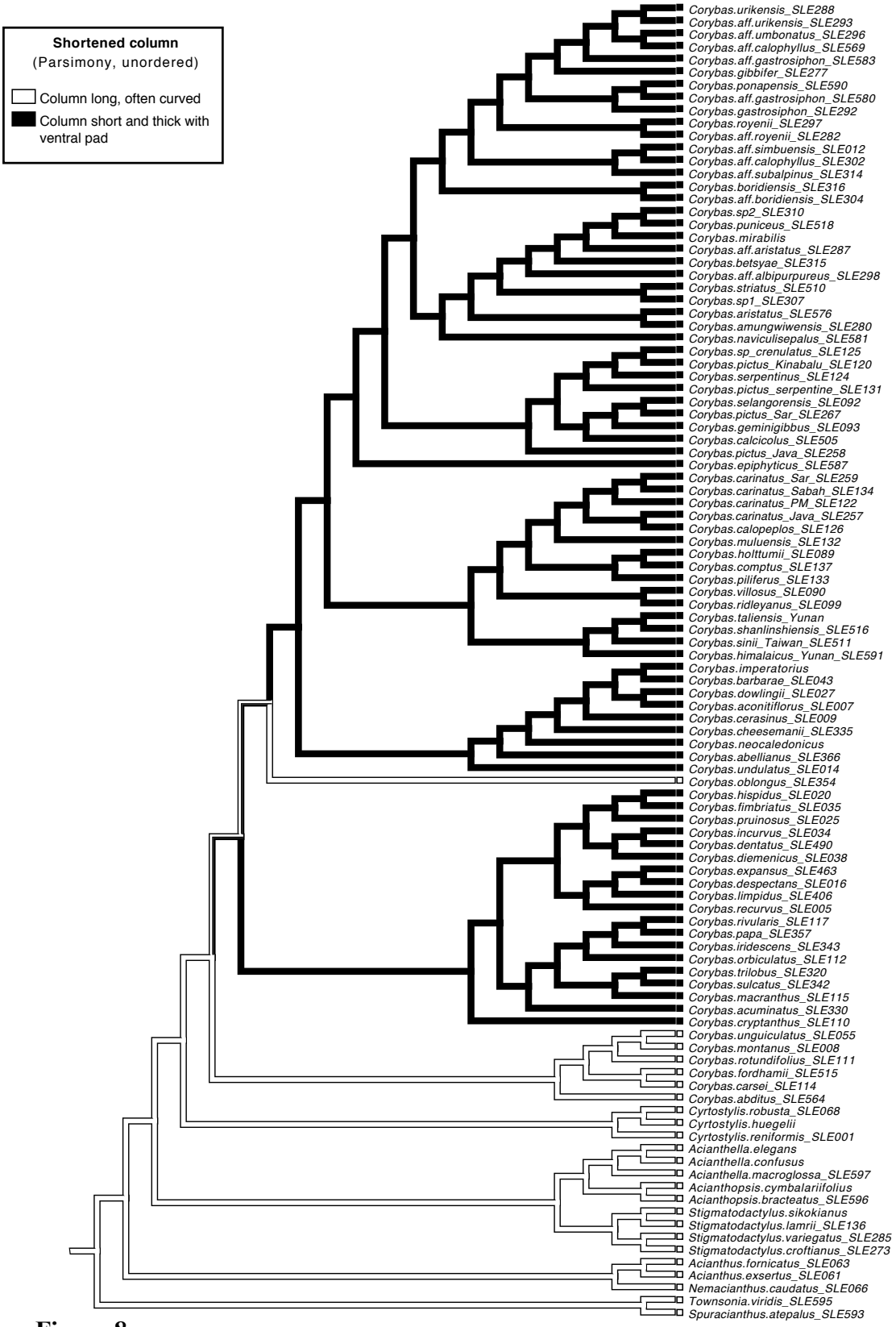
Shortened column
(Parsimony, unordered)
Column long, often curved
Column short and thick with ventral pad
Corybas.urikensis_SLE288
Corybas.aff.urikensis_SLE293
Corybas.aff.umbonatus_SLE296
Corybas.aff.calophyllus_SLE569
Corybas.aff.gastrosiphon_SLE583
Corybas.gibbifer_SLE277
Corybas.ponapensis_SLE590
Corybas.aff.gastrosiphon_SLE580
Corybas.gastrosiphon_SLE292
Corybas.royenii_SLE297
Corybas.aff.royenii_SLE282
Corybas.aff.simbuensis_SLE012
Corybas.aff.calophyllus_SLE302
Corybas.aff.subalpinus_SLE314
Corybas.boridiensis_SLE316
Corybas.aff.boridiensis_SLE304
Corybas.sp2_SLE310
Corybas.puniceus_SLE518
Corybas.mirabilis
Corybas.aff.aristatus_SLE287
Corybas.betsyae_SLE315
Corybas.aff.albipurpureus_SLE298
Corybas.striatus_SLE510
Corybas.sp1_SLE307
Corybas.aristatus_SLE576
Corybas.amungwiwensis_SLE280
Corybas.naviculisepalus_SLE581
Corybas.sp_crenulatus_SLE125
Corybas.pictus_Kinabalu_SLE120
Corybas.serpentinus_SLE124
Corybas.pictus_serpentine_SLE131
Corybas.selangorensis_SLE092
Corybas.pictus_Sar_SLE267
Corybas.geminigibbus_SLE093
Corybas.calcicolus_SLE505
Corybas.pictus_Java_SLE258
Corybas.epiphyticus_SLE587
Corybas.carinatus_Sar_SLE259
Corybas.carinatus_Sabah_SLE134
Corybas.carinatus_PM_SLE122
Corybas.carinatus_Java_SLE257
Corybas.calopeplos_SLE126
Corybas.muluensis_SLE132
Corybas.holttumii_SLE089
Corybas.comptus_SLE137
Corybas.piliferus_SLE133
Corybas.villosus_SLE090
Corybas.ridleyanus_SLE099
Corybas.taliensis_Yunan
Corybas.shanlinshiensis_SLE516
Corybas.sinii_Taiwan_SLE511
Corybas.himalaicus_Yunan_SLE591
Corybas.imperatorius
Corybas.barbarae_SLE043
Corybas.dowlingii_SLE027
Corybas.aconitiflorus_SLE007
Corybas.cerasinus_SLE009
Corybas.cheesemanii_SLE335
Corybas.neocaledonicus
Corybas.abellianus_SLE366
Corybas.undulatus_SLE014
Corybas.oblongus_SLE354
Corybas.hispidus_SLE020
Corybas.fimbriatus_SLE035
Corybas.pruinosus_SLE025
Corybas.incurvus_SLE034
Corybas.dentatus_SLE490
Corybas.diemenicus_SLE038
Corybas.expansus_SLE463
Corybas.despectans_SLE016
Corybas.limpidus_SLE406
Corybas.recurvus_SLE005
Corybas.rivularis_SLE117
Corybas.papa_SLE357
Corybas.iridescens_SLE343
Corybas.orbiculatus_SLE112
Corybas.trilobus_SLE320
Corybas.sulcatus_SLE342
Corybas.macranthus_SLE115
Corybas.acuminatus_SLE330
Corybas.cryptanthus_SLE110
Corybas.unguiculatus_SLE055
Corybas.montanus_SLE008
Corybas.rotundifolius_SLE111
Corybas.fordhamii_SLE515
Corybas.carsei_SLE114
Corybas.abditus_SLE564
Cyrtostylis.robusta_SLE068
Cyrtostylis.huegelii
Cyrtostylis.reniformis_SLE001
Acianthella.elegans
Acianthella.confusus
Acianthella.macroglossa_SLE597
Acianthopsis.cymbalariifolius
Acianthopsis.bracteatus_SLE596
Stigmatodactylus.sikokianus
Stigmatodactylus.lamrii_SLE136
Stigmatodactylus.variegatus_SLE285
Stigmatodactylus.croftianus_SLE273
Acianthus.fornicatus_SLE063
Acianthus.exsertus_SLE061
Nemacianthus.caudatus_SLE066
Townsonia.viridis_SLE595
Spuracianthus.atepalus_SLE593

Figure 8.

Figure 9.

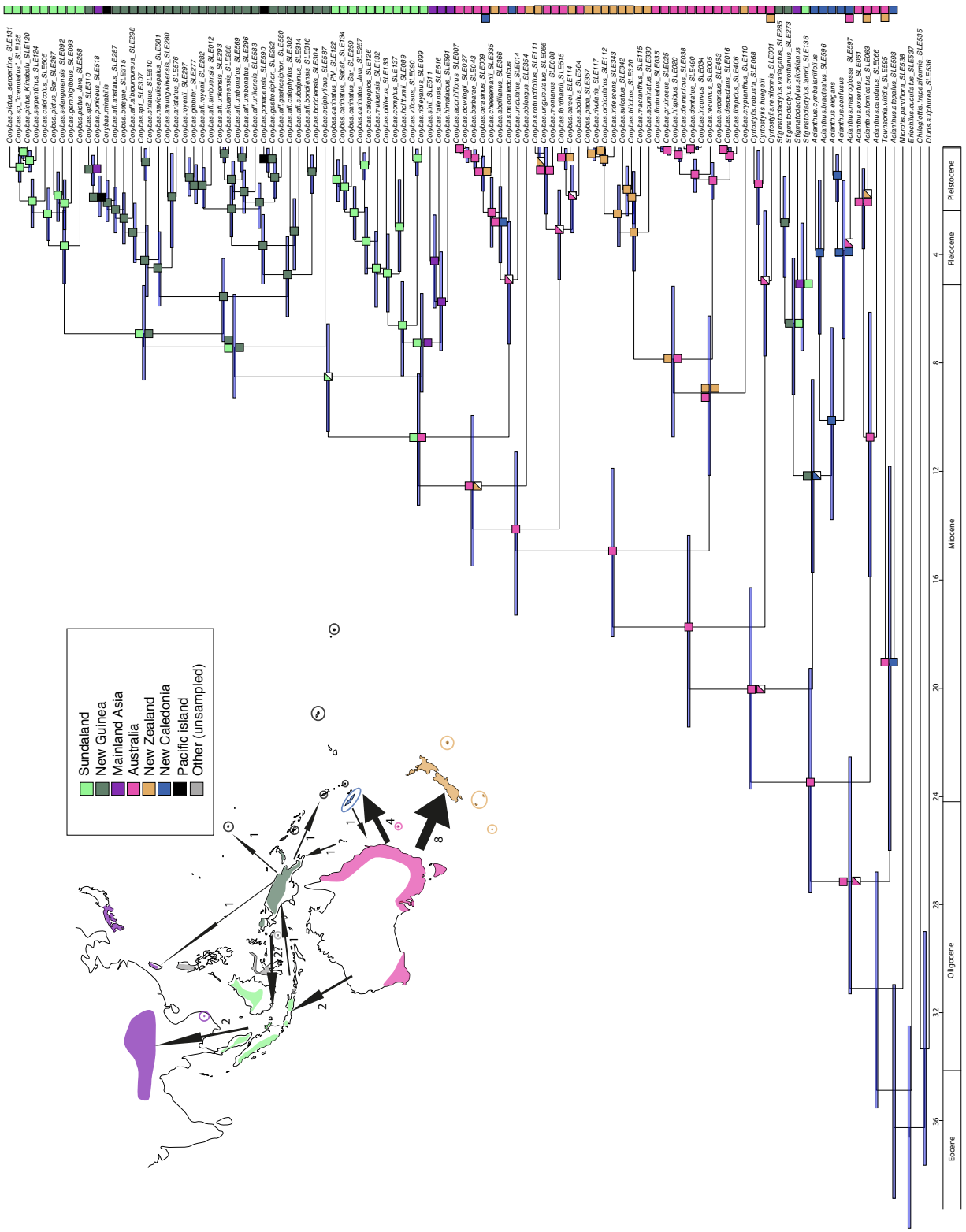
Sundaland
New Guinea
Mainland Asia
Australia
New Zealand
New Caledonia
Pacific island
Other (unsampled)
Eocene
Oligocene
Miocene
Pliocene
Pleistocene
36
32
28
24
20
16
12
8
4

Figure 10.

Chapter 2: Phylogeny and species delimitation in the Corysanthes clade

Abstract

Problems with elucidating species boundaries and evolutionary history abound in groups that have high rates of speciation, such as orchids. In order to reconstruct the phylogenetic and phylogeographic history in a recently radiated, Australian endemic clade (the Corysanthes clade) in the terrestrial orchid genus *Corybas* (Acianthinae, Diurideae), we used both sequencing of standard molecular markers (plastid spacers, nrITS, and the single-copy nuclear gene phytochrome C) as well as a form of reduced representation library sequencing, genotyping-by-sequencing (GBS). Phylogenetic analyses of the GBS data provided an unprecedented degree of resolution within this group, while remaining highly congruent with the results from the standard molecular regions. Based on our findings, we recommend reducing the number of species in this clade from 10-12, to seven monophyletic, strongly supported, morphologically distinct species (*Corybas fimbriatus*, *C. hispidus*, *C. pruinosus*, *C. diemenicus*, *C. incurvus*, *C. recurvus*, and *C. despectans*). *Corybas dentatus* appears to be a hybrid of *C. diemenicus* and *C. incurvus*. *Corybas limpidus* forms a basal grade relative to *C. despectans*, and *C. expansus* appears embedded within *C. despectans*. On the other hand, several morphological forms recognized by local botanists do appear to represent distinct evolutionary lineages and should be protected to preserve infraspecific genetic diversity and evolutionary potential.

Introduction

Systematists face perpetual challenges in elucidating species boundaries and evolutionary history in recently evolved groups. These problems abound in groups that have high rates of speciation. One such group is the Orchidaceae, likely the largest plant family in the world, with some 26,000 described species and an estimated 30,000 in total (Joppa et al., 2011). There are various explanations for the high rate of speciation in this family. These include specialized interactions with pollinators and fungi (Darwin 1885; Dressler 1981; Hapeman and Inouye, 1997; Johnson et al. 1998; Schiestl and Schlüter, 2009), deceit in interactions with mutualists (Cozzolino and Widmer, 2005; Jersacova et al., 2006), epiphytism and associated adaptations (Gentry and Dodson, 1987; Gravendeel et al., 2004; Silvera et al., 2009), and highly skewed reproductive success rates leading to small effective population sizes and high levels of genetic differentiation (Tremblay et al., 2004; cf. Phillips et al., 2012).

Perhaps not surprisingly, debates about species delimitation abound in orchid taxonomy, and are particularly common in well-studied terrestrial taxa such as *Ophrys* (Devey et al., 2008) and *Dactylorrhiza* (Devos et al., 2006). Because of the charismatic nature of orchids—combined with the asymmetry in the number of trained taxonomists working in temperate, developed, but species-poor regions, as compared to speciose, poor tropic ones—there is a recognized problem with taxonomic exaggeration bias (Pillon and Chase, 2007). In some cases, though, morphological variation may represent real genetic or even epigenetic (Paun et al., 2010) variation. Protecting this variation, whether or not it warrants recognition at the species level, is important to ensure persistence and

adaptability of taxa. This is especially so when in response to changing environmental conditions.

When the rates of morphological diversification are high, researchers have frequently found it difficult to achieve phylogenetic resolution at the species level—let alone the intraspecific level—using standard phylogenetic markers (e.g. Mant et al., 2002; Farrington et al., 2009; Howard et al., 2014). Lack of resolution also hinders efforts to understand processes and patterns of diversification, and hence our ability to understand the high rates of diversification in Orchidaceae. Researchers have had variable success using population-genetic markers such as AFLPs (Hedren et al., 2001; Mant et al., 2005; Devey et al., 2009; Indsto et al., 2009), but these are not always reproducible, there are concerns about homology of fragments especially as genetic distances increase, and major variation in genome size across orchids make such techniques unsuitable for groups with large nuclear genomes (Fay et al., 2005).

Recent advances in technology, particularly the advent of next generation sequencing (NGS), have opened up the possibility of obtaining large amounts of genetic information for systematic studies in non-model organisms (Lemmon and Lemmon, 2013; Lexer et al., 2013; McCormack et al., 2013). These techniques are quite new, and have thus far been used in relatively few empirical studies, but have shown enormous promise for resolving phylogeny on the infraspecific level (Emerson et al., 2010; Reitzel et al., 2013), the species level (Eaton and Ree, 2013; Wagner et al., 2013; Cruaud et al., 2014), and even the family level (Viricel et al., 2013).

Corybas, is a diverse genus of tiny, terrestrial orchids. As part of our systematic

studies of this genus, we tackle these issues of fine-scale phylogeny and species delimitation in a clade that showed little variation with traditional plastid and nuclear sequence data. Our hope was that NGS data could resolve the very recent divergence within that clade and serve as a model for future studies in *Corybas* and other taxonomically complicated groups. The Corysanthes clade (Fig. 1), identified by Clements et al. (2002) and described as a separate genus by Jones et al. (2002), is endemic to Australia and occurs in all temperate states including Tasmania. It is the only major clade in the genus endemic to a single, well-studied continent. This allowed thorough sampling within and among species. The clade separated from its closest extant relatives 6-7 million years ago, and existing species appear to have arisen within the last 1-2 million years (Chapter 1). The Corysanthes clade is generally considered to contain at least 10 species (WCSP, 2014). Some authorities recognize more (Jones 2007, Jones 2009) and additional morphological forms have been proposed as distinct species (Jones, 1993; Jeanes and Backhouse, 2006; Brown et al., 2008; Bates, 2011). Table 1 shows a list of recognized species and tag names, together with a comparison of their morphological features. Half these species have been described in the last 40 years. Habitat preferences in this group range widely, from coastal sand dunes to temperate rain forest and from elevations at sea level to ca. 1000 meters. *Corybas dentatus* and *C. aff. diemenicus* "Tea tree swamp" are federally protected. Species boundaries are uncertain, especially in the *C. diemenicus* and *C. despectans* complexes. One hybrid has been described, and the species *C. dentatus* appears to be morphologically intermediate between two other species (Bates 2009).

We wished to reconstruct phylogenetic and phylogeographic history in this group and understand patterns of genetic diversity to revise it. To do so, we used both sequencing of standard molecular markers (plastid spacers, nrITS, and the single-copy nuclear gene *PhyC* as well as a form of reduced representation library sequencing (genotyping-by-sequencing, or GBS, (Elshire et al., 2011)). We then relate these patterns to the morphology, ploidy, and habitat preferences of these taxa. Furthermore, we examine their history in the context of geological and climatology history within Australia. We also make recommendations for the classification of this group.

Methods

Sampling

For each population sampled, we collected at least two plants, often five or more, and sampled as broadly across the population as possible. Particularly in cases where multiple species of *Corybas* occur, we sampled flowering plants or plants immediately next to flowering plants. *Corybas* reproduces clonally (Pridgeon and Chase, 1995), though the connection between mother and daughter shoots only lasts for one growing season. While clone size is not easily determined, discrete patches of plants are often found within populations. We collected one or two plants per patch, leaving tubers in the ground to allow plants to regenerate. Leaf tissues were removed and dried in silica gel or a lyophizer soon after harvesting.

Almost all samples were collected between June 2008 and August 2011, mostly

on collecting trips by the first two authors. A network of professional and skilled amateur botanists (the Orchid Research Group [ORG]) in South Australia, Victoria, Tasmania and New South Wales provided many additional vouchered samples. We collected plant tissue from multiple populations of all 10 species in the Corysanthes clade recognized by the Kew World Checklist (WCSP, 2014), as well as most of the recognized morphological forms (Tables 1, 2). In selecting sampling areas for each species, we attempted to collect from as broad a geographical area as possible. Despite multiple attempts, we were unable to obtain material from two important regions/groups: the northern Queensland disjunct population of *C. fimbriatus*, and the Southern Tablelands form of *C. diemenicus* (*Corysanthes grumula* Jones).

Flow cytometry

We used flow cytometry to determine genome size for a handful of accessions across the Corysanthes clade. Previous attempts at AFLPs had shown a distinctive pattern of several large peaks swamping the signals of numerous smaller peaks. This suggests a large genome (Fay et al., 2005), thus it was important to understand genome size before planning for genomic-level work. Reported chromosome counts vary across the genus (Mehra and Sehgal, 1976; Peakall and James, 1989; Dawson, 2000; Dawson et al., 2007). In the Corysanthes clade, the only available count is from *C. recurvus* (2N=54). The two clades closest to Corysanthes, Nematoceras and Molloybas (*C. cryptanthus),* have 2N values of 34 and 36 (occasional tetraploids with 72), respectively, suggesting that cytological evolution may have been important in this group.

Sample preparation for flow cytometry followed the procedure described in (Doležel et al., 1989), using 0.5 mL of buffer for chopping, and eliminating centrifugation to avoid damaging nuclei. One silica-dried leaf was chopped using a razor blade along with 1 cm^2 of fresh pea standard in LB01 buffer (Doležel et al., 1989) in a Petri dish. The suspension was filtered through CellTrics 30 μm filters (Partec, Swedesboro, NJ) and incubated on ice for five minutes to one hour while other sample preparation continued. Propidium iodide and RNase (each at a final concentration of 50 μg/mL) were added to each sample 5 minutes before the sample was analyzed in the flow cytometer.

Samples were run on an Accuri™ C6 Flow Cytometer (BD Bioscience, San Jose, California) in the Abbott Lab in the Engineering Department of the University of Wisconsin-Madison, with a 488 nm 50 mW solid state laser. Pulse area was detected using a FL2-A detector (585 nm mean/42 nm bandwidth). We used the software "CFlow Plus" (BD Bioscience) to visualize histogram peaks and calculate means. DNA content was calculated in reference to the genome size of *Pisum sativum* (1C=4.88, Kew Plant DNA C-values Database).

DNA extraction and Sanger sequencing

We extracted genomic DNA using either the EZNA Plant Mini Kit (Omega Bio-tek, Norcross, GA) or DNEasy Plant Mini Kit (QIAGEN, Germantown, MD). A single individual from three or more populations per species was selected for sequencing, with

additional individuals added when there were potentially questionable results. For each of these individuals, we sequenced four highly variable, rapidly evolving intergenic chloroplast spacers (*psbJ-petA*, *rps16-trnQ*, *psbD-trnT*, *trnL-trnF*), nrITS, and *PhyC* (phytochrome C). DNA amplification and sequencing followed Shaw et al. (2007) for the chloroplast spacers. For ITS, we used the standard primers of White et al. (1990), but substituted the ITS5A primer developed by (Downie and Katz-Downie, 1996) to correct for 2 base-pair substitutions specific to the angiosperms. For *PhyC*, we followed the PCR program of Russell et al. (2010), but used primers developed specifically for orchid tribe Diurideae (Chapter 1). PCR products were imaged on a 1.5 % agarose gel, cleaned with ExoSAP (Affymetrix, Santa Clara, CA), and sequenced using ABI BigDye technology (Life Technologies, Grand Island, NY). Before submitting sequences to the UW-Madison Biotech Center for sequencing, reactions products were cleaned over a Sephadex column in a Millipore plate (EMD Millipore, Billerica, MA).

Sequences were assembled and edited using Geneious Pro v 5.4.3. Alignments were generated using the ClustalX (Larkin et al., 2007) or Consensus Align algorithms as implemented in (Biomatters Ltd., Auckland, New Zealand), and adjusted manually as necessary to account for the misplacement of indels using these algorithms. With ITS and *PhyC*, some sequences showed a small percentage of polymorphic bases. These were coded using the standard IUPAC nucleotide ambiguity codes. The incongruence length difference test (ILD) (Farris et al., 1994), as implemented in PAUP 4.0d102 (Swofford, 2003), was used to test for congruence among data sets. There is a tendency for this test to incorrectly reject the hypothesis of congruence in certain circumstances (Darlu and

Lecointre, 2002). Consequently, we also visually inspected our trees for individual partitions and analyzed our concatenated data when the individual analyses did not yield any hard incongruences.

We used maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) to reconstruct relationships in the Corysanthes clade using sequence data. MP analyses employed PAUP 4.0d102, using 100 repetitions of random sequence addition and TBR branch swapping to search for multiple islands of equally parsimonious trees. Parsimony bootstrap analyses used 100 repetitions, each using TBR and 10 random starting trees.

The optimal model for nucleotide evolution for each partition was chosen using jModelTest (Posada, 2008)—in all cases the optimal model was determined to be GTR+G. Maximum likelihood and Bayesian analyses were run via the CIPRES science gateway V3.3 (Miller et al., 2010), using RAxML 7.2.7 (Stamatakis, 2014) and MrBayes 3.2.2 (Ronquist et al., 2012), respectively. RAxML ran 100 ML bootstrap replicates for each analysis. For the combined data, we ran our ML analysis using GARLI 2.01 (Zwickl, 2006) allowing for optimization of parameters separately for each partition, also implemented on the CIPRES gateway, but used RAxML for bootstrap analyses. Two simultaneous runs were implemented for each MrBayes analysis, using four chains each, standard prior settings, up to one million generations (sampled every 1000 generations and discarding the first 25% as burn-in) and automatically stopped when the two runs had sufficiently converged. Posterior probabilities for clades were estimated as the frequency of trees containing a particular clade in the post-burn-in fraction of sampled trees.

GBS (genotyping by sequencing)

We generated reduced representation genomic libraries for Illumina sequencing using the genotyping-by-sequencing (GBS) method described by Elshire et al. (2011). The method is similar to the more commonly used RADseq technique (Baird et al., 2008; Hohenlohe et al., 2010, 2012; Peterson et al. 2012), in which genomic DNA is digested with a restriction enzyme, ligated to barcoded adapters, amplified, and – after additional processing -- sequenced in a flow cell using Illumina's sequencing-by-synthesis technology (Illumina Inc., San Diego, CA). The process differs from traditional RADseq in that it skips the random shearing and size selection steps. Because sequences are restricted to the ends of fragments and the single PCR step strongly biases the fragment pool towards shorter fragments, the pool of amplicons tends to be smaller with GBS than with RADseq. However, a smaller fragment pool leads to greater depth of sequencing, and in our case where we had concerns about genome size and were multiplexing 96 samples per lane, this was seen to be an advantage of the technique.

The University of Wisconsin-Madison Biotech center conducted the library preparation and Illumina sequencing. Before submitting samples for processing, DNA concentrations of selected extracts were quantified using a Qubit 2.0 fluorometer (Life Technologies, Grand Island, NY) and standardized across the plate. A subset of samples was digested with a restriction enzyme to confirm that inhibitory substances would not prevent digestion during library preparation. One hundred and seventy-nine DNA extracts were selected for processing on the basis of having relatively intact genomic DNA. We tried to include three individuals per population, representing at least one

(usually three or more) population of each species or distinct form. This was not always possible due to minimal sampling in some cases (particularly those samples sent in from the ORG network) and/or poor quality extracts; thus several populations are represented by only one or two individuals. Nine of these extracts were repeated on a different plate, and four water blanks were added as controls. This resulted in two full plates, each of which was run on one lane of a flow cell.

To digest samples, we used the enzyme *ApeK1*, which is less likely to cut at methylated CG-rich regions. After the digest, sticky fragment ends were ligated to adapters that included one of the Illumina adapter sequences (GBS does not use standard Y-adapters), the Illumina sequencing primer, and a 4-8 base pair adapter. These ligated samples were pooled (96 samples per plate), amplified, cleaned using magnetic bead to remove any fragments smaller than 100 bp (including adapter dimers), and sequenced on the HiSeq2000 using single-end, 100 bp reads.

After initial processing at the Biotech center, the resulting sequences were demultiplexed, barcodes were removed, and files were renamed to reflect sequence identity using a combination of cutadapt (Martin 2011) and shell scripts. We then processed sequences for each sample through the Stacks pipeline (Catchen et al. 2011) using the UW-Madison HPC cluster at the Center for High Throughput Computing. In forming unique stacks for each individual (ustacks module), we specified a minimum read depth of three, a maximum distance of two base pairs, and allowed Stacks to break up or remove "lumberjack" stacks—that is, stacks over-represented in a sample likely resulting from regions of highly repetitive DNA. To create the catalog of loci used to

score individual samples (cstacks module), we used 133 of the 174 non-water samples that yielded stacks. We excluded samples with an unusually large number of tags from catalog creation. We suspect the large number of tags in some of these samples may have resulted from random environmental or laboratory contamination and, since we only used loci present in at least one-third of populations (see below), would have been dropped from further analyses in any case. Furthermore, inclusion of all these loci in the catalog vastly increased analysis time and was not feasible due to resource constraints. These samples were scored, however, for all cataloged loci. In scoring loci, Stacks automatically excludes tags that match to more than one locus in the database, as well as any loci that appear to have any more than two alleles in a given individual.

After samples were scored for the presence/absence and genotype of all loci in the catalog, samples were processed through the Populations module of Stacks. Of the 72 populations that were included in the final analysis, SNPs had to be present in at least one individual from a minimum of 25 populations. Once again, we set a minimum read depth of three to score alleles at a particular locus. Various population cutoffs were tested; this seemed to provide a reasonable balance between maximizing phylogenetic information while allowing for fast processing and avoiding potential artifacts related to random environmental contamination. A previous analysis (Wagner et al., 2013) presented a strong case for using as many SNPs as possible to maximize phylogenetic resolution, even when this resulted in a large amount of missing data.

For phylogenetic analyses, we pooled data for the one to three individuals within each population. Variable loci within populations/individuals were included in

phylogenetic analyses and scored using standard ambiguity codes. We analyzed concatenated SNP data using both maximum likelihood implemented in RaxML 7.2.7 (Stamatakis, 2014) and maximum parsimony implemented in PAUP 4.0d102 (Swofford, 2003) to elucidate phylogenetic and phylogeographic history. Search parameters were the same as those described above. Because model-based phylogenetic approaches are intended for analyses of full sequence data (whereas the standard phylogenetic output from Stacks excludes all invariant sites), the use of maximum likelihood might be inappropriate. However, by using a GTR+G model of evolution, thus allowing for optimization of substitution rates and variation in rates among sites, the ML analysis showed more reasonable of populations with substantial amounts of missing data. The MP analyses tended to place them, with weak support, as sister to all other populations within the clade. In any case, results from the two analyses were largely congruent.

Rooting the trees generated by analyses of the GBS data proved somewhat problematic. Because the Corysanthes clade is on a relatively long branch and our use of the Stacks pipeline set fairly stringent requirements for determining homologous loci, we did not include material from neighboring clades in our initial GBS runs. In retrospect, this was a mistake, as even a relatively small number of scored loci in a small number of outgroup taxa would probably have allowed us to root the tree with some confidence. In addition, our analyses of nuclear and chloroplast genes were not particularly effective in resolving relationships at the root of this clade. However, our phylogenetic trees for the chloroplast and combined data sets, rooted with several outgroup taxa, show support for a clade consisting of the exclusively eastern taxa (*C. incurvus*, *C. diemenicus s.l.*, *C.*

hispidus, *C. fimbriatus*, and *C. pruinosus*) and two primarily western clades (*C. recurvus* and *C. despectans/limpidus/expansus*). Analyses of the chloroplast data showed moderate to strong support for the sister relationship of *C. recurvus* to the eastern clade, and since no other analyses supported any other arrangement, this is how we rooted our GBS trees.

Results

Flow cytometry:

As shown in Table 3, all analyzed accessions had an estimated 1C value around 9.5 pg. This confirmed that our genome sizes were large, and validated our choice of a methylation-sensitive enzyme. We also found no evidence of variation in genome sizes across the Corysanthes clade, though admittedly our sampling was limited.

Sanger sequencing:

Figures 3 through 6 display phylogenetic trees constructed from chloroplast, ITS, *PhyC*, and combined data, respectively. In at least some of these trees, we see evidence of six main clades: 1) a *C. despectans/limpidus/expansus* (*C. despectans s.l.*) group, 2) a *C. diemenicus/dilatatus/aff. diemenicus* (*C. diemenicus s.l.*) group and 3) a closely related *C. incurvus/dentatus* group, 4) a C. *recurvus* group, 5) a *C. hispidus/fimbriatus* group, and 6) a *C. pruinosus* clade. No single region provided support for all of these groups, however.

In regards to the C. *diemenicus s.l.* and *C. incurvus/dentatus* groups, chloroplast data provided no resolution, but both ITS and *PhyC* sequences largely differentiated these two clades, admittedly with limited support. However, there is evidence of gene flow between them at one site in South Australia (Sandy Creek Conservation Park). Specimens identified as *Corybas dentatus* from Sandy Creek group with *C. diemenicus s.l.* using ITS (Fig. 4) but not *PhyC* (Fig. 5). One specimen of *C. "dilatatus"* from Sandy Creek groups with *C. dentatus* using *PhyC* but not ITS. In addition, the other two specimens from this site show highly polymorphic *PhyC* sequences. *Corybas dentatus* has been suggested to have a hybrid origin (Bates, 2011), possibly the result of a *C. dilatatus* x *C. incurvus* cross. The *C. "dilatatus*" *PhyC* sequence clustering with *C. dentatus* may represent an additional case of introgression not resulting in significant morphological change.

Even with samples of *C. "dilatatus"* from Sandy Creek and all accessions of *C. dentatus* removed from the analyses, the ILD test still suggested significant incongruence among data partitions (p=0.04). However, as the individual gene trees lacked any regions of hard incongruence, we also analyzed this combined data set. The combined analysis (Fig. 6) yielded strong support for all six clades, and resolved most of the relationships among them. Relationships at the base of the Corysanthes clade were still essentially unresolved, with a polytomy consisting of *C. recurvus*, *C. despectans*, and a clade consisting of all the exclusively eastern taxa. The chloroplast data (Fig. 3) supported a sister relationship between C. recurvus and the eastern taxa, though this was not uncovered in the combined analysis. Within the eastern taxa, the combined analysis showed strong support for sister relationships between *C. incurvus* and *C. diemenicus s.l.*,

and between *C. pruinosus* and *C. fimbriatus* + *C. hispidus*. The lack of differentiation between C. *hispidus* and C. *fimbriatus* was surprising, as they are both morphologically and ecologically distinct (Table 1).

Little phylogenetic structure was evident within the *C. despectans* group, though there was some support for distinct *C. expansus* and eastern (South Australian=SA) *C. despectans* clades. However, all accessions from Western Australia (WA) plus *Corybas aff. limpidus* "fat dwarf" from South Australia formed an unresolved grade relative to these two moderately supported clades. Within *C. diemenicus s.l.*, some accessions contained a small number of unique SNPs—particularly *C. "longitubus", C. aff. diemenicus* "tea tree swamp", and the Tasmanian accessions—but these provided no phylogenetic signal. In the combined analysis, there was weak support for a sister relationship between *C. "longitubus"* and the rest of *C. diemenicus s.l.* (excluding, of course, the *C. "dilatatus"* accessions from Sandy Creek, which were removed due to suspicion of introgression from *C. incurvus*).

GBS

Both plates submitted for GBS library preparation and sequencing had between 130 and 140 million reads. Despite efforts to standardize DNA concentrations across samples, the number of reads per sample was highly variable, ranging from less than a thousand to several million. All water controls had very few reads, and even fewer stacks (between 0 and 10). Unfortunately, several samples also yielded few to no stacks, and we

had to exclude them from our analyses. Generally, these were older, poorer quality DNA extracts. All three samples from one population of *C. incurvus*, at Conimbla National Park in NSW, failed to yield any usable data. In this particular case, we suspect that the very young, not fully expanded leaf tissue may have contained inhibitory compounds that prevented *ApeK1* from cutting. Ultimately 160 samples (of the initial 179 unique samples) were included in phylogenetic analyses of populations.

After restricting our data set to loci that were present in at least 25 of the 72 populations but allowing for polymorphisms within individuals and populations, we obtained a matrix of 118,587 bases (SNPs) from 32,206 loci. Phylogenetic analyses of the 72 distinct populations (where a population consisted of between 1 and 3 individuals of a particular taxon or morphological form at a given site) yielded highly resolved trees (Figures 5 and 6). Results were largely congruent between maximum likelihood (ML) and maximum parsimony (MP) analyses.

Both analyses provided strong support for at least seven taxa: *C. hispidus*, *C. fimbriatus*, *C. pruinosus*, *C. diemenicus s.l.*, *C. incurvus* including *C. dentatus*, *C. recurvus*, and *C. despectans s.l* (Fig. 7 and 8). A good deal of genetic structure was evident within each of these clades as well, though not many relationships among populations were strongly supported. Within *C. diemenicus s.l.*, both analyses found strong support for a sister relationship between "*C. longitubus"* from the Barrington Tops and the rest of the clade, as well as strong support for a clade consisting of all sampled *C. aff. diemenicus* "Tea tree swamp" populations. Within the *C. despectans s.l.* clade, both analyses showed strong support for a clade consisting of all *C. despectans* and *C.*

expansus populations from South Australia, excluding the unusual *C. aff. limpidus* "fat dwarf" from Newland Head which formed part of the otherwise Western Australian grade relative to this set of populations. *Corybas expansus* accessions and South Australian *C. despectans* accessions formed two distinct, well-supported clades, with the exception of one population assigned to *C. expansus* that grouped with *C. despectans*. The one other consistently well-supported relationship between populations was found within *C. hispidus*, in which the collection from Mihi Gorge near Armidale, NSW and the collection from Mt. Hamilton, Victoria were strongly supported as sister. Despite the geographical distance between them, both of these populations contain plants with particularly large flowers.

Discussion

Comparison of methods

We are unaware of any papers that have used the GBS protocol to generate data for molecular phylogenetics. A handful of studies have used the related RADseq protocol (Eaton and Ree, 2013; Wagner et al., 2013; Cruaud et al., 2014), however. One known problem, especially when multiplexing at this level, is that many loci are poorly represented across samples (Wagner et al., 2013). For instance, when we specified 50 rather than 25 as the minimal number of populations in which a locus had to be found in order to be included in our analyses, the number of included loci dropped from over 10,000 to under 300. Many of these "missing" data points may represent real losses of

restriction enzyme cutting sites. They could potentially serve as characters for phylogenetic analyses in and of themselves. However, often the missing data appeared to be randomly distributed across taxa and populations. Given the large variance in the number of reads per sample, likely many fragments simply did not amplify.

Despite the substantial proportion of missing data, GBS data vastly improved tree resolution and support values relative to sequence data. Doubtless, this related to our ability to include tens of thousands of variable sites as opposed to, for instance, only 58 (of 3858) variable sites within the ingroup of our concatenated 5-gene data set. For instance, the GBS data provided clear separation of *C. hispidus* and *C. fimbriatus*, which, despite their clear morphological and ecological differences, had proven essentially genetically identical at every standard locus sequenced. Analysis of the GBS data set also yielded clear, strongly supported resolution of relationships among taxa, and supported several distinct evolutionary lineages within taxa—including several morphological forms that local botanists had recognized as unique.

The results of our GBS analyses were also largely congruent with the tree we obtained by concatenating chloroplast, ITS, and *PhyC* data. This 5-gene data set did not provide strong support of structure within taxa. That said, there were hints of, for instance, *C. expansus* and the eastern populations of *C. despectans* forming distinct clades, and indications that *C. diemenicus* accessions from Tasmania, *C. aff. diemenicus* "tea tree swamp", and *C. "longitubus"* were genetically distinct. The sister relationships of taxa, at least when our GBS trees are rooting according to results from the 5-gene analyses, are also preserved.

The "broom-and-handle" phylogeny that characterizes the Corysanthes clade—i.e. a long, naked branch ending in an essentially unresolved "comb" of recently divergence taxa—is likely the result of extinction and/or increased speciation resulting from changing climates in Australia, especially following the onset of extreme aridity at the beginning of the Pleistocene (Crisp and Cook, 2007, 2009; Byrne et al., 2011). Many other Australian orchids, at well as other lineages that have tended to rely on mesic conditions, exhibit this phylogenetic pattern. Researchers have faced problems in resolving phylogeny and species boundaries in essentially every terrestrial Australian orchid genus (e.g. Clements et al., 2002, 2011; Mant et al., 2002; Farrington et al., 2009; Indsto et al., 2009; Janes et al., 2010). Techniques such as GBS show enormous promise for generating data capable of unraveling evolutionary history in a manner that is relatively quick, inexpensive, and not technically challenging.

Taxon delimitation

Using criteria of reciprocal monophyly, support values, and diagnosability, we propose that seven species in this clade. They are: an expanded concept of *Corybas despectans* Jones, *Corybas recurvus* Jones, *Corybas diemenicus* (Lind.) Rupp & Nicholls, *Corybas incurvus* Jones & Clements, *Corybas pruinosus* (R. Cunn.) Reichb., *Corybas fimbriatus* (Br.) Reichb., and *Corybas hispidus* Jones. While this would entail synonymizing or reducing in rank (to the level of variety) three taxa that have been recognized as distinct for over 20 years—*C. expansus*, *C. limpidus*, and *C. dentatus*—we

do not feel there is sufficient evidence to maintain these as distinct species. We discuss particular groups below.

In the *C. despectans* complex, *C. limpidus* populations form a grade relative to *C. expansus* and *C. despectans*. Indeed, despite being easily separable at their morphological extremes—*C. limpidus* has larger, more open flowers and a substantially wider dorsal sepal—in populations such as Ledge Beach in Gull Rock National Park, WA the two taxa are found growing interspersed on the dunes with morphological intermediates. It was thus not surprising that the sampled "*C. limpidus*" population from this locality fell into the WA *C. despectans* clade. A preliminary population structure analysis (not shown) indicated that that particular population showed evidence of admixture. A primarily WA *C. despectans* clade was moderately supported as sister to a *C. expansus* plus SA *C. despectans* clade, indicating that C. *despectans* was not monophyletic. There are some minor differences between the SA and WA forms of *C. despectans*—the WA populations tend to have paler markings on the labellum and slightly wider dorsal sepals. An unpublished manuscript from David Jones we found at the Adelaide herbarium revealed that at one point he had considered publishing WA *C. "despectans"* as a distinct species. However, given the apparent non-monophyly of *C. limpidus* (not to mention the morphological variation seen in both SA and WA *C. despectans*), this would not solve the taxonomic problems in this group in any case.

C. expansus, differentiated from *C. despectans* by an expanded labellum blade and slight different coloration, also appears to intergrade with *C. despectans* within individual populations (Bates, 2011). At Aldinga Scrub in SA, the *C. "expansus"*

population was found to group with SA *C. despectans* in our analyses. However, the individuals classified as *C. expansus* were growing just a few meters away from individuals of the more typical *C. despectans* form, and once again preliminary population structure analyses (not shown) indicate admixture in this population. The very small amount of genetic variation seen within SA *C. despectans*, and, for the most part, its clear separation from *C. expansus*, may arise from its strong tendency to self-pollinate. Observations of the column structure in *C. despectans* at Newland Head show an essentially nonfunctional viscidium and poor separation of the pollinia and stigmatic surface, so many individuals may be obligately self-pollinating. Furthermore, perhaps due to the exposed coastal conditions in which many *C. despectans* populations grow, the flowers often fully open and hence are essentially cleistogamous. Interestingly, the *C. despectans* populations in WA seem to be primarily outcrossing, while small flowered, self-pollinated forms on the exposed Leeuwin-Naturaliste ridge have distinct tag names (Brown et al., 2008).

Finally, *C. aff. limpidus* "fat dwarf" does indeed appear to represent a separate dispersal to SA. We note, however, that this population had many missing data. This probably explained why MP analysis grouped it as sister to the rest of the C. *despectans* s.l. clade, whereas ML placed it within the WA C. *despectans* clade).

Thus, despite our recommendation that *C. expansus*, *C. limpidus*, *C. aff. limpidus* "fat dwarf" be synonymized with *C. despectans*, the degree to which these names represent real genetic differences is surprising. It points to the keen observation of local botanists and conservation officers. For instance, Newland Head in SA contains three

distinct lineages, all within a relatively small area. (*C. aff. despectans* "red eyed dwarf", on the other hand, appears to be nothing other than an unusual color variant of *C. despectans*). Certainly recognizing and protecting the less common lineages is important for preserving genetic diversity and evolutionary potential of the species, and efforts with SA to protect these discrete varieties should continue. It is also important, both for evolutionary biologists and for conservationists, to understand this diversity within the broader context. Western Australia, in this case, contains many more distinct evolutionary units than South Australia.

The nature of *C. "dentatus*" is still not clear. In none of our analyses does it form a distinctive, well-supported clade. Analyses repeatedly show its close affinity with *C. incurvus*. There is also evidence, at least in the ITS sequences, of introgression with *C. diemenicus s.l.*. It does form a clade in the ML analysis of our GBS data. This suggests that it may be a distinct lineage in a broader *C. incurvus* clade, but a *C. dentatus* clade is not well supported and is not at all apparent in the MP analysis. Furthermore, *C. dentatus* is morphologically intermediate between *C. diemenicus s.l,*. It is only found in areas where the two putative parents co-occur and bloom at the same time, and, while it often appears distinctive within a population, seems to vary across populations. There is even considerable discrepancy between the formal description and the characters used by local botanists and conservation officers to recognize it in the field. This suggests a more likely possibility that *C. dentatus* represents a hybrid of the sister species *C. incurvus* and *C. diemenicus*, likely with strong degree of backcrossing with *C. incurvus*. A preliminary population structure analysis (not shown) also suggested the existence of only two

interbreeding lineages (*C. incurvus* and *C. diemenicus s.l.*), with *C. dentatus* grouping with *C. incurvus* and at least a small amount of introgression evident in accessions of all three.

The consistent finding of *C. recurvus* as a distinct lineage across all our analyses was somewhat surprising. Before 1991, it was considered to be merely a western variety of *C. diemenicus.* The morphological characters that separate it from *C. diemenicus*—very dark, recurved flowers with pronounced downward pointing teeth in the labellum tube—are somewhat cryptic, though they are very consistent across populations. In fact, *C. recurvus* appears to be quite genetically homogeneous, perhaps suggesting a recent population bottleneck followed by dispersal into its current range. Southwestern Australia has a very high rate of plant species endemism—around 50%—and is considered a global biodiversity hotspot (Hopper and Gioia, 2004). It is also quite geographically isolated: between it and southeastern Australia, the central arid zone extends all the way to the coast in the form of the highly calcareous Nullarbor Plain. This forms a strong dispersal barrier to the great majority of plant species. Along with the Western Australian endemic *Corybas abditus*, *C. recurvus* seems to provide yet another example of the uniqueness of the southwestern Australian flora.

There is also substantial genetic variation *within* the *C. diemenicus* complex. Of all the taxa in the Corysanthes complex with ambiguous status, *C. "longitubus"* would be the one lineage to recognize as distinct, a possible eighth species, based on its genetic distinctiveness. It is not particularly morphologically distinctive, however, and the characters used to separate it from the rest of the clade are somewhat subjective. They

are variable within the Barrington Tops area and overlap substantially with the characters that are supposed to distinguish *C. grumulus* in Australian Alps region to the south. Unfortunately, we were unable to sample any of *C. grumulus* populations, but it is possible that with their inclusion, *C. "longitubus"* might appear less distinctive. *Corybas "longitubus"* does represent a majorly disjunct population, however, and it is quite likely that it has a distinct evolutionary history from the rest of the clade. In a situation analogous to that seen in the *C. despectans s.l.* clade, Victoria appears to contain several distinct lineages, and once again local botanists and conservation officials have applied names to these groups. For instance, *C*. aff. *diemenicus* "Tea tree swamp" clearly represents a discrete evolutionary unit, and should be protected as such. There is some support for separate lineages of narrow and wide-flowered forms of *C. diemenicus*. The latter would consist of those labeled *C*. "*dilatatus.* This does not entirely hold up because the Sandy Creek samples appear sister to the narrow-flowered forms, but it is possible its unusual placement could reflect the detected gene flow between *C*. "*dilatatus*" and *C. incurvus* at that particular site. While the ML and the MP GBS analyses do not strongly supported this division, the topology is at least consistent between the two trees.

The correspondence between distinct morphological groups and the names applied to them quickly breaks down when we consider the accessions from Tasmania. We had relatively poor representation of Tasmanian populations in our sampling. However, within the three populations for which we obtained at least some GBS data there seems to be a substantial amount of genetic variation. These accessions are interspersed with much better sampled populations from Victoria and SA. Indeed, other

researchers have also commented on the amount of morphological variation in Tasmania (Jones, 1999). Many forms that appear discrete within Victoria — for instance both dark and pale-bossed forms — are not only also found in Tasmania, but may often be found growing interspersed within populations. While the relationship of the *C. aff. diemenicus* "Tea tree swamp" populations to the Port Sorrell and E Shelley Beach populations was not strongly supported, this topology was consistent between the MP and ML analyses, and it is interesting that the Port Sorrell population primarily consisted of plants with narrow flowers with a dark boss bordered by a broad hispid band—the same characters that define *C. aff. diemenicus* "Tea tree swamp".

Strong phylogeographical connections between Victoria and Tasmania are known to exist (Nevill et al., 2010; Byrne et al., 2011). While researchers have detected ice age refugia in both areas, Tasmania often contains a larger number of distinct, genetically divergent populations. While substantially more sampling would be requited to test this hypothesis, we suspect that the three distinct forms we have sampled from SA and Victoria may represent discrete dispersal events from Tasmania leading to the establishment of morphologically and genetically distinct forms on the mainland. With the exception of the Barrington Tops and possibly Australian Alps populations, Tasmania may have served as an important mesic refugium for much of the *C. diemenicus s.l.* genetic variation observed in mainland Australia today.

C. hispidus, *C. fimbriatus*, and *C. pruinosus* form a well supported clade in our analyses of both the 5-gene data and the GBS data. All share a distinctive morphological trait. Rather than the denticulate or broadly dentate margins of other taxa in the

Corysanthes group, these species have long fimbriae along the labellum margins. These taxa are also all restricted to the eastern edge of Australia. While *C. pruinosus* appeared genetically distinct in the 5-gene data set, *C. hispidus* and *C. fimbriatus* could not be distinguished except in the GBS analyses, where they formed two strongly supported, separate clades. This appears to have been a very recent speciation event. The two lineages clearly warrant recognition at the species level. Each has a number of distinctive morphological traits and the two show clear separation of habitat preference and range.

The lack of obvious geographical structure within many taxa may reflect recent speciation or high rates of gene flow among populations. However, it may also reflect the climatological history of Australia. While Australia was not glaciated, the climate became cooler and extremely arid at each glacial maximum, causing range contractions (Byrne, 2008). Most Australian taxa appear to have weathered these changes in multiple localized refugia as evidenced by highly localized genetic diversity, but certainly not all (Byrne, 2008; Byrne et al., 2011). Given its propensity for moist conditions and its delicate morphology, *Corybas* likely faced quite drastic range contractions. Individual species may have been restricted to one or a few refugia during glacial maxima, and the isolation may actually have prompted differentiation and ultimately speciation.

The proposed taxonomic treatment of the Corysanthes clade may be seen as conservative. It is important to be aware of the effects of taxonomic exaggeration on conservation efforts, however (Pillon and Chase, 2007). The rank of species may be an arbitrary, subjective designation in many cases. Nonetheless, scientists, administrators, and conservationist removed from the field of systematics are often not aware of that. It is

important to be consistent in setting criteria for species delimitation, at least within a group. An examination of the genus as a whole shows how little genetic diversity is contained within this group. Certainly rapid speciation is a real phenomenon, and, to a degree, appears to have taken place in the Corysanthes clade, with at least seven highly distinct lineages arising within about 1 million years. However, in the absence of evidence for reproductive isolation, ecological differentiation, or clear, consistent, diagnosable morphological traits, it simply does not make sense to recognize every separate lineage detected in a phylogeny, or morphological variety detected within the field, as a distinct species. Consider New Guinea where sampling is particularly poor and we regularly encounter new species of *Corybas* (Chapter 1), or the *C. pictus* complex on the Malay archipelago, where we have detected cryptic speciation in response to edaphic variation and extended periods of isolation. The amount of genetic and morphological variation contained within the Corysanthes clade pales in comparison. That said, at the state level, it is still important to recognize and protect genetic diversity in the native flora—but conservation planning efforts can and should be based around infraspecific genetic variation and not just species designations.

Literature Cited

BATES, R. 2011. South Australia's Native Orchids. Australian Orchid Foundation.

BROWN, A., P. DUNDAS, K. DIXON, AND S. HOPPER. 2008. Orchids of Western Australia. University of Western Australia Press.

BYRNE, M. 2008. Evidence for multiple refugia at different time scales during Pleistocene climatic oscillations in southern Australia inferred from phylogeography. *Quaternary Science Reviews* 27: 2576–2585.

BYRNE, M., D.A. STEANE, L. JOSEPH, D.K. YEATES, G.J. JORDAN, D. CRAYN, K. APLIN, ET AL. 2011. Decline of a biome: evolution, contraction, fragmentation, extinction and invasion of the Australian mesic zone biota. *Journal of Biogeography* 38: 1635–1656.

CLEMENTS, M.A., D.L. JONES, I.K. SHARMA, M.E. NIGHTINGALE, M.J. GARRATT, K.. FITZGERALD, A.M. MACKENZIE, AND B.P.J. MOLLOY. 2002. Phylogenetics of Diurideae (Orchidaceae) based on the internal transcribed spacer (ITS) regions of nuclear ribosomal DNA. *Lindleyana* 17: 135–171.

CLEMENTS, M.A., J.T. OTERO, AND J.T. MILLER. 2011. Phylogenetic relationships in Pterostylidinae (Cranichideae: Orchidaceae): combined evidence from nuclear ribomsomal and plastid DNA sequences. *Australian Journal of Botany* 59: 99–117.

CRISP, M.D., AND L.G. COOK. 2007. A congruent molecular signature of vicariance across multiple plant lineages. *Molecular Phylogenetics and Evolution* 43: 1106–1117.

CRISP, M.D., AND L.G. COOK. 2009. Explosive radiation or cryptic mass extinction? Interpreting signatures in molecular phylogenies. *Evolution* 63: 2257–2265.

CRUAUD, A., M. GAUTIER, M. GALAN, J. FOUCAUD, L. SAUNÉ, G. GENSON, E. DUBOIS, ET AL. 2014. Empirical assessment of RAD sequencing for interspecific phylogeny. *Molecular Biology and Evolution*msu063.

DARLU, P., AND G. LECOINTRE. 2002. When does the incongruence length difference test fail? *Molecular Biology and Evolution* 19: 432–437.

DAWSON, M.I. 2000. Index of chromosome numbers of indigenous New Zealand spermatophytes. *New Zealand Journal of Botany* 38: 47–150.

DAWSON, M.I., B.P.J. MOLLOY, AND E.. BEUZENBERG. 2007. Contributions to a chromosome atlas of the New Zealand flora–39. Orchidaceae. *New Zealand Journal of Botany* 45: 611–684.

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: 385–402.

DEVEY, D.S., R.M. BATEMAN, M.F. FAY, AND J.A. HAWKINS. 2009. Genetic structure and systematic relationships within the Ophrys fuciflora aggregate (Orchidaceae: Orchidinae): high diversity in Kent and a wind-induced discontinuity bisecting the Adriatic. *Annals of Botany* 104: 483–495.

DEVOS, N., O. RASPÉ, S.-H. OH, D. TYTECA, AND A.-L. JACQUEMART. 2006. The evolution of Dactylorhiza (Orchidaceae) allotetraploid complex: insights from

nrDNA sequences and cpDNA PCR-RFLP data. *Molecular phylogenetics and evolution* 38: 767–778.

DOLEŽEL, J., P. BINAROVÁ, AND S. LCRETTI. 1989. Analysis of Nuclear DNA content in plant cells by Flow cytometry. *Biologia Plantarum* 31: 113–120.

DOWNIE, S.R., AND D.S. KATZ-DOWNIE. 1996. A molecular phylogeny of Apiaceae subfamily Apioideae: evidence from nuclear ribosomal DNA internal transcribed spacer sequences. *American Journal of Botany* 83: 234–251.

EATON, D.A.R., AND R.H. REE. 2013. Inferring phylogeny and introgression using RADseq data: An example from flowering plants (Pedicularis: Orobanchaceae). *Systematic Biology* 62: 689–706.

ELSHIRE, R.J., J.C. GLAUBITZ, Q. SUN, J.A. POLAND, K. KAWAMOTO, E.S. BUCKLER, AND S.E. MITCHELL. 2011. A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. *PloS one* 6: e19379.

EMERSON, K.J., C.R. MERZ, J.M. CATCHEN, P.A. HOHENLOHE, W.A. CRESKO, W.E. BRADSHAW, AND C.M. HOLZAPFEL. 2010. Resolving postglacial phylogeography using high-throughput sequencing. *Proceedings of the National Academy of Sciences* 107: 16196–16200.

FARRINGTON, L., P. MACGILLIVRAY, R. FAAST, AND A.D. AUSTIN. 2009. Investigating DNA barcoding options for the identification of Caladenia (Orchidaceae) species. *Australian Journal of Botany* 57: 276–286.

FARRIS, J.S., M. KÄLLERSJÖ, A.G. KLUGE, AND C. BULT. 1994. Testing Significance of Incongruence. *Cladistics* 10: 315–319.

FAY, M.F., R.S. COWAN, AND I. LEITCH. 2005. The effects of nuclear DNA content (C-value) on the quality and utility of AFLP fingerprints. *Annals of Botany* 95: 237–246.

HEDREN, M., M.F. FAY, AND M.W. CHASE. 2001. Amplified fragment length polymorphisms (AFLP) reveal details of polyploid evolution in Dactylorhiza (Orchidaceae). *American Journal of Botany* 88: 1868–1880.

HOPPER, S.D., AND P. GIOIA. 2004. The southwest Australian floristic region: evolution and conservation of a global hot spot of biodiversity. *Annual Review of Ecology, Evolution, and Systematics* 35: 623–650.

INDSTO, J.O., P.H. WESTON, AND M.A. CLEMENTS. 2009. A molecular phylogenetic analysis of Diuris (Orchidaceae) based on AFLP and ITS reveals three major clades and a basal species. *Australian Systematic Botany* 22: 1–15.

JANES, J., D. STEANE, R. VAILLANCOURT, AND M.F. DURETTO. 2010. A molecular phylogeny of the subtribe Pterostylidinae (Orchidaceae): resolving the taxonomic confusion. *Australian Systematic Botany* 23: 248–259.

JEANES, J., AND G.N. BACKHOUSE. 2006. Wild Orchids of Victoria, Australia. Aquatic Photographics.

JONES, D.L. 1993. Corybas. *In* Flora of New South Wales, 212–215. UNSW Press.

JONES, D.L. 1999. The orchids of Tasmania. Miegunyah Press.

JONES, D.L., M.A. CLEMENTS, I.K. SHARMA, A.M. MACKENZIE, AND B.P.J. MOLLOY. 2002. Nomenclature notes arising from studies intro the Tribe Diurideae (Orchidaceae). *Orchadian* 13: 437–468, 502–503.

JOPPA, L.N., D.L. ROBERTS, AND S.L. PIMM. 2011. How many species of flowering plants are there? *Proceedings of the Royal Society B: Biological Sciences* 278: 554–559.

LARKIN, M.A., G. BLACKSHIELDS, N.P. BROWN, R. CHENNA, P.A. MCGETTIGAN, H. MCWILLIAM, F. VALENTIN, ET AL. 2007. Clustal W and Clustal X version 2.0. *Bioinformatics* 23: 2947–2948.

LEMMON, E.M., AND A.R. LEMMON. 2013. High-Throughput Genomic Data in Systematics and Phylogenetics. *Annual Review of Ecology, Evolution, and Systematics* 44: 99–121.

LEXER, C., S. MANGILI, E. BOSSOLINI, F. FOREST, K.N. STÖLTING, P.B. PEARMAN, N.E. ZIMMERMANN, AND N. SALAMIN. 2013. "Next generation" biogeography: towards understanding the drivers of species diversification and persistence. *Journal of Biogeography* 40: 1013–1022.

MANT, J.G., R. PEAKALL, AND P.H. WESTON. 2005. Specific pollinator attraction and the diversification of sexually deceptive Chiloglottis (Orchidaceae). *Plant Systematics and Evolution* 253: 185–200.

MANT, J.G., F.P. SCHIESTL, R. PEAKALL, AND P.H. WESTON. 2002. A phylogenetic study of pollinator conservatism among sexually deceptive orchids. *Evolution*888–898.

MCCORMACK, J.E., S.M. HIRD, A.J. ZELLMER, B.C. CARSTENS, AND R.T. BRUMFIELD. 2013. Applications of next-generation sequencing to phylogeography and phylogenetics. *Molecular Phylogenetics and Evolution* 66: 526–538.

MEHRA, P.N., AND R.N. SEHGAL. 1976. IOPB chromosome number reports. *Taxon* 25: 483–500.

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 Oleans, LA, 1–8.

NEVILL, P.G., G. BOSSINGER, AND P.K. ADES. 2010. Phylogeography of the world's tallest angiosperm, Eucalyptus regnans: evidence for multiple isolated Quaternary refugia. *Journal of Biogeography* 37: 179–192.

PAUN, O., R.M. BATEMAN, M.F. FAY, M. HEDRÉN, L. CIVEYREL, AND M.W. CHASE. 2010. Stable Epigenetic Effects Impact Adaptation in Allopolyploid Orchids (Dactylorhiza: Orchidaceae). *Molecular Biology and Evolution* 27: 2465–2473.

PEAKALL, R., AND S.H. JAMES. 1989. Chromosome numbers of some Australian terrestrial orchids. *Lindleyana* 4: 85–88.

PHILLIPS, R.D., K.W. DIXON, AND R. PEAKALL. 2012. Low population genetic differentiation in the Orchidaceae: implications for the diversification of the family. *Molecular Ecology* 21: 5208–5220.

PILLON, Y., AND M.W. CHASE. 2007. Taxonomic exaggeration and its effects on orchid conservation. *Conservation Biology* 21: 263–265.

POSADA, D. 2008. jModelTest: Phylogenetic Model Averaging. *Molecular Biology and Evolution* 25: 1253–1256.

PRIDGEON, A.M., AND M.W. CHASE. 1995. Subterranean axes in tribe Diurideae (Orchidaceae): Morphology, anatomy, and systematic significance. *American Journal of Botany* 82: 1473–1495.

REITZEL, A.M., S. HERRERA, M.J. LAYDEN, M.Q. MARTINDALE, AND T.M. SHANK. 2013. Going where traditional markers have not gone before: utility of and promise for RAD sequencing in marine invertebrate phylogeography and population genomics. *Molecular Ecology* 22: 2953–2970.

RONQUIST, F., M. TESLENKO, P. VAN DER MARK, D.L. AYRES, A. DARLING, S. HÖHNA, B. LARGET, ET AL. 2012. MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* 61: 539–542.

RUSSELL, A., R. SAMUEL, 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.

STAMATAKIS, A. 2014. RAxML Version 8: A tool for Phylogenetic Analysis and Post-Analysis of Large Phylogenies. *Bioinformatics*btu033.

SWOFFORD, D. 2003. PAUP*: phylogenetic analysis using parsimony, version 4.0b10.

TREMBLAY, R.L., J.D. ACKERMAN, J.K. ZIMMERMAN, AND R.N. CALVO. 2004. Variation in sexual reproduction in orchids and its evolutionary consequences: a spasmodic journey to diversification. *Botanical Journal of the Linnean Society* 84: 1–54.

VIRICEL, A., E. PANTE, W. DABIN, AND B. SIMON-BOUHET. 2013. Applicability of RAD-tag genotyping for interfamilial comparisons: empirical data from two cetaceans. *Molecular Ecology Resources*n/a–n/a.

WAGNER, C.E., I. KELLER, S. WITTWER, O.M. SELZ, S. MWAIKO, L. GREUTER, A. SIVASUNDAR, AND O. SEEHAUSEN. 2013. Genome-wide RAD sequence data provide unprecedented resolution of species boundaries and relationships in the Lake Victoria cichlid adaptive radiation. *Molecular Ecology* 22: 787–798.

WCSP. 2014. World Checklist of Selected Plant Families. Facilitated by the Royal Botanic Gardens, Kew. Available at: http://apps.kew.org/wcsp/ [Accessed April 5, 2014].

WHITE, T.J., T.D. BRUNS, S. LEE, AND J.W. TAYLOR. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR-Protocols : A guide to methods and applications*.

ZWICKL, D.J. 2006. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. Ph.D. dissertation. The University of Texas at Austin.

Figures and Tables

Figure 1. Members of the Corysanthes clade. **A.** *Corybas pruinosus* (Royal NP); **B.** *Corybas hispidus* (cult, photo by R. van Vugt); **C.** *Corybas fimbriatus* (Oatley NP); **D.** *Corybas recurvu*s (Beedelup NP); **E.** *Corybas diemenicus* (E Shelley Beach), **F.** *Corybas "dilatatus"* (Nixon-Skinner CP); **G.** *Corybas "longitubus"* (Barrington Tops, photo by M. Clements); **H.** *Corybas dentatus* (Scott CP); **I.** *Corybas incurvus* (cult, photo by R. van Vugt); **J.** *Corybas limpidus*, showing the wider dorsal sepal (Hopetoun); **K.** *Corybas aff. limpidus* "fat dwarf" (Newland Head); **L.** *Corybas expansus* (Stenhouse Bay, photo by M. Clements); **M.** *Corybas depectans* (Aldinga Scrub). Unless otherwise noted, photos by S. Lyon. All other images used with permission.

Table 1. Comparison of traits used to distinguish taxa in the Corysanthes clade. Data are derived from various sources listed in the text.

Figure 2. Elevation map of Australia showing collection localities for each Corysanthes taxon collected in this study.

Table 2. Collection localities for each taxon, with the number of individuals sequenced for standard regions (chloroplast spacers, ITS, *PhyC*) using Sanger sequencing ("Sang.") and the number of individuals included in the GBS analyses.

Table 3. Flow cytometry data for the Corysanthes clade, with approximate 1C values calculated using pea standard.

Figure 3. Maximum likelihood phylogeny of concatenated chloroplast data for the Corysanthes clade. Values above the branch indicate maximum parsimony/maximum likelihood bootstrap support values for each clade, below the branch show posterior probabilities from Bayesian analysis.

Figure 4. Maximum likelihood phylogeny of ITS data for the Corysanthes clade. Values above the branch indicate maximum parsimony/maximum likelihood bootstrap support values for each clade, below the branch show posterior probabilities from Bayesian analysis.

Figure 5. Maximum likelihood phylogeny of *PhyC* data for the Corysanthes clade. Values above the branch indicate maximum parsimony/maximum likelihood bootstrap support values for each clade, below the branch show posterior probabilities from Bayesian analysis.

Figure 6. Maximum likelihood phylogeny of combined chloroplast, ITS, and *PhyC* data sets, excluding taxa suspected to be hybrids and collections showing indication of hybridization. Maximum likelihood phylogeny of ITS data for the Corysanthes clade. Values above the branch indicate maximum parsimony/maximum likelihood bootstrap

support values for each clade, below the branch show posterior probabilities from Bayesian analysis. Six distinct, well-supported lineages are colored for reference.

Figure 7. One of 16 most parsimonious trees obtained in analysis of concatenated SNP data from genotyping-by-sequencing, including variable sites. Arrows indicate nodes that collapse in strict consensus. Support values derived from 100 bootstrap replicates.

Figure 8. Maximum likelihood phylogeny of concatenated SNP data from genotyping-by-sequencing, including variable sites. Support values derived from 100 bootstrap replicates.

Figure 1.

Table 1.

Taxon	Dorsal sepal	Labellum margin	Labellum boss	Labellum tube	Labellum surface	Flower orientation	Other	Distribution
C. despectans Jones	very narrow, keeled, short, pale green, erect and not hooded	shortly toothed, incurved, suffused maroon in broad band (sparser markings in WA)	narrow, weakly mounded, squarish channel	shorter than blade, auricles concealed	granulose, raised vertical ridges, or with flattened, downwards-pointing teeth	erect to slightly nodding	"aff. despectans" in WA with very reduced flowers, cleistogamous; "red-eyed dwarf" in SA with green labellum and deep red column and tube; minutely winged column	Vic, SA, WA, coastal scrub
C. expansus Jones	longer, narrowly spathulate, pale green	finely toothed, flared especially on upper margins, broad maroon band on sides	moderately mounded with squarish channel, large white central region with yellow center	shorter than blade, auricles concealed	numerous papillae and appressed triangular calli	upright to slightly recurved	seems to intergrade with SA C. despectans; more prominently winged column	SA, sheltered areas on coastal dunes
C. limpidus Jones	spathulate, broad	blunt irregular teeth, slightly incurved; maroon blotches, with translucent areas	slightly mounded, squarish channel	shorter than blade, auricles concealed	dense mass of inward sloping calli	upright	"aff. limpidus" in WA smaller with less granular labellum; minutely winged column	WA, sheltered areas on coastal dunes
C. aff. limpidus "fat dwarf"	wider, spathulate, pale green with sparse red markings, apiculate	toothed incurved margins, red markings on margin	mounded, squarish channel suffused orange	shorter than blade, auricles concealed		upright	elongated ovary	SA, coastal scrub
C. diemenicus (Lind.) Rupp & Nicholls	spathulate, broad, red	coarsely toothed, upper margins incurved or flared, prominently veined red	mounded, white to purplish	longer than blade	sparsely to heavily hispid around boss, sometimes hairs extending onto boss	upright to slightly recurved	variable	Tas, Vic; applied to all populations in Tasmania
*C. dilatatus (*Rupp)	spathulate, broad, dark red	coarsely toothed, flared, blotchy maroon rays	mounded, broad, white/cream	very slightly longer than blade	minutely hispid around boss	upright	large, broad flowers	applied to SA and some Vic populations
*C. grumulus (*Jones) Backhouse	spathulate, broad, dark red	coarsely toothed, upper margins incurved, distinct maroon rays	strongly mounded, whitish, suffused with red	longer than blade	hispid band around boss	upright	smaller flowers	Vic, ACT, southern NSW
C. longitubus Jones MS (as Corysanthes longituba)	spathulate, broad, grey with reddish markings	coarsely toothed,upper margins incurved, maroon blotches not forming distinct rays	mounded, white	distinctly longer than blade	minutely hispid around boss	upright	smaller, paler flowers	Barrington Tops, NSW
C. aff. diemenicus "tea tree swamp"	spathulate, broad dark red	coarsely toothed,upper margins incurved, thick maroon/purple rays	mounded, round, maroon/purple	very slightly longer than blade	broad hispid band below and extending into boss	upright	**protected**	Coastal woolly tea tree swamps in Vic, SA
C. incurvus Jones & Clements	spathulate, shorter than diemenicus, translucent green with purple markings	finely toothed, strongly incurved, broad band of purple just inside margin, labellum jutting forward	relatively flat, cream to yellow, notched to varying degrees	shorter than blade, auricles concealed	hispid in band around boss (purple); a few downwards sloping teeth in throat	slightly recurved		SA, Vic, Tas, NSW
C. dentatus Jones	spathulate and broad, pale with varying degrees of purple mottling	more coarsely toothed, incurved or slightly spreading; not as jutting, white edged teeth	as described by Jones: flat and purplish; as described by Bates: often notched	shorter than blade, auricles concealed	narrow hispid band, flattened calli near apex of tube	upright	**protected,** highly variable	SA
C. x miscellus Jones	spathulate, pale with purple markings	finely to coarsely toothed, slightly incurved but with teeth expanding	as described by Bates: flat; as described by Jones: notched, prominent and white	longer than blade	broad to narrow hispid band (purple to white), irregular, inwards sloping teeth in opening of tube	upright	variable	SA
C. recurvus Jones	spathulate, broad, dark purple (pale at base with purple markings)	coarsely toothed (not as broad as diemenicus), slightly incurved to flared, solidly dark purple with darker veins	mounded but less pronounced than in diemenicus group; pale purple-red	slightly longer than blade	coarse, downward-pointing teeth lining labellum tube; short black teeth prominent around boss	strongly recurved		WA
C. pruinosus (R. Cunningham) Reichb.	spathulate, broad, pale but opaque, with sparse red markings	fimbriate, slightly incurved, margins maroon/purple but with pale fringe	broad, weakly mounded, cream with purple center	slightly longer than blade	hispid around boss	recurved		NSW; wet sclerophyll forests on coast and nearby ranges
C. hispidus Jones	spathulate, obtuse, translucent and speckled maroon; extending out beyond labellum, slightly keeled, sometimes with short-acuminate apex	fimbriate; spreading or incurved; speckled or solidly maroon	broad, flat, deeply notched, cream-colored	shorter than blade, auricles concealed	densely hispid across surface	recurved	variation in floral size, some flowers can be quite large; column winged	Vic to S Qld; drier inland mountainous areas in locally moist locales
*C. fimbriatus (*Br.) Reichb.	spathulate, broad; translucent and speckled red	fimbriate; speckled red with red fimbriae	strongly mounded, translucent red/purple, speckled red	much shorter than blade, auricles concealed	smooth or shortly scabrous below boss	recurved	column not winged	Vic,Tas, NSW, Qld up to Atherton area; wet sclerophyll forests, often on sandy soils close to coast

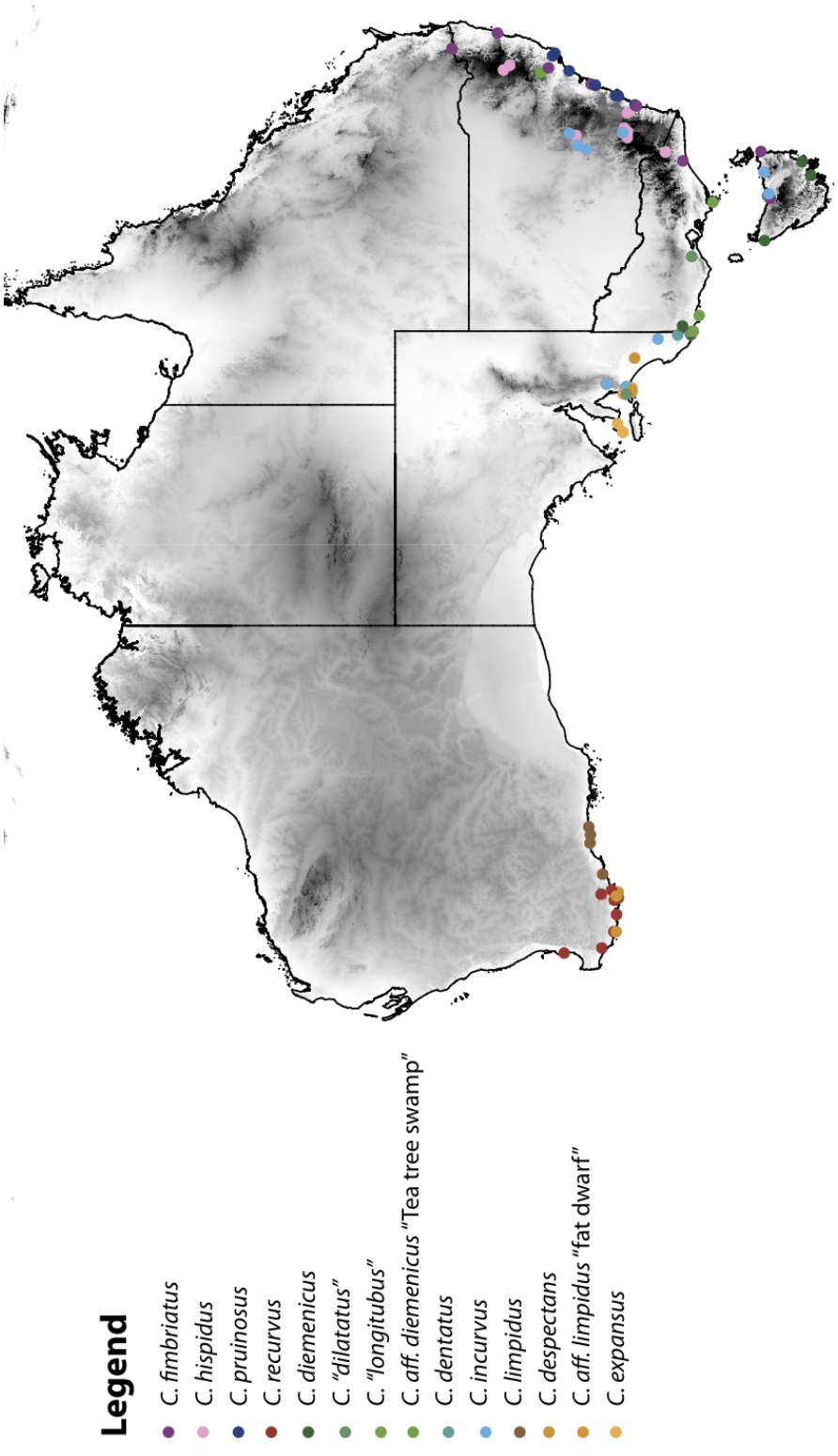
Legend
C. fimbriatus
C. hispidus
C. pruinosus
C. recurvus
C. diemenicus
C. "dilatatus"
C. "longitubus"
C. aff. diemenicus "Tea tree swamp"
C. dentatus
C. incurvus
C. limpidus
C. despectans
C. aff. limpidus "fat dwarf"
C. expansus

Figure 2.

Table 2.

Species	Site	Sang	GBS
C. despectans	Aldinga Scrub, SA	-	3
	Coonalpyn, SA	1	3
	D'Entrecasteaux NP, WA	1	3
	Gull Rock, WA	-	2
	Innes NP, SA	-	3
	Nanarup Beach, WA	1	3
	Newland head, SA	1	3
	Sandy Creek, SA	-	2
C. aff. despectans "red-eyed dwarf"	Newland head, SA	-	3
C. expansus	Aldinga Scrub, SA	-	3
	Innes NP, SA	1	3
	Newland head, SA	1	3
	Sandy Creek, SA	-	3
	Stenhouse Bay, SA	-	3
C.limpidus	Gull Rock, WA	1	3
	Hopetoun, WA	-	3
	Mason Bay, WA	-	2
	Millers Point, WA	1	3
	Munglinup Beach, WA	1	2
C. aff. limpidus "fat dwarf"	Newland head, SA	1	2
C. fimbriatus	Barrington Tops, NSW	-	3
	Bruthen, Vic	-	1
	Henry Somerset CA, Tas	1	-
	Mt William NP, Tas	1	1
	Mt. Clunie Rd, NSW	-	1
	Oatley Park, NSW	-	3
	Orara E SF, NSW	-	2
C. hispidus	Black Mt, ACT	1	3
	Gibralter Falls, ACT	-	3
	Mihi Gorge, NSW	-	1
	Molonglo Gorge, ACT	-	1
	Mt. Canobolas, NSW	-	3
	Mt. Duval, NSW	1	1
	Mt. Hamilton, Vic	-	1
	nr Braidwood, NSW	-	1
	Tidbinbilla, ACT	1	3
C. pruinosus	Alum Mt, NSW	1	3
	Bateman's Bay, NSW	1	1
	Falls Creek, NSW	1	2
	Lake Macquarie CA	1	2
	Myall Lakes, NSW	-	2
	Pelican Pt, NSW	-	3
	Royal NP, NSW	1	3

Species	Site	Sang	GBS
C. diemenicus	Arthur River, Tas	1	-
	E Shelley Beach, Tas	-	1
	Point Danger, Vic	-	2
	Port Sorell, Tas	1	1
	Truganini Res, Tas	1	1
	Wilkin FFR, Vic	-	1
C. "dilatatus"	Inverleigh, Vic	1	1
	Lower Glenelg NP, Vic	1	1
	Sandy Creek, SA	3	2
	Scott CP, SA	-	1
C. "longitubus"	Barrington Tops, NSW	1	3
C. aff. diemenicus "Tea tree"	Cotters Lake, Vic	1	1
	nr mouth of Glenelg R, Vic	-	2
	Picanninie Ponds, Vic	-	1
C. dentatus	Frome Rd, SA	1	3
	Nangawarry NFR, SA	1	3
	Sandy Creek, SA	3	3
	Scott CP, SA	-	3
C. incurvus	Black Mt, ACT	1	4
	Harford, Tas	1	-
	Mullion Creek, NSW	-	3
	Nangar NP, NSW	-	3
	Sandy Creek, SA	-	2
	Scott CP, SA	1	2
	The Gap, SA	-	3
C. recurvus	Beedelup NP, WA	1	3
	D'Entrecasteaux NP, WA	-	3
	Gull Rock, WA	-	3
	Stirling Range NP, WA	-	2
	Two Peoples Bay, WA	1	1
	Williams Bay, WA	-	1
	Yalgorup NP, WA	1	2

Table 3.

Sample	Collection	Pea standard (1C = 4.88) FL2-A	Sample FL2-A	1C (pg)
Corybas aff. diemenicus, Barrington Tops, NSW	Dowling 521	398245	778957	9.55
Corybas diemenicus, Inverleigh, Vic	ORG6297	345918	685458	9.67
Corybas hispidus, Molonglo Gorge, ACT	Otero 1219	327383	653140	9.74
Corybas incurvus, Conimbla NP, NSW	SPL213	341063	677082	9.69
Corybas limpidus, Hopetoun, WA	SPL234	363513	705164	9.47
Corybas pruinosus, Pelican Pt, NSW	SPL207	362513	698339	9.4
Corybas recurvus, Stirling Range, WA	SPL230	348873	674047	9.43
Corybas recurvus, Williams Bay, WA	SPL264	380413	751306	9.64
Mean				**9.57**

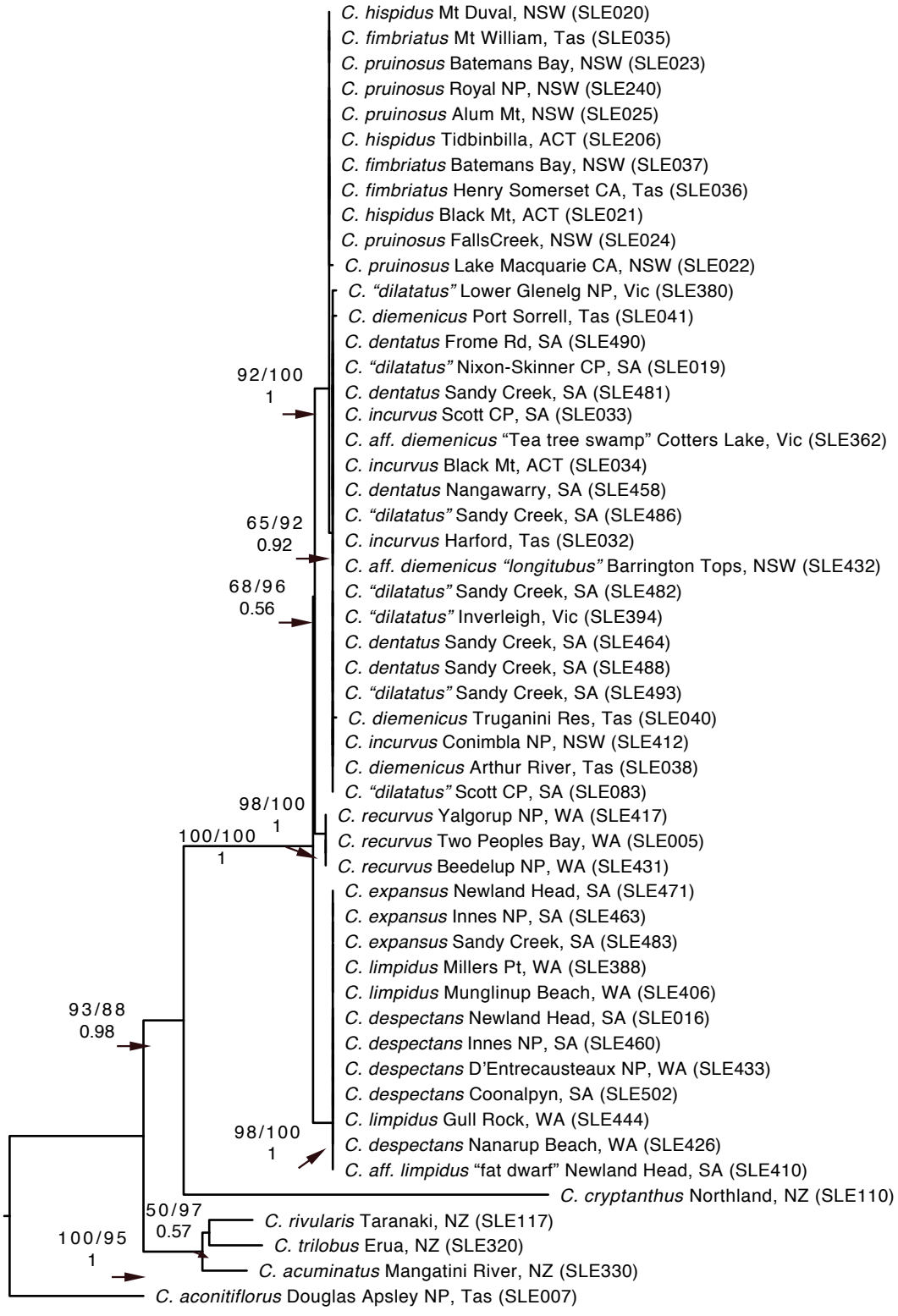
C. hispidus Mt Duval, NSW (SLE020)
C. fimbriatus Mt William, Tas (SLE035)
C. pruinosus Batemans Bay, NSW (SLE023)
C. pruinosus Royal NP, NSW (SLE240)
C. pruinosus Alum Mt, NSW (SLE025)
C. hispidus Tidbinbilla, ACT (SLE206)
C. fimbriatus Batemans Bay, NSW (SLE037)
C. fimbriatus Henry Somerset CA, Tas (SLE036)
C. hispidus Black Mt, ACT (SLE021)
C. pruinosus FallsCreek, NSW (SLE024)
C. pruinosus Lake Macquarie CA, NSW (SLE022)
C. "dilatatus" Lower Glenelg NP, Vic (SLE380)
C. diemenicus Port Sorrell, Tas (SLE041)
C. dentatus Frome Rd, SA (SLE490)
C. "dilatatus" Nixon-Skinner CP, SA (SLE019)
C. dentatus Sandy Creek, SA (SLE481)
C. incurvus Scott CP, SA (SLE033)
C. aff. diemenicus "Tea tree swamp" Cotters Lake, Vic (SLE362)
C. incurvus Black Mt, ACT (SLE034)
C. dentatus Nangawarry, SA (SLE458)
C. "dilatatus" Sandy Creek, SA (SLE486)
C. incurvus Harford, Tas (SLE032)
C. aff. diemenicus "longitubus" Barrington Tops, NSW (SLE432)
C. "dilatatus" Sandy Creek, SA (SLE482)
C. "dilatatus" Inverleigh, Vic (SLE394)
C. dentatus Sandy Creek, SA (SLE464)
C. dentatus Sandy Creek, SA (SLE488)
C. "dilatatus" Sandy Creek, SA (SLE493)
C. diemenicus Truganini Res, Tas (SLE040)
C. incurvus Conimbla NP, NSW (SLE412)
C. diemenicus Arthur River, Tas (SLE038)
C. "dilatatus" Scott CP, SA (SLE083)
C. recurvus Yalgorup NP, WA (SLE417)
C. recurvus Two Peoples Bay, WA (SLE005)
C. recurvus Beedelup NP, WA (SLE431)
C. expansus Newland Head, SA (SLE471)
C. expansus Innes NP, SA (SLE463)
C. expansus Sandy Creek, SA (SLE483)
C. limpidus Millers Pt, WA (SLE388)
C. limpidus Munglinup Beach, WA (SLE406)
C. despectans Newland Head, SA (SLE016)
C. despectans Innes NP, SA (SLE460)
C. despectans D'Entrecausteaux NP, WA (SLE433)
C. despectans Coonalpyn, SA (SLE502)
C. limpidus Gull Rock, WA (SLE444)
C. despectans Nanarup Beach, WA (SLE426)
C. aff. limpidus "fat dwarf" Newland Head, SA (SLE410)
C. cryptanthus Northland, NZ (SLE110)
C. rivularis Taranaki, NZ (SLE117)
C. trilobus Erua, NZ (SLE320)
C. acuminatus Mangatini River, NZ (SLE330)
C. aconitiflorus Douglas Apsley NP, Tas (SLE007)
92/100
1
65/92
0.92
68/96
0.56
98/100
1
100/100
1
93/88
0.98
98/100
1
50/97
0.57
100/95
1
0.0080

Figure 3.

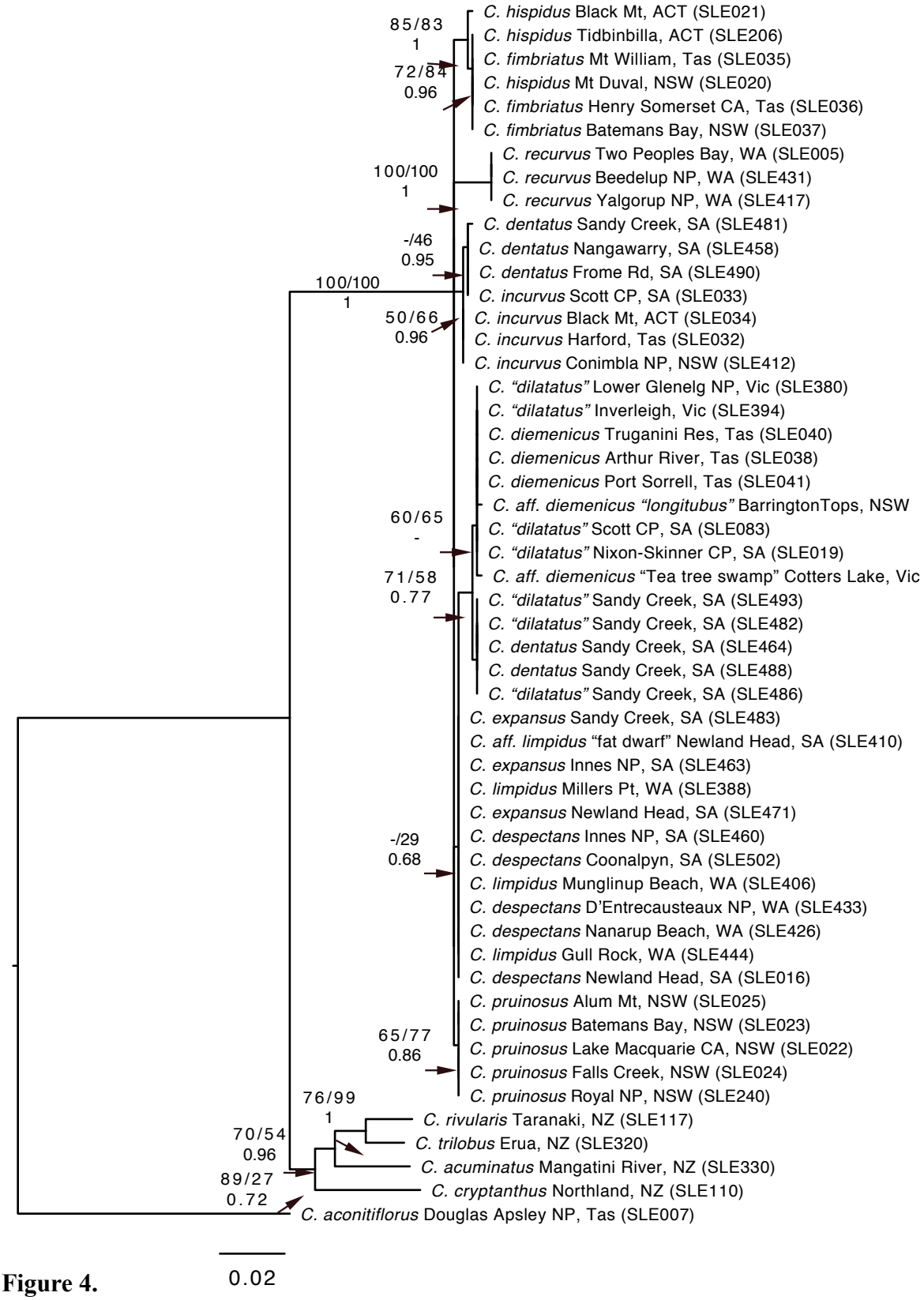
85/83
1
72/84
0.96
100/100
1
-/46
0.95
100/100
1
50/66
0.96
60/65
-
71/58
0.77
-/29
0.68
65/77
0.86
76/99
1
70/54
0.96
89/27
0.72
C. hispidus Black Mt, ACT (SLE021)
C. hispidus Tidbinbilla, ACT (SLE206)
C. fimbriatus Mt William, Tas (SLE035)
C. hispidus Mt Duval, NSW (SLE020)
C. fimbriatus Henry Somerset CA, Tas (SLE036)
C. fimbriatus Batemans Bay, NSW (SLE037)
C. recurvus Two Peoples Bay, WA (SLE005)
C. recurvus Beedelup NP, WA (SLE431)
C. recurvus Yalgorup NP, WA (SLE417)
C. dentatus Sandy Creek, SA (SLE481)
C. dentatus Nangawarry, SA (SLE458)
C. dentatus Frome Rd, SA (SLE490)
C. incurvus Scott CP, SA (SLE033)
C. incurvus Black Mt, ACT (SLE034)
C. incurvus Harford, Tas (SLE032)
C. incurvus Conimbla NP, NSW (SLE412)
C. "dilatatus" Lower Glenelg NP, Vic (SLE380)
C. "dilatatus" Inverleigh, Vic (SLE394)
C. diemenicus Truganini Res, Tas (SLE040)
C. diemenicus Arthur River, Tas (SLE038)
C. diemenicus Port Sorrell, Tas (SLE041)
C. aff. diemenicus "longitubus" BarringtonTops, NSW
C. "dilatatus" Scott CP, SA (SLE083)
C. "dilatatus" Nixon-Skinner CP, SA (SLE019)
C. aff. diemenicus "Tea tree swamp" Cotters Lake, Vic
C. "dilatatus" Sandy Creek, SA (SLE493)
C. "dilatatus" Sandy Creek, SA (SLE482)
C. dentatus Sandy Creek, SA (SLE464)
C. dentatus Sandy Creek, SA (SLE488)
C. "dilatatus" Sandy Creek, SA (SLE486)
C. expansus Sandy Creek, SA (SLE483)
C. aff. limpidus "fat dwarf" Newland Head, SA (SLE410)
C. expansus Innes NP, SA (SLE463)
C. limpidus Millers Pt, WA (SLE388)
C. expansus Newland Head, SA (SLE471)
C. despectans Innes NP, SA (SLE460)
C. despectans Coonalpyn, SA (SLE502)
C. limpidus Munglinup Beach, WA (SLE406)
C. despectans D'Entrecausteaux NP, WA (SLE433)
C. despectans Nanarup Beach, WA (SLE426)
C. limpidus Gull Rock, WA (SLE444)
C. despectans Newland Head, SA (SLE016)
C. pruinosus Alum Mt, NSW (SLE025)
C. pruinosus Batemans Bay, NSW (SLE023)
C. pruinosus Lake Macquarie CA, NSW (SLE022)
C. pruinosus Falls Creek, NSW (SLE024)
C. pruinosus Royal NP, NSW (SLE240)
C. rivularis Taranaki, NZ (SLE117)
C. trilobus Erua, NZ (SLE320)
C. acuminatus Mangatini River, NZ (SLE330)
C. cryptanthus Northland, NZ (SLE110)
C. aconitiflorus Douglas Apsley NP, Tas (SLE007)
0.02

Figure 4.

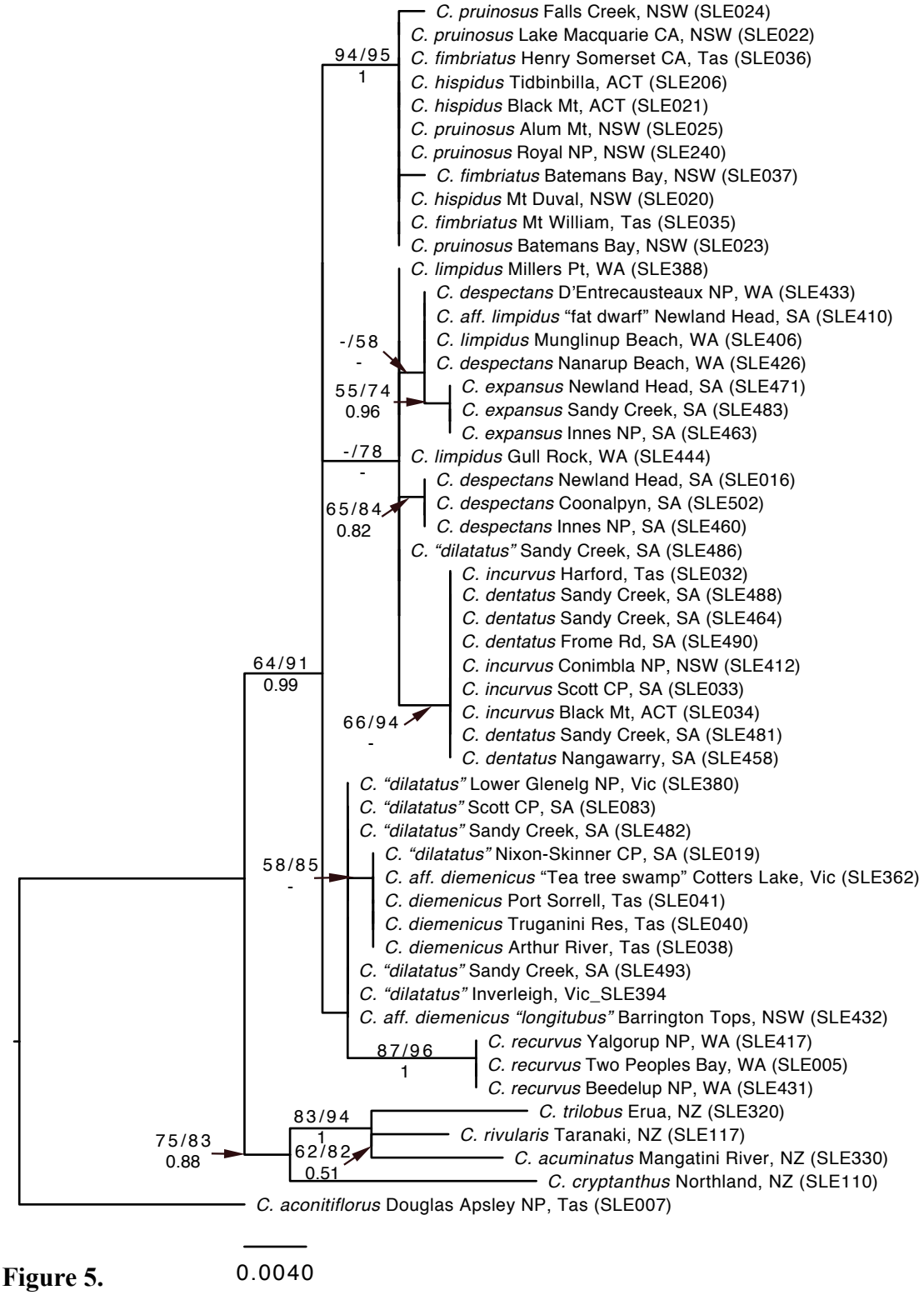
C. pruinosus Falls Creek, NSW (SLE024)
C. pruinosus Lake Macquarie CA, NSW (SLE022)
94/95
1
C. fimbriatus Henry Somerset CA, Tas (SLE036)
C. hispidus Tidbinbilla, ACT (SLE206)
C. hispidus Black Mt, ACT (SLE021)
C. pruinosus Alum Mt, NSW (SLE025)
C. pruinosus Royal NP, NSW (SLE240)
C. fimbriatus Batemans Bay, NSW (SLE037)
C. hispidus Mt Duval, NSW (SLE020)
C. fimbriatus Mt William, Tas (SLE035)
C. pruinosus Batemans Bay, NSW (SLE023)
C. limpidus Millers Pt, WA (SLE388)
C. despectans D'Entrecausteaux NP, WA (SLE433)
C. aff. limpidus "fat dwarf" Newland Head, SA (SLE410)
-/58
-
C. limpidus Munglinup Beach, WA (SLE406)
C. despectans Nanarup Beach, WA (SLE426)
55/74
0.96
C. expansus Newland Head, SA (SLE471)
C. expansus Sandy Creek, SA (SLE483)
C. expansus Innes NP, SA (SLE463)
-/78
-
C. limpidus Gull Rock, WA (SLE444)
C. despectans Newland Head, SA (SLE016)
65/84
0.82
C. despectans Coonalpyn, SA (SLE502)
C. despectans Innes NP, SA (SLE460)
C. "dilatatus" Sandy Creek, SA (SLE486)
C. incurvus Harford, Tas (SLE032)
C. dentatus Sandy Creek, SA (SLE488)
C. dentatus Sandy Creek, SA (SLE464)
C. dentatus Frome Rd, SA (SLE490)
64/91
0.99
C. incurvus Conimbla NP, NSW (SLE412)
C. incurvus Scott CP, SA (SLE033)
C. incurvus Black Mt, ACT (SLE034)
66/94
-
C. dentatus Sandy Creek, SA (SLE481)
C. dentatus Nangawarry, SA (SLE458)
C. "dilatatus" Lower Glenelg NP, Vic (SLE380)
C. "dilatatus" Scott CP, SA (SLE083)
C. "dilatatus" Sandy Creek, SA (SLE482)
58/85
-
C. "dilatatus" Nixon-Skinner CP, SA (SLE019)
C. aff. diemenicus "Tea tree swamp" Cotters Lake, Vic (SLE362)
C. diemenicus Port Sorrell, Tas (SLE041)
C. diemenicus Truganini Res, Tas (SLE040)
C. diemenicus Arthur River, Tas (SLE038)
C. "dilatatus" Sandy Creek, SA (SLE493)
C. "dilatatus" Inverleigh, Vic_SLE394
C. aff. diemenicus "longitubus" Barrington Tops, NSW (SLE432)
87/96
1
C. recurvus Yalgorup NP, WA (SLE417)
C. recurvus Two Peoples Bay, WA (SLE005)
C. recurvus Beedelup NP, WA (SLE431)
83/94
1
C. trilobus Erua, NZ (SLE320)
75/83
0.88
C. rivularis Taranaki, NZ (SLE117)
62/82
0.51
C. acuminatus Mangatini River, NZ (SLE330)
C. cryptanthus Northland, NZ (SLE110)
C. aconitiflorus Douglas Apsley NP, Tas (SLE007)
0.0040

Figure 5.

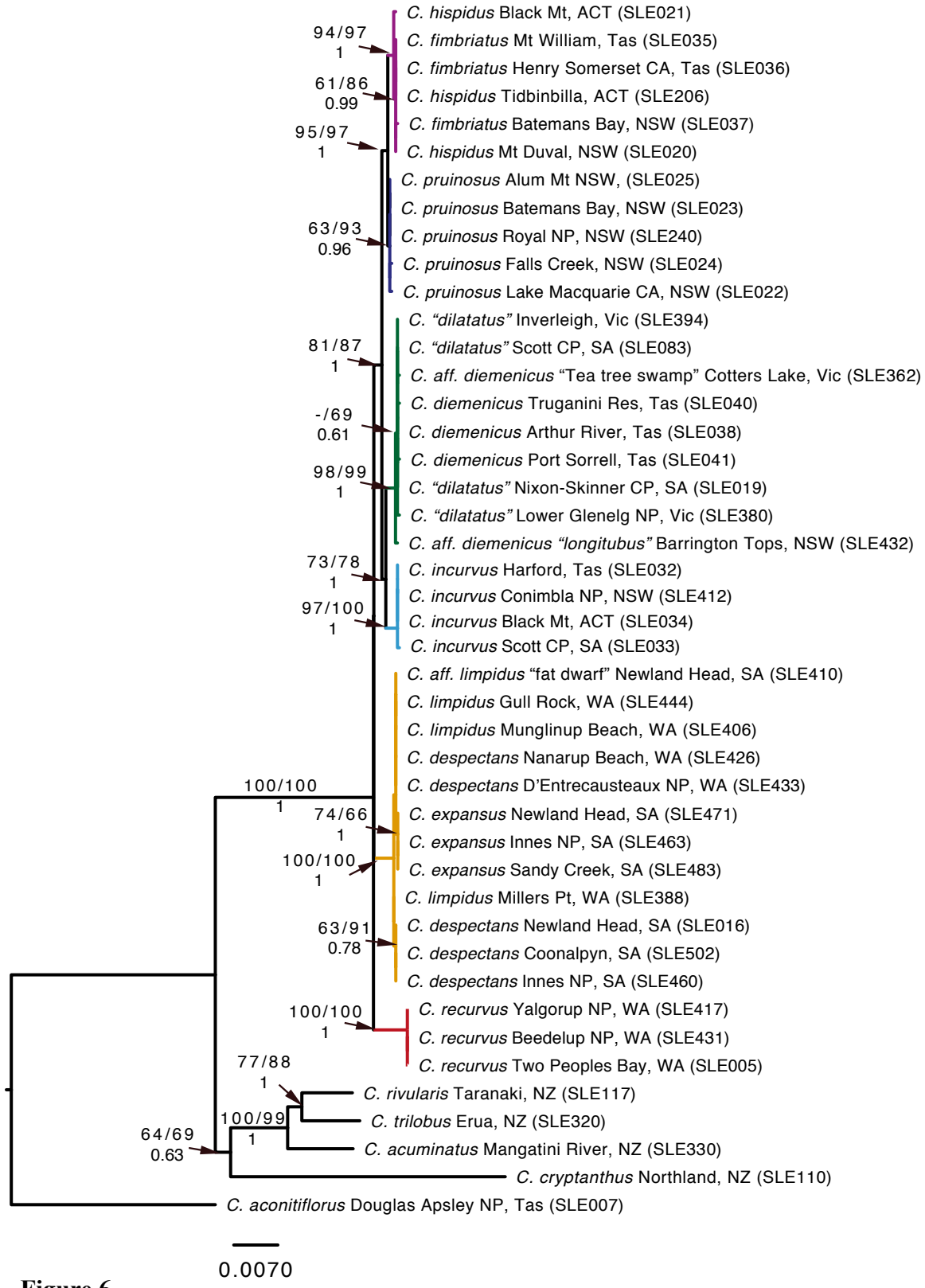
C. hispidus Black Mt, ACT (SLE021)
94/97
1
C. fimbriatus Mt William, Tas (SLE035)
C. fimbriatus Henry Somerset CA, Tas (SLE036)
61/86
0.99
C. hispidus Tidbinbilla, ACT (SLE206)
C. fimbriatus Batemans Bay, NSW (SLE037)
95/97
1
C. hispidus Mt Duval, NSW (SLE020)
C. pruinosus Alum Mt NSW, (SLE025)
C. pruinosus Batemans Bay, NSW (SLE023)
63/93
0.96
C. pruinosus Royal NP, NSW (SLE240)
C. pruinosus Falls Creek, NSW (SLE024)
C. pruinosus Lake Macquarie CA, NSW (SLE022)
C. "dilatatus" Inverleigh, Vic (SLE394)
81/87
1
C. "dilatatus" Scott CP, SA (SLE083)
C. aff. diemenicus "Tea tree swamp" Cotters Lake, Vic (SLE362)
C. diemenicus Truganini Res, Tas (SLE040)
-/69
0.61
C. diemenicus Arthur River, Tas (SLE038)
C. diemenicus Port Sorrell, Tas (SLE041)
98/99
1
C. "dilatatus" Nixon-Skinner CP, SA (SLE019)
C. "dilatatus" Lower Glenelg NP, Vic (SLE380)
C. aff. diemenicus "longitubus" Barrington Tops, NSW (SLE432)
73/78
1
C. incurvus Harford, Tas (SLE032)
C. incurvus Conimbla NP, NSW (SLE412)
97/100
1
C. incurvus Black Mt, ACT (SLE034)
C. incurvus Scott CP, SA (SLE033)
C. aff. limpidus "fat dwarf" Newland Head, SA (SLE410)
C. limpidus Gull Rock, WA (SLE444)
C. limpidus Munglinup Beach, WA (SLE406)
C. despectans Nanarup Beach, WA (SLE426)
C. despectans D'Entrecausteaux NP, WA (SLE433)
100/100
1
74/66
1
C. expansus Newland Head, SA (SLE471)
C. expansus Innes NP, SA (SLE463)
100/100
1
C. expansus Sandy Creek, SA (SLE483)
C. limpidus Millers Pt, WA (SLE388)
63/91
0.78
C. despectans Newland Head, SA (SLE016)
C. despectans Coonalpyn, SA (SLE502)
C. despectans Innes NP, SA (SLE460)
100/100
1
C. recurvus Yalgorup NP, WA (SLE417)
C. recurvus Beedelup NP, WA (SLE431)
C. recurvus Two Peoples Bay, WA (SLE005)
77/88
1
C. rivularis Taranaki, NZ (SLE117)
100/99
1
C. trilobus Erua, NZ (SLE320)
64/69
0.63
C. acuminatus Mangatini River, NZ (SLE330)
C. cryptanthus Northland, NZ (SLE110)
C. aconitiflorus Douglas Apsley NP, Tas (SLE007)
0.0070

Figure 6.

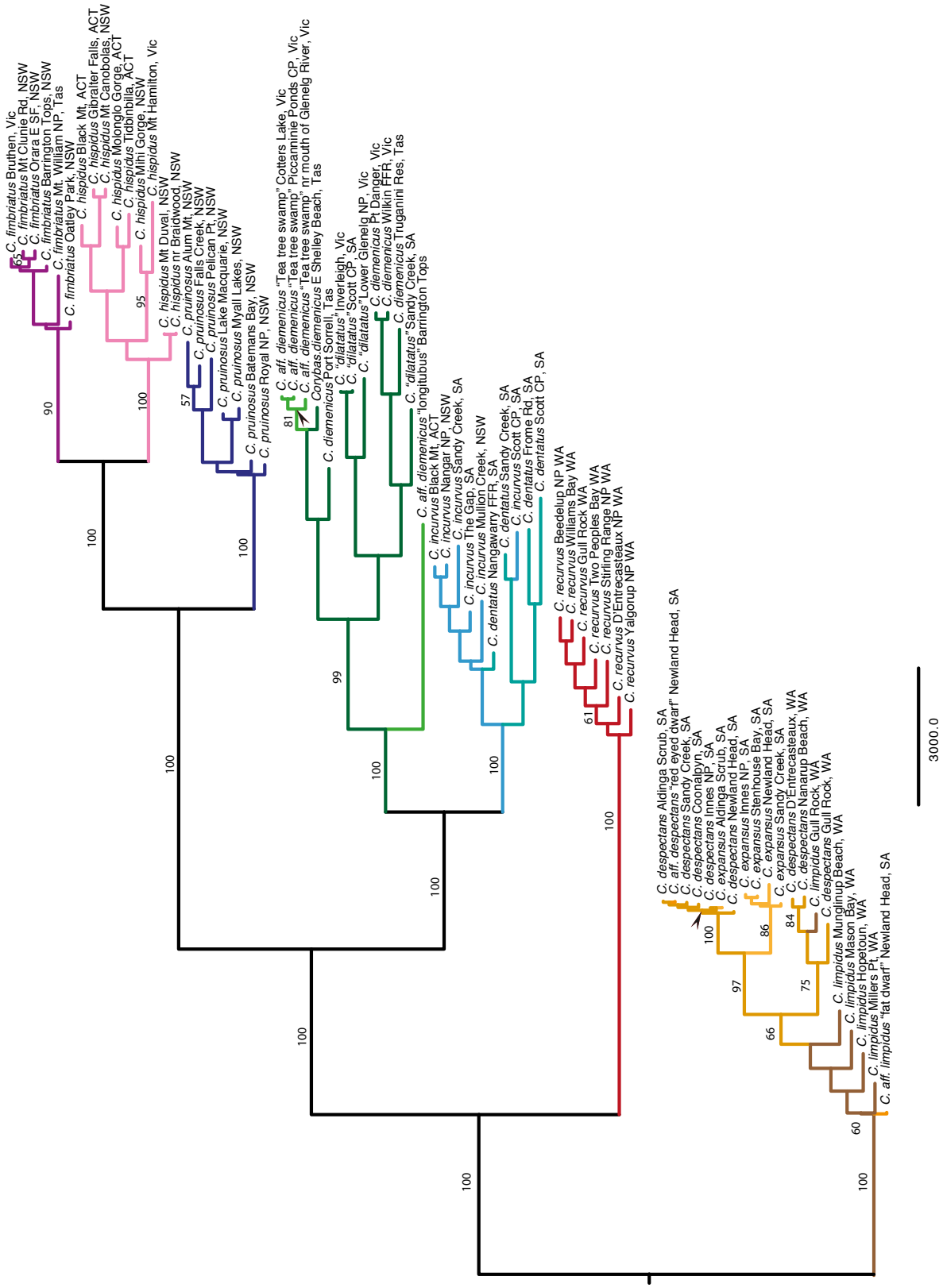
C. fimbriatus Bruthen, Vic
C. fimbriatus Mt Clunie Rd, NSW
C. fimbriatus Orara E SF, NSW
C. fimbriatus Barrington Tops, NSW
C. fimbriatus Mt. William NP, Tas
C. fimbriatus Oatley Park, NSW
C. hispidus Black Mt, ACT
C. hispidus Gibralter Falls, ACT
C. hispidus Mt Canobolas, NSW
C. hispidus Molonglo Gorge, ACT
C. hispidus Tidbinbilla, ACT
C. hispidus Mihi Gorge, NSW
C. hispidus Mt Hamilton, Vic
C. hispidus Mt Duval, NSW
C. hispidus nr Braidwood, NSW
C. pruinosus Alum Mt, NSW
C. pruinosus Falls Creek, NSW
C. pruinosus Pelican Pt, NSW
C. pruinosus Lake Macquarie, NSW
C. pruinosus Myall Lakes, NSW
C. pruinosus Batemans Bay, NSW
C. pruinosus Royal NP, NSW
C. aff. diemenicus "Tea tree swamp" Cotters Lake, Vic
C. aff. diemenicus "Tea tree swamp" Piccaninnie Ponds CP, Vic
C. aff. diemenicus "Tea tree swamp" nr mouth of Glenelg River, Vic
Conybas diemenicus E Shelley Beach, Tas
C. diemenicus Port Sorrell, Tas
C. "dilatatus" Inverleigh, Vic
C. "dilatatus" Scott CP, SA
C. "dilatatus" Lower Glenelg NP, Vic
C. diemenicus Pt Danger, Vic
C. diemenicus Wilkin FFR, Vic
C. diemenicus Truganini Res, Tas
C. "dilatatus" Sandy Creek, SA
C. aff. diemenicus "longitubus" Barrington Tops
C. incurvus Black Mt, ACT
C. incurvus Nangar NP, NSW
C. incurvus Sandy Creek, SA
C. incurvus The Gap, SA
C. incurvus Mullion Creek, NSW
C. dentatus Nangawarry FFR, SA
C. dentatus Sandy Creek, SA
C. incurvus Scott CP, SA
C. dentatus Frome Rd, SA
C. dentatus Scott CP, SA
C. recurvus Beedelup NP WA
C. recurvus Williams Bay WA
C. recurvus Gull Rock WA
C. recurvus Two Peoples Bay WA
C. recurvus Stirling Range NP WA
C. recurvus D'Entrecasteaux NP WA
C. recurvus Yalgorup NP WA
C. despectans Aldinga Scrub, SA
C. aff. despectans "red eyed dwarf" Newland Head, SA
C. despectans Sandy Creek, SA
C. despectans Coonalpyn, SA
C. despectans Innes NP, SA
C. expansus Aldinga Scrub, SA
C. despectans Newland Head, SA
C. expansus Innes NP, SA
C. expansus Stenhouse Bay, SA
C. expansus Newland Head, SA
C. expansus Sandy Creek, SA
C. despectans D'Entrecasteaux, WA
C. despectans Nanarup Beach, WA
C. limpidus Gull Rock, WA
C. despectans Gull Rock, WA
C. limpidus Munglinup Beach, WA
C. limpidus Mason Bay, WA
C. limpidus Hopetoun, WA
C. limpidus Millers Pt, WA
C. aff. limpidus "fat dwarf" Newland Head, SA
3000.0

Figure 7.

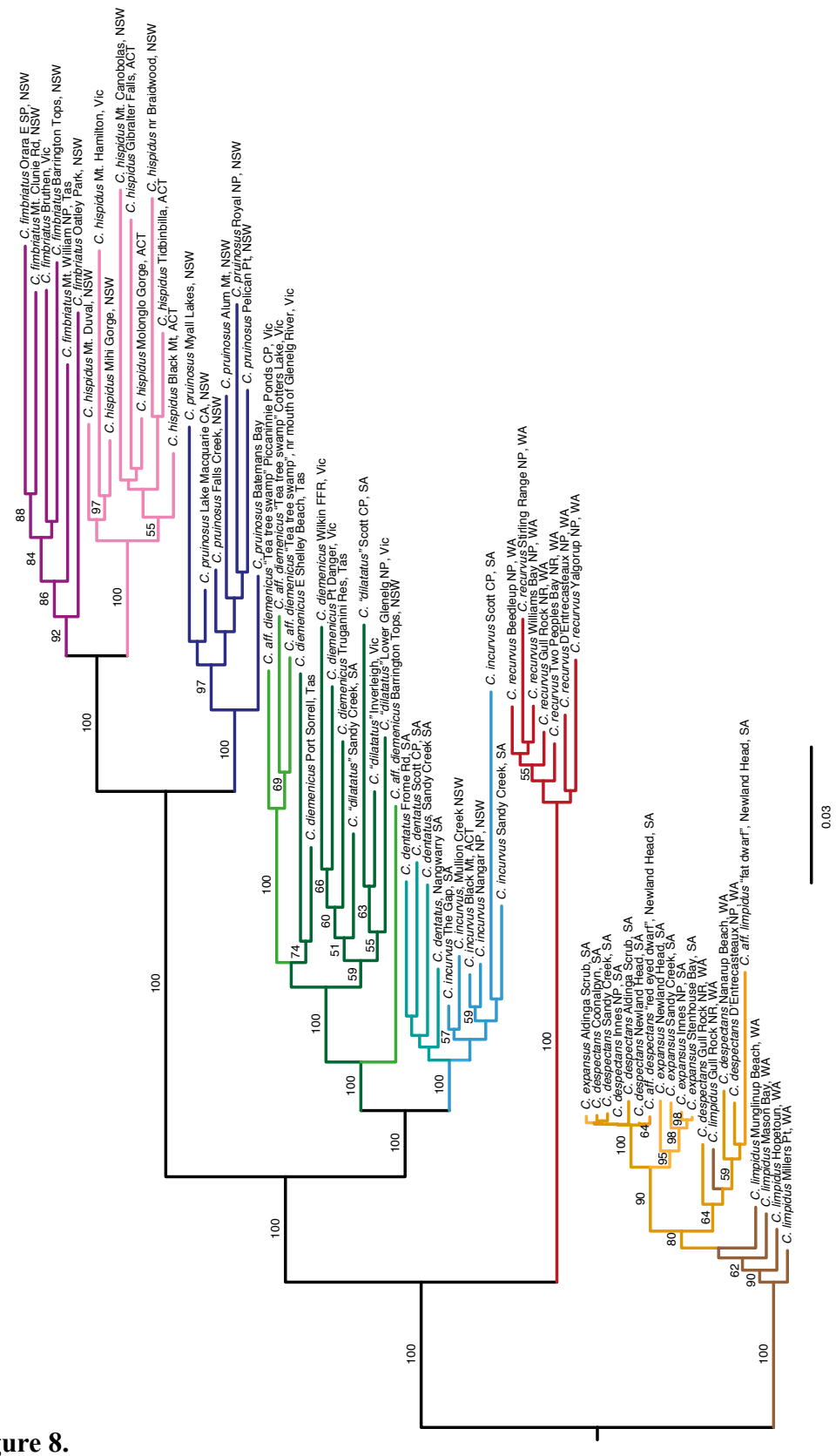
C. fimbriatus Orara E SP, NSW
C. fimbriatus Mt. Currie Rd, NSW
C. fimbriatus Bruthen, Vic
C. fimbriatus Barrington Tops, NSW
C. fimbriatus Mt. William NP, Tas
C. fimbriatus Oatley Park, NSW
C. hispidus Mt. Duval, NSW
C. hispidus Mt. Hamilton, Vic
C. hispidus Mihi Gorge, NSW
C. hispidus Mt. Canobolas, NSW
C. hispidus Molonglo Gorge, ACT
C. hispidus Gibraltar Falls, ACT
C. hispidus nr Braidwood, NSW
C. hispidus Tidbinbilla, ACT
C. hispidus Black Mt, ACT
C. pruinosus Myall Lakes, NSW
C. pruinosus Lake Macquarie CA, NSW
C. pruinosus Alum Mt, NSW
C. pruinosus Falls Creek, NSW
C. pruinosus Royal NP, NSW
C. pruinosus Pelican Pt, NSW
C. pruinosus Batemans Bay
C. aff. diemenicus "Tea tree swamp" Ponds CP, Vic
C. aff. diemenicus "Tea tree swamp" Cotters Lake, Vic
C. aff. diemenicus "Tea tree swamp", nr mouth of Glenelg River, Vic
C. diemenicus E Shelley Beach, Tas
C. diemenicus Port Sorrell, Tas
C. diemenicus Wilkin FFR, Vic
C. diemenicus Pt Danger, Vic
C. diemenicus Truganini Res, Tas
C. "dilatatus" Sandy Creek, SA
C. "dilatatus" Scott CP, SA
C. "dilatatus" Inverleigh, Vic
C. "dilatatus" Lower Glenelg NP, Vic
C. aff. diemenicus Barrington Tops, NSW
C. dentatus Frome Rd, SA
C. dentatus Scott CP, SA
C. dentatus Nangwarry SA
C. dentatus Sandy Creek SA
C. incurvus The Gap, SA
C. incurvus, Mullion Creek NSW
C. incurvus Black Mt, ACT
C. incurvus Nangar NP, NSW
C. incurvus Sandy Creek, SA
C. incurvus Scott CP, SA
C. recurvus Beedelup NP, WA
C. recurvus Stirling Range NP, WA
C. recurvus Williams Bay NP, WA
C. recurvus Gull Rock NR, WA
C. recurvus Two Peoples Bay NR, WA
C. recurvus D'Entrecasteaux NP, WA
C. recurvus Yalgorup NP, WA
C. expansus Aldinga Scrub, SA
C. despectans Aldinga Scrub, SA
C. despectans Sandy Creek, SA
C. despectans Innes NP, SA
C. despectans Aldinga Scrub, SA
C. despectans Newland Head, SA
C. aff. despectans "red eyed dwarf", Newland Head, SA
C. expansus Newland Head, SA
C. expansus Sandy Creek, SA
C. expansus Innes NP, SA
C. expansus Stenhouse Bay, SA
C. despectans Gull Rock NR, WA
C. limpidus Gull Rock NR, WA
C. despectans Nanarup Beach, WA
C. despectans D'Entrecasteaux NP, WA
C. aff. limpidus "fat dwarf", Newland Head, SA
C. limpidus Munglinup Beach, WA
C. limpidus Mason Bay, WA
C. limpidus Hopetoun, WA
C. limpidus Millers Pt, WA
0.03

Figure 8.

Chapter 3: Mycorrhizal interactions in the Corysanthes clade: Specificity, effects of phylogeny and environment, and relationship to range size

Abstract

In order to assess the role of mycorrhizal associations in the recent radiation of an Australian endemic clade (Corysanthes) of the orchid genus *Corybas* (Acianthinae, Diurideae), we investigated the identity and specificity of all major lineages within this group. We tested whether these traits were significantly constrained by phylogeny or more likely to be divergent among closely related taxa than expected by chance. We also examined correlations between environmental conditions and fungal community composition. Because fungal association may also affect dispersal ability, and in turn rates of diversification, we also examined the relationship between range size and specificity in a phylogenetic context. Members of the Corysanthes clade primarily utilize five fungal operational taxonomic units (OTUs) in the genus *Tulasnella*. None is a close match to any described species of *Tulasnella*. Additional, occasional symbionts were detected from the genera *Tulasnella*, *Ceratobasidium*, *Sebacina*, and *Tomentella*. Each species of orchid utilized a distinct suite of fungal associates, but there was substantial overlap among these. All orchid species associated strongly with one particular *Tulasnella* species ("*Tulasnella* 8"). Species also differed in their degree of specificity towards fungi, both as measured using the full suite of detected mycorrhizal fungi (including the rare, occasional symbionts), and as measured by the suite of Tulasnella OTUs utilized. For the most part, we were unable to detect a phylogenetic signal in fungal community usage or specificity. However, there was a significant phylogenetic signal in the usage of two fungal OTUs ("*Tulasnella* 5" and "*Tulasnella* 1"). This also correlated with the coastal dune habitats and seasonally high precipitation that are more typical for the two western—and earliest diverging—lineages. Character states related to fungal association also did not appear significantly phylogenetically over-dispersed. Several soil and climatic factors significantly correlated with the fungal community ordination axes—the first axis strongly correlated with seasonality of precipitation and soil conditions ranging from deep sands to more structured soils with higher clay content.

The second axis strongly correlated with altitude and seasonality of temperature. This suggests a direct effect of environmental conditions on patterns of mycorrhizal association. That said, co-occurring orchid species tended to utilize slightly different suites of fungi. Orchid species — as a factor — explained more variation in fungal communities than any one environmental variable. Species with a larger phylogenetic breadth of fungal associations did not tend to have larger ranges; if anything, the pattern appeared to be the opposite. We discuss potential reasons for this counterintuitive result, as well as the potential for promiscuity in fungal relationships to allow for dispersal out of Australia and immediately adjacent areas.

Introduction

Ecological and evolutionary interactions with mycorrhizal fungi are among the many processes that may help drive diversification in the exceptionally species-rich family Orchidaceae (Taylor et al., 2004; Shefferson et al., 2007; Waterman and Bidartondo, 2008). All orchids pass through a mycoheterotrophic stage after germination. During this time, they rely on fixed carbon obtained by digesting fungi that have colonized their roots. Most orchids later become photosynthetic, although the association with mycorrhizal fungi persists in many terrestrial genera along with the potential to receive carbon from these fungi. The nature of relationships between photosynthetic orchids and their mycorrhizal fungi – and their position on the continuum from parasitism to mutualism, and from mycoheterotrophy to autotrophy — is actively debated (Bidartondo et al., 2004; Girlanda et al., 2006; Cameron et al., 2007, 2008; Rasmussen and Rasmussen, 2009). Strong dependence on a resource, especially in deceptive or manipulative relationships, may favor high specificity in order to overcome host defenses (Thompson, 1994). Mycoheterotrophic orchids tend to be highly specialized on their fungal hosts. These hosts likely form ectomycorrhizae with nearby woody plants and obtain carbon thereby. Photosynthetic orchids have fungal associations that range from highly specific (McCormick et al. 2004; Shefferson et al. 2005; Irwin et al. 2007; Roche et al. 2010; Wright et al. 2010) to more generalized (Bonnardeaux et al. 2007; Shefferson et al. 2007; Roy et al. 2009).

Interactions with fungi may affect orchid diversification, but their role remains unclear. When mycorrhizal associations are highly specific, suitable orchid microsites

may be limited. This may help promote premating isolation among populations that diverge in their fungal partners. Combined with low fruit set in many taxa, this can reduce effective population sizes and increase the power of drift, perhaps punctuated by bouts of intense selection (Tremblay et al., 2004). Drift might increase rates of diversification, although an initial review of the literature failed to find the degree of population differentiation expected under strong drift (Phillips et al. 2012). Shifts to new fungal partners may promote speciation in both photosynthetic (Shefferson et al. 2007) and mycoheterotrophic orchids (Taylor et al., 2004; Barrett et al., 2010). While Roche et al. (2010) found extremely high fungal specificity in the Australian photosynthetic orchid genus *Chiloglottis*, all sampled species used the same mycorrhizal species of *Tulasnella*. Similar levels of specificity and conservatism appear in the related genus *Drakaea* (Phillips et al., 2011).

In order to assess the role of mycorrhizal associations in orchid diversification, we need to understand phylogenetic relationships within one or more orchid lineages, and to have data on the identity, variety, and relationships among mycorrhizal partners for the members of those lineages. Few authors have studied relationships within a fully sampled orchid clade. Several studies have reported phylogenetic signal (i.e. generally conserved relationships) in the identity of symbionts (Shefferson et al., 2007, 2010; Jacquemyn et al., 2011; Waterman et al., 2011; Ogura-Tsujita et al., 2012) and in the breadth of association with different orchid species (Shefferson et al., 2007, 2010; Jacquemyn et al., 2011). Without seed baiting or other kinds of environmental sampling for fungi, it can be difficult to separate the availability of fungi in particular environments from genetically

controlled preferences in different orchid species for particular fungi. In addition, particular environmental conditions might determine the suitability of particular fungi in specific contexts. Environmental factors play a role in shaping the relationships between orchids and their mycorrhizal fungi (Phillips et al., 2011; Martos et al., 2012; McCormick et al., 2012; Long et al., 2013; Pandey et al., 2013). In some cases, purported shifts in fungal association may result more from changes in habitat association or dispersal to new areas than from changes in orchid fungal preference. However, it is often the case that when related orchids co-occur within sites, they maintain (or even strengthen) their different fungal preferences (Taylor et al., 2004; Shefferson et al., 2007; Waterman and Bidartondo, 2008).

If the availability of particular fungi limits successful dispersal and establishment in distant areas, one might hypothesize that orchid species compatible with a broader range of fungi should have broader geographic and/or ecological ranges. In recent years, a number of studies have analyzed the relationship between rarity and mycorrhizal specificity more explicitly. In a few cases, wide-ranging weedy orchids have a much broader suite of fungal associates (Bonnardeaux et al., 2007; Long et al., 2013) than narrowly endemic taxa (Swarts et al., 2010). To the extent there is a pattern, it is often the reverse of what would be expected. For example, Bailarote et al. (2012) compared the fungal associates of one rare orchid undergoing population decline and a more common species. The common species used a much narrower range of fungi than the rare species. A review of the existing literature by Pandey et al. (2013) found that the rarest orchid taxa tended not to be particularly specialized, whereas those orchids that are highly

specialized on their fungi tend to have moderately sized to large ranges. In *Orchis*, Jacquemyn et al. (2010, 2011) noted significant nestedness in interaction networks with mycorrhizal fungi. Orchid species with highly specialized fungal interactions used fungi that were utilized by a wide range of other orchids. The fungi that were rarely detected were only used by generalist orchids. The more common fungal associates also appeared to have wider geographical ranges.

The genus *Corybas* (Orchidaceae, subfamily Orchidoideae) provides ideal material for testing whether recently formed species specialize on different mycorrhizal fungi, or whether differences in fungal associates appears to be more related to the different ecological conditions occupied by sister species than to their divergence in fungal associates within the same habitat. *Corybas* (ca. 135 spp.) consists almost exclusively of small, terrestrial species with single leaves and flowers that grow in populations that are clonal patches. The genus likely originated in Australia ~ 15 million years ago and then showed remarkable dispersal ability, colonizing areas as distant as the Himalayas and Tahiti (Chapter 1). Only one lineage – initially identified by Clements et al. (2002) and here referred to as the Corysanthes clade – is wholly endemic to Australia, where it is restricted to areas close to the southeastern and southwestern coasts as well as Tasmania (Fig.1). The Corysanthes clade is generally thought to contain at least 10 species (Kew 2014), although Jones (2007) recognizes more. Additional forms have been proposed as distinct species (Jones, 1993, 2008; Jeanes and Backhouse, 2006; Brown et al., 2008; Bates, 2011). Species within this clade all appear to have diverged from each other during the last 1-2 million years, following roughly 6.5 million years of divergence between

their ancestor and their closest relatives within the genus (Chapter 1). Habitat preferences in this group range widely, from coastal sand dunes to temperate rain forest and from elevations at sea level to around 1000 meters. Two species are restricted to Western Australia, two are narrow endemics in South Australia, and one is a narrow endemic in SE Australia. Species delimitations are uncertain, especially in the *C. diemenicus* complex. One hybrid has been described, and the species *C. dentatus* appears to be morphologically intermediate between two other species (Bates 2009). Very limited data currently exist regarding the identity and specificity of fungal symbionts of *Corybas*, though pre-molecular studies of the Australian taxa by Warcup (1981) indicated that the genus appeared to associate with the fungal genus *Tulasnella* (Heterobasidomycetes: Cantharellales: Tulasnellaceae).

Here, we use DNA sequences to identify the mycorrhizal associates of all species in the Corysanthes clade. We assess the specificity of such associations from both the fungal and plant perspectives and test whether the identity and specificity of mycorrhizal symbionts are more divergent within lineages than expected. We examine the role of environmental conditions in shaping the mycorrhizal communities associated with species in the Corysanthes clade and conduct a phylogenetically structured test of whether the range size of individual orchid species increases with the breadth of fungal associates.

Methods

Sampling and sample processing

We sampled multiple populations for all described taxa in the Corysanthes group apart from *Corybas grumulus* (Jones, 2008). Populations were also sampled for several tag names that have been proposed as distinct taxa. Figure 1 and Table 1 show sample locations along with the particular species sampled at a given site and the number of individuals of each species from which we obtained mycorrhizal fungi sequences.

We initially included material of supposed non-flowering *C. grumulus* from Black Mountain. These individuals turned out to be genetically identical to *C. incurvus* from the same location, and we believe they were misidentified. There are close morphological similarities of *C. grumulus* to other members of the *C. diemenicus* complex and it is often considered synonymous with *C. diemenicus*. We do not believe that the inclusion of populations classified as *C. grumulus* would significantly change our results because none of the other supposed segregates of the *C. diemenicus* complex appear to be genetically distinct (as based on standard regions for phylogenetic analyses) and the complex as a whole appears to be quite consistent in its mycorrhizal associations across a range of environmental conditions.

For each population sampled, we collected 4-6 whole plants, sampled as broadly across the population as possible. In cases where multiple species of *Corybas* occur, we preferentially sampled flowering plants or plants immediately next to flowering plants. A few additional populations with more limited sampling were also included in our

analyses, especially when they represented unusual plant locations or morphologies. Typically, whole living plants were collected in small amounts of substrate and stored in a plastic bag until they could be processed (within 1-2 days). In some cases, roots were immediately cleaned under running water, and whole plants were stored in a clean plastic bag on a damp paper towel in the refrigerator until they could be processed. Leaf tissues from these same plants were removed and dried for use in phylogenetic studies of the Corysanthes group.

We initially identified a few mycorrhizal symbionts via culturing. In June-July 2009, we sampled numerous individuals of various species of *Corybas* and the related genera *Acianthus* and *Cyrtostylis*. Within the *Corysanthes* group, we attempted to culture fungi from *Corybas diemenicus*, *C. despectans*, *C. pruinosus*, *C. incurvus*, *C. fimbriatus*, and *C. hispidus*. Root and collar tissues were thoroughly washed, and the epidermis, external hyphae, soil particles were removed. Under a laminar flow hood, segments of peleton-containing tissues were macerated, and individual peletons were transferred though five washes in sterile water following the protocol described in McCormick et al. (2004). We individually plated the 5-6 healthiest looking peletons, each in less than 1 μL of water from the final wash, on Fungal Isolating Medium (FIM) (Clements et al., 1986). Hyphae from each germinating peleton were subcultured onto fresh, solid FIM, and then subsequently subcultured into liquid FIM. Cultures grown successfully in liquid FIM were then rinsed, the central agar plug was removed, and tissue was dried in a lyophilizer. While isolates were easily obtained from almost all sampled individuals of *Acianthus* and *Cyrtostylis*, as well as several individuals *of Corybas aconitiflorus s.l.* and *Corybas*

unguiculatus, only 3 individuals (all *C. pruinosus*) from the Corysanthes group yielded any fungal isolates. Upon sequencing, we identified all these cultures as members of *Tulasnella calospora* clade ("*Tulasnella* 2"). This was also the determination for the majority of *Acianthus* and *C. aconitiflorus s.l.* cultures.

For all other samples, we identified mycorrhizal fungi through direct amplification of mycorrhizal tissues. These samples were processed as follows: the collar (swollen region at the interface of root and stem) and the thicker portions of the root were scrubbed under tap water with a brush until no visible dirt remained. The samples were then surface sterilized with 0.25% NaClO for 2 minutes and washed three times with sterile distilled deionized water. Samples were then examined under a dissecting microscope. Any remaining dirt or external hyphae were removed, together with as much of the epidermis as possible without discarding too much of the area of fungal colonization (often directly below a very thin epidermis). Peleton-containing regions were excised, transferred to a microcentrifuge tube with a ventilated lid, and quickly dried in silica gel or with a lyophilizer.

DNA extraction, PCR, cloning and sequencing

We extracted DNA from the preserved mycorrhizal tissues of 268 plants in the Corysanthes groups, as well as from several cultures and mycorrhizal tissues from related orchids. Tissues were ground using a TissueLyser (QIAGEN, Germantown, MD) at 30 Hz for 2 minutes. Various extraction kits were used early in the study, including the

DNEasy Plant Mini Kit (QIAGEN, Germantown, MD), the Extract N Amp Plant Kit (Sigma-Aldrich, St. Louis, MO), the EZNA Fungi Mini Kit and the EZNA Fungi High Performance Mini Kit (Omega Bio-Tek, Norcross, GA). The EZNA Fungi High Performance Mini Kit was ultimately chosen for use in the great majority of samples (~250). The combination of a CTAB-based extraction method modified to precipitate fungal polysaccharides (which inhibited amplification of extraction products from some fungal cultures) and a column cleanup worked well for our purposes. We extended the initial lysis period to 45-60 minutes, and eluted in only 100-150 µL of TE buffer.

DNA analyses utilized the nuclear ribosomal internal transcribed spacer region (nrITS). This region is the primary barcoding region for fungi (Schoch et al., 2012), and a recent study indicated that with the genus *Tulasnella* (which makes up the majority of samples included here), nrITS alone yields nearly identical results to phylogenetic analyses of multiple low-copy nuclear genes (Linde et al., 2014). Five µL of extracted DNA were added to each 25 µL PCR reaction. We attempted to amplify the fungal ITS region(s) for each sample using two different primer combinations: ITS-OF1 with ITS-OF4, and ITS-1 with ITS-4Tul (Taylor and McCormick, 2008). PCR programs followed Taylor and McCormick (2008), but used 35 cycles, as our DNA concentrations were often low. The OF primers are designed to amplify all major groups of known orchid symbionts, including *Tulasnella*. However, due to high rates of molecular evolution in the ITS region of *Tulasnella*, more general primers may not work and genus-specific primers are often necessary (Taylor and McCormick, 2008).

Prior to sequencing, PCR products were imaged on a 1.5% agarose gel. A handful of reactions showed obvious double bands. These separate bands were gel-extracted using either the ZymoClean Gel DNA Recovery Kit (Orange, CA) or the QIAquick Gel Extraction Kit (QIAGEN, Germantown, MD, reamplified, and sequenced separately. PCR reactions were cleaned using ExoSAP (Affymetrix, Santa Clara, CA), and sequencing reactions were cleaned using a Sephadex column in a Millipore plate (EMD Millipore, Billerica, MA). We sequenced all reactions using ABI BigDye technology (Life Technologies, Grand Island, NY) at the UW-Madison Biotech Center.

Sequences were assembled using Geneious Pro v 5.4.3 (Biomatters Ltd., Auckland, New Zealand). Occasional SNP polymorphisms were detected in these sequences, affecting less than 1% of bases in a given sequence—these were coded with the IUPAC ambiguity codes. This should not impact the assignment of fungal symbionts to OTUs. Nineteen reactions yielded sequences around 2200 bp (the core clade of "*Tulasnella 5*"), and additional internal sequencing primers were designed to capture the full sequence. While this is abnormally long for the ITS region, *Tulasnella* fungi are well known for their high rates of ITS sequence evolution (Taylor and McCormick, 2008; Linde et al., 2014). About a quarter of the initial sequences showed base ambiguities beyond occasional SNPs. We reran these reactions and cloned using the pGEM-T vector system (Promega, Madison, WI). We attempted to select eight colonies for re-amplification; in most cases cloning reactions yielded at least four colonies containing inserts of the correct size. The ITS region was amplified directly from the selected colonies, and sequenced as with the other samples.

Analyses

We initially aligned sequences using Clustal X (Larkin et al., 2007) and the Consensus Align algorithms implemented in Geneious Pro v 5.4.3. Representatives of distinct clusters of sequences were searched using megablast (Altschul et al., 1990) against the NCBI GenBank nucleotide and UNITE fungal ITS databases (Kõljalg et al., 2013), to provide preliminary identifications to genus. Close matches for each major group of fungal sequences, especially those identified to species and those isolated from other orchid species, were incorporated into alignments. Because of the large number of highly divergent *Tulasnella* sequences obtained, we performed the final *Tulasnella* alignment using Muscle (Edgar, 2004) as implemented on the CIPRES science gateway, then manually adjusted the alignment in Geneious. Maximum likelihood analyses were conducted for *Tulasnella* and all other mycorrhizal fungi separately. We ran analyses on the CIPRES science gateway using GARLI 2.01 (Zwickl, 2006), under a GTR+G+I model of sequence evolution. Bootstrap analyses in RAxML used the same model, with the automatic stopping criterion implemented. Fungal OTUs were initially identified using the criteria of reciprocal monophyly and relative branch lengths, but also generally met the < 3% divergence in ITS criterion for recognizing distinct fungal species (Barrett et al., 2010; Roche et al., 2010; Linde et al., 2014). In a few cases, a slightly more distant lineage was grouped into an OTU when the relationship was strongly supported, to avoid an excess of singleton taxa.

Detailed analyses of phylogenetic and phylogeographic patterns in the Corysanthes group are the subject of a separate paper (Chapter 2). At the time of writing,

we had conducted analyses only of plastid spacer (*trnL-trnF*, *psbJ-petA*, and *rps16-trnQ*), nrITS, and phytochrome C (*PhyC*) sequences. These analyses identified only six distinct, moderately to strongly supported clades in the Corysanthes group. These are: 1) *Corybas despectans s.l.* (including both eastern and western *C. despectans*, *C. limpidus*, *C. expansus*, and the tag name *C. aff. limpidus* "fat dwarf" from South Australia), 2) *Corybas diemenicus s.l.* (including *C. "longitubus"* [Jones MS] from the Barrington Tops, the western populations with wider flowers sometimes called *C. "dilatatus"*, and the standard form of *C. diemenicus*), 3) *Corybas incurvus*, 4) *Corybas pruinosus*, 5) *Corybas recurvus*, and 6) a clade consisting of both *Corybas fimbriatus* and *C. hispidus*. While we detected no genetic differences between *C. fimbriatus* and *C. hispidus* at these particular loci, these two taxa have distinctive floral morphologies with no obvious intermediate forms (Jones, 1973). Moreover, they have distinct habitat preferences and essentially no range overlap. On a 0.5 x 0.5 degree grid overlaid on the range maps for each species, only three cells contained records for both species, and these were always found at different elevations. As a result, we treated them as distinct species in our analyses. *Corybas dentatus*, a highly localized endemic from South Australia, variously appears as genetically identical to either *C. incurvus* or *C. diemenicus*, depending on the site and the locus sequenced. This supports the hypothesis that *C. dentatus* is a hybrid between *C. incurvus* and *C. diemenicus*. Indeed its morphology is intermediate between the two and it lives almost exclusively in areas where the two likely parents co-occur. While we report the fungal associates of this taxon, we excluded it from all phylogenetically structured analyses. To construct a general tree of the Corysanthes clade for phylogenetically structured analyses, we ran a partitioned analysis of combined

plastid, nrITS, and PhyC data using GARLI 2.01 implemented on CIPRES, with all partitions using a GTR+G+I model (as determined with jModelTest (Posada, 2008)). Using Mesquite (Maddison and Maddison, 2011), we arbitrarily pruned the resulting tree to a single representative accession for each plant species. Because of the lack of differentiation between *C. hispidus* and *C. fimbriatus* and the near-zero ($1x10^{-8}$) branch length joining *C. recurvus* to the eastern taxa, these branches were set to 0.0001, about an order of magnitude shorter than other branch lengths within the tree.

For each orchid species, we estimated fungal specificity following Shefferson et al. (2007). This index of specificity calculates the average molecular distance from each fungal sequence found in an individual of a given orchid species to every other fungal sequences obtained for that species. Genetic distances are first averaged by population and then those average population distances are averaged by species. In cases where we obtained multiple sequences from a single individual, genetic distances were averaged for the individual first. This index down-weights the distances of rare symbionts within a population, but populations themselves are given relatively high weight. To avoid bias, we excluded populations where only a single plant yielded mycorrhizal sequences. Genetic distances were calculated in two different ways. First, we used an alignment of the 5.8S region for all mycorrhizal sequences—the ITS region in its entirety was too variable to align across fungal genera. Second, we used an alignment of the full ITS region for all *Tulasnella* sequences (which accounted for the great majority of fungal sequences obtained). We used genetic distances calculated using the GTR+G model in PAUP (Swofford, 2003).

We ordinated mycorrhizal communities using NMDS, implemented in the vegan package in R (Oksanen et al., 2013) using the metaMDS function on Bray distances. We then plotted correlations with numerous environmental variables and the abundance of different fungal OTUs, and calculated the centroids for each orchid taxon. Elevation came directly from GPS readings made in the field or, if unavailable, using a fine-scale elevation map from WorldClim (Hijmans et al., 2005). We included latitude and longitude and obtained annual precipitation, precipitation seasonality (standard deviation), mean annual temperature, and temperature seasonality (coefficient of variation) from additional WorldClim GIS layers. The Australian Soil Atlas provided soil data (ASRIS, 2011), specifically characteristics for the dominant soil type within a given area. Exploratory analyses revealed that several of the soil variables were strongly correlated with one another. Consequently, we focused primarily on: A horizon median clay content, A horizon median structure (degree of pedality calculated on a scale of 1 to 5, with 5 being the most highly structured), A horizon median thickness, A horizon water holding capacity per unit depth, A horizon median saturated hydraulic conductivity (K_{sat}), solum (soil plus subsoil layers sharing the same weathering history) median thickness, and nutrient content (measured as responsiveness to N/P/K fertilizer on a scale of 1 to 3, with 1 being the most responsive).

To estimate range size, we obtained collection records for each orchid species from the Australian Virtual Herbarium (AVH, 2014), with additional records provided by the Western Australian herbarium and our own work. Because of past taxonomic confusion (Jones and Clements 1988), we discarded all *C. diemenicus* records prior to

1980 and all other records before 1960. These records accounted for a relatively small proportion of the total, generally overlapped with more recent records, and tended to have poor or missing locality information in any case. Any records with suspected errors in georeferencing (e.g., locations mapping offshore or well into the dry interior) were carefully scrutinized and usually discarded as well. Range size was calculated as the number of 0.5 by 0.5 degree grid cells occupied by a given orchid taxon. For GIS analyses we used QGIS (QGIS Development Team, 2013).

Fungal specificity (calculated using both the combined 5.8S data set and the Tulasnella ITS data set) and Axes 1 and 2 scores for each orchid species centroid in the fungal ordination were mapped onto the phylogeny for the Corysanthes group. We reconstructed ancestral states using the parsimony criterion in Mesquite. Data consisting of range size, combined fungal 5.8S specificity, Tulasnella ITS specificity, Axis 1 score, and Axis 2 score for each orchid species, together with the phylogeny, were analyzed using PhyloCom (Webb et al., 2008). We used the AOT (Analysis of Traits) module to test for phylogenetic signal in each of these characters and to examine whether the mean and standard deviation of descendent nodes differed from expected based on randomization (test of phylogenetic conservatism and/or over-dispersion). We also used the AOT module to analyze correlations among phylogenetic independent contrasts of range size and specificity.

As an additional test of the relationship between fungal usage and relatedness of orchid taxa, we also calculated fungal overlap between each pair of orchid species using the Schoener (1970) index, and assessed whether it showed a significant positive or

negative correlation with phylogenetic distance between orchid species using linear regression in R. The Schoener index calculates the distance between two species as: $1 - 0.5 \times \Sigma (N_{iA}-N_{iB})$, where A and B are the two species and N_i is the proportion of individuals using, in this case, the i^{th} fungal taxon. Distances between orchid species were calculated as the average molecular distance (calculated under a GTR+G model) from each individual of species A to each individual of species B.

Results

Sequencing results

Of the 268 individuals from which we attempted to amplify the fungal ITS region, 195 yielded sequences of potential mycorrhizal fungi. Of these, 180 individuals were associated exclusively with *Tulasnella*, seven yielded both *Tulasnella* and some other mycorrhizal sequences, and eight were found to contain only non-*Tulasnella* fungi. Figure 2 labels the major clades identified and treated as fungal OTUs in the characterization of fungal communities.

We omitted a small number of fungal sequences obtained from subsequent analyses as probable contaminants or pathogens. All were found in only one or two individuals. These probably contaminant sequences were identified as *Nigrospora sp.*, *Clitopilus hobsonii*, *Ilyonectria cyclaminicola*, *Cercophora sp.*, *Fusarium sp.*, and *Mycena sp.,* and an unidentified zygomycete. Of these, only *Mycena* might possibly be a mycorrhizal symbiont, as it has been found in association with the unrelated orchids

Dendrobium officinale (Zhang et al., 2012) and the adult stage of *Gastrodia elata* (Ogura-Tsujita et al., 2009). However, given that we detected this genus in only a single individual, and that it co-occurred with another highly unusual fungus, it seems more likely that this sample was not properly cleaned or surface-sterilized and hence the DNA extract may have been contaminated with soil fungi.

The genera *Ceratobasidium*, *Sebacina*, and *Tomentella* are all known groups of orchid mycorrhizal fungi and were amplified from multiple individuals in this study. *Ceratobasidium* and *Sebacina* have been cultured or directly sequenced from other *Corybas* accessions (S. Lyon, unpublished data; Clements et al., 2011; Watkins, 2012), and are major symbionts of a large number of Australian orchid taxa (Warcup, 1981). Seven of the eight *Ceratobasidium* sequences in this study were less than 0.5% divergent from the sequence of the unidentified mycorrhizal symbiont of the underground orchid *Rhizanthella gardneri* (Bougoure et al. 2010). The eighth was clearly related, but about 10% divergent from the other taxa. The 3% sequence divergence criterion is often applied in defining fungal OTUs (Barrett et al., 2010; Roche et al., 2010; Linde et al., 2014), though researchers have noted that individual fungal species may actually contain more variation, especially in rapidly evolving groups (Nilsson et al., 2008).

Among the *Sebacina* sequences, there were three distinct groups detected in members of the Corysanthes groups. The *Sebacina* 1 clade was detected in a single individual each of *C. hispidus*, *C. fimbriatus*, *C. pruinosus*, and *C. despectans*. All sequences were a maximum of 2.4% divergent from each other. No named species of *Sebacina* were closely related to this clade: an unidentified fungus from *Dactylorrhiza*

AM697889 was the closest BLAST hit. The clade appears embedded within a group containing multiple fungi considered part of the *Sebacina vermifera* complex. *Sebacina* 3, detected in a single individual of *Corybas recurvus*, was also cultured from *Cyrtostylis robusta* from South Australia and appears genetically very close to Warcup's isolates from *Cyrtostylis "reniformis"* (probably *C. robusta*). These sequences were all closely related to *Sebacina vermifera* FN663145 isolated from *Eriochilus scaber*. *Sebacina* 4 was detected in *C. fimbriatus* (OFE159) and *C. pruinosus* (OFE290), the former of which is closely related to *Sebacina vermifera* AF202728, and though the two sequences are about 6.8% divergent they are treated as part of a single OTU in this study.

Tomentella has not previously been detected as a symbiont in any Australian orchids, but in our study was found in three different taxa ranging from northern NSW to far western SA. Its occasional presence in samples in this study could possibly be a result of contamination from soil fungi. However, it is a major group of symbionts in other orchids (McCormick et al., 2004; Matsuda et al., 2009; Barrett et al., 2010), and an important ectomycorrhizal group in Australia (Chambers et al., 2005; Midgley et al., 2007). Furthermore, all three sequences grouped with *Tomentella sublilacina*, a known ectomycorrhizal *Tomentella* (Lilleskov and Bruns, 2005). Thus *Tomentella* was treated as an occasional, likely opportunistic mycorrhizal fungus for this group. The sequences were at most 6.6% divergent from one another, but were treated as a single OTU for the purposes of characterizing fungal communities.

Most sequences obtained were classified as *Tulasnella.* Only one of these clades yielded a relatively close BLAST match to a named *Tulasnella* species—*Tulasnella*

calospora (*Tulasnella* 2). We detected this clade in a few individuals of *Corybas pruinosus*, including from three cultures obtained from Alum Mt. in NSW, and a single individual of *Corybas hispidus*. All sequences in this clade were less than 0.4% divergent from one another. Several other major lineages encountered in this study were related to this clade. *Tulasnella* 3, detected in a single individual of *Corybas dentatus*, was genetically very close to the Warcup isolate *T. violea* DQ520097, though the species concept of *T. violea* has some problems (Suárez et al., 2006). *Tulasnella* 6, also detected in a single individual of *C. dentatus*, was strongly supported as a member of a clade containing unidentified fungi from *Cymbidium* (AB506850), *Gymnadenia* (KC243955), and *Piperia* (JQ994405), and somewhat distantly related to the *Tulasnella pruinosa* (DQ457642). Finally, *Tulasnella* 1, detected only in the *Corybas despectans* complex on coastal dunes, included several fungal sequences from *Cypripedium californicum* (DQ925493-5) and a sequence from an Ecuadorian orchid (HM451667). While this clade contains more genetic variation than any other fungal OTU displayed in Figure 2, the actual sequences obtained from the Corysanthes group display less than 0.5% divergence from one another, except for a single sequence from Corybas despectans (OFE197, max. 5% divergence) that is embedded in the remaining Corysanthes sequences and is weakly supported as sister to the sequences obtained from GenBank.

All the remaining fungal sequences fell into a second large clade of *Tulasnella*. Within this clade, a single fungal OTU, *Tulasnella 8* accounted for over half of the fungal sequences obtained, and was found in association with at least 25-60% of the individuals sampled for each species in the Corysanthes group. There was some structure within this

group, often corresponding to orchid species or population. Sequences within this clade typically were less than 2.9% divergent from other clade members. The exceptions were 13 deeply embedded sequences that had a maximum of 4.5% divergence from other members of the clade. It is interesting to note that, despite being the major mycorrhizal associate of the Corysanthes group, *Tulasnella* 8 had no particularly close matches in GenBank, and has not been detected in any other groups of Corybas to date.

Tulasnella 8 is strongly supported as sister to *Tulasnella* 7, which itself is sister to an unidentified fungus from *Cymbidium goeringii* (AB506858). Two of the *Tulasnella* 7 sequences, one each from *C. pruinosus* and *C. fimbriatus*, show only 0.1% sequence divergence while the third, from *C. diemenicus,* is 5% divergent. These two clades are placed as sister to a clade consisting of *T. tomaculum* (KC152380), the *Tulasnella* species used by *Chiloglottis spp.* (e.g. HM196792), and an unidentified orchid fungus from Reunion Is. (JF691085), but this sister relationships is not supported.

Tulasnella 5, shown as sister to the aforementioned clades in this second major group of *Tulasnella* (but again without support), was found only in members of the *C. despectans* complex, particularly on coastal dunes in western and south Australia, as well as in two individuals of *C. recurvus* growing in a sand flat only a short distance from the dunes. This clade is highly unusual in that most of these accession yielded ITS sequences that were ~2200 base pairs in length (2-3 times longer than typical fungal ITS sequences). All sequences were less than 1.3% divergent from one another, though four sequences contained substantial gaps and were not excessively long. The tendency for *C. despectans* to associate with this group may explain why the majority of the mycorrhizal

samples that failed to yield fungal samples were from the *C. despectans* complex on coastal dunes—such a long ITS region may be subject to degradation in poorly preserved samples.

The final major *Tulasnella* clade, *Tulasnella* 4, could be subdivided into three distinct subclades. As a whole, the clade is genetically closest to an unidentified fungus from *Corybas trilobus* (in the sister group to the Corysanthes clade) in New Zealand (HM802323) and an unidentified fungus from *Gymnadenia* (KC243936). Most sequences fell into *Tulasnella* 4B, which represented the second most abundant symbiont for the Corysanthes group as a whole. Interestingly, it also tended to yield amplicons requiring cloning, due to the presence of multiple, closely related ITS strains found within a single individual. All sequences within *Tulasnella* 4B were less than 2.7% divergent from one another. *Tulasnella* 4C contained sequences all displaying less than 2.2% divergence, though the seven sequences from *C. recurvus* formed a separate, well supported subclade. Finally, *Tulasnella* 4A consisted of only 3 sequences. The two from *C. pruinosus*, from the same population, were nearly genetically identical, whereas the third sequence, from *C. recurvus*, was slightly more than 3% divergent from the other two.

Figure 2 also displays the patterns of association for each major clade in the Corysanthes group. Each has a slightly different profile. For *C. despectans*, the primary symbiont was *Tul.* 5, found in 43% of sequenced individuals. *Tul*. 8 was found in 36% of individuals, with *Tul.*1 as a minor symbiont (here defined as present in 10-25% of individuals. Occasional symbionts were found in fewer than 10% of sampled individuals, and are displayed in Figure 2 with dashed lines, but are not mentioned here. As

previously mentioned, *C. despectans* is unique in its associations with *Tul. 1* and is the only Corysanthes taxon to heavily utilize *Tul. 5*. While *C. hispidus* and *C. fimbriatus* are shown as a terminal primarily associating with *Tul. 8* with *Tul.* 4B as a minor symbiont, the two taxa have different fungal profiles. *Corybas hispidus* showed similar rates of association with *Tul. 8* (46%) and *Tul. 4B* (41%), while *Corybas fimbriatus* was found almost exclusively in association with *Tul. 8* (82%). *Corybas pruinosus* was also fairly strongly associated with *Tul.* 8 (64%), with *Tul. 2* as a minor symbiont. *Corybas incurvus* was nearly equally split in its association with *Tul. 8* (29%), *Tul. 4B* (35%) and *Tul. 4C* (35%), whereas *C. diemenicus* was strongly associated with *Tul. 8* (76%) with *Tul. 4B* as a minor symbiont. *Corybas recurvus* was similarly split in its association with *Tul. 8* (39%) and *Tul. 4C* (40%). The probable hybrid *C. dentatus* had, perhaps not surprisingly, a fungal usage profile somewhat intermediate between *C. incurvus* and *C. diemenicus*, fairly strongly associating with *Tul. 8* (63%), with 25% of individuals utilizing *Tul. 4C*. *Ceratobasidium* appears as a minor symbiont, though found in only two individuals, as our total sample size was only sixteen.

The NMDS ordination of fungal communities in two dimensions converged on a solution with a stress of 0.075, and achieved reasonably good separation of the fungal communities (Fig 5). Vectors for each of the fungal OTUs that were significantly correlated ($p<0.05$) with ordination scores are shown in Figure 5A, with correlation values shown in Table 2. Centroids for each sampled orchid species are plotted in Figure 5C, and species as a variable was highly significant (Table 2). The placement of centroids, based on populations, essentially match the patterns described above, based on

individuals. *Corybas despectans* has the highest score on Axis 1, for instance, which is highly correlated with usage of *Tul. 5* and *Tul. 1*.

Specificity

As calculated using the 5.8S region from all fungi, mean phylogenetic breadth of fungal associates ranged from 0.05 to 0.15. The lowest value was in *C. incurvus*. Despite being similarly split among the three largest groups of *Tulasnella*, *C. incurvus* was restricted to those three related clades. The highest values were in *C. despectans* and *C. pruinosus*. *Corybas despectans* had not only a phylogenetically wide range of *Tulasnella* associates, but also quite a few occasional associates from all three other groups (*Sebacina*, *Ceratobasidium*, and *Tomentella*). *Corybas pruinosus*, though using *Tul.* 8 to a large extent, also associated with the phylogenetically distant *Tulasnella calospora* (*Tul.* 2), and had very occasional associations with *Tomentella* and *Sebacina*.

As calculated using only *Tulasnella*, mean phylogenetic breadth of fungal associates ranged from 0.06 to 0.30. The lowest value was in *C. fimbriatus,* found only in association with *Tul.* 8 and the closely related *Tul.* 7. The highest value was 0.30 in *C. despectans*. Despite these calculations being limited to a particular genus of fungus, the values are higher (i.e. less specificity) because the entire ITS regions has been used to calculate specificity, rather than just the small, highly conserved 5.8S region.

Phylogenetic structure

Figure 3 shows parsimony-based reconstructions of ancestral character states for both specificity measures as well as axes scores. The only variable for which a significant phylogenetic signature was detected at the tree level, based on a minimal standard deviation of standardized contrasts, was Axis 1 score ($p=0.038$ based on randomization tests). Again, this primarily correlates with usage of *Tul. 1* and *Tul.* 5. However, as we will discuss in the next section, this axis is also significantly correlated with several environmental variables as well as longitude, and may reflect geography rather than phylogeny *per se*. Range, specificity using both *Tulasnella* and 5.8S data, and Axis 2 score were, at the tree level, neither more phylogenetically conserved nor over-dispersed than expected at random. Furthermore, we detected no correlation between phylogenetic distance and fungal community usage as calculated with the Schoener index (Figure 4A). The trend was slightly negative, but there were both phylogenetically close and phylogenetically distant taxa with very similar fungal communities, while those at intermediate phylogenetic distances tended to be less similar.

However, at some particular nodes, reconstructions of subsequent nodes did differ significantly from random. PhyloCom calculates two sets of divergence statistics at a node. The first set of values (T = terminal) are based on the average and standard deviation of trait values for all terminal taxa descended from a node, whereas the second set of values (A = ancestral averaging) are based on average and standard deviation of the descendent (daughter) nodes and take branch lengths into account. Because A statistics weight the values of terminal taxa inversely in proportion to the diversity of

encompassing subclades, they are considered a more direct measure of evolutionary divergence. For *Tulasnella* specificity, the A value at the root was higher than expected (p=0.005), indicating a shift towards increased specificity at subsequent nodes. For combined fungal 5.8S specificity, the A value for the *C. diemenicus* plus *C. incurvus* node was lower than expected (p=0.026), whereas the variance in both T and A values for the *C. fimbriatus* plus *C. hispidus* node were both lower than expected (p=0.035, these two taxa had exactly the same 5.8S specificity, despite using different sets of fungi). For Axis 1 scores (primarily corresponding to the use of *Tulasnella* 1 and 5, and *Tulasnella* 4C to a lesser extent), the root once again had a higher A value than expected (p=0.004), whereas T and variance in T were lower than expected for the eastern clade (both p=0.045). Finally, for Axis 2 score (largely corresponding to increased usage of *Tulasnella* 8, and lower usage of *Tulasnella* 4B and to a lesser extent *Tulasnella* 4C), the A value was higher than expected (p=0.044) for the node for the fringed clade (*C. fimbriatus*, *C. hispidus*, and *C. pruinosus*).

Environmental correlations

The following environmental variables were significantly correlated with fungal community composition: annual temperature, A horizon thickness, precipitation seasonality, A horizon K_{sat}, altitude, temperature seasonality, A horizon clay content, solum thickness, A horizon pedality (degree of structure), soil nutrients, and longitude (but, interestingly, not latitude). Figure 5B plots vectors for examined variables with a p-value of 0.05 or lower. Table 2 lists axis scores, R^2 and p-values

The environmental variable vectors clustered into 2 groups: those strongly correlated with axis 1, and those correlated with both axes. The former group consisted primarily of soil variables together with precipitation seasonality and, interestingly, longitude. Sites where the dominant soil type had higher clay content (and hence lower water conductance at saturation) and nutrient content had lower scores on axis 1, whereas sandier sites (where the A horizon tended to be thick and unstructured, with either little in the way of subsoil or subsoil layers with a different weathering history) had higher scores. The sandier sites also tended to have more seasonal rainfall, which itself seemed to be negatively correlated with longitude: i.e. those sites on thick, unstructured sand that received very seasonal precipitation tended to be more westerly distributed. In fact, the sites in Western Australia and western South Australia tended to be coastal sand dunes.

The remaining significant variables were tied to altitude, with the vectors for altitude and temperature seasonality both opposite to mean annual temperature. This is not surprising given that these sites were mild temperate to subtropical and largely found close to the coast. This group of variables was not independent of the soil attributes, as the sandy sites were also generally at very low altitudes. The altitude gradient does seem to separate community composition in a way that seems independent of soil quality.

Comparing the environmental variables to the species variables, we find *Tulasnella* 1 and 5 in sandy sites with highly seasonal precipitation, with *Tulasnella* 1 perhaps more common in warmer, low altitude sites. *Tulasnella* 4B tended to be found in those sites at higher altitude, whereas *Tulasnella* 4C seemed to be associated with a combination of somewhat higher altitude and more seasonal rainfall. *Tulasnella* 8, found

as a major symbiont in all species and in a wide range of habitats, seems to have particular dominance in sites that are intermediate in elevation and temperature, with less seasonal rainfall and somewhat richer, more structured soils.

While environmental variables appear to play a very strong role in structuring local fungal communities, even when split out by the species with which they associate, it is informative to compare the fungal community compositions of different species growing together at the same site. At the Black mountain site (ACT), where *C. incurvus* and *C. hispidus* grow in close proximity (with *C. hispidus* slightly higher up the gully in slightly moister conditions), both species associated in part with *Tulasnella* 4B, but while *C. hispidus* utilized *Tulasnella* 8, *C. incurvus* utilized *Tulasnella* 4C. At Gull Rock in Western Australia, *C. despectans* (including *C. limpidus*) utilized *Tulasnella* 1 and 5, whereas *C. recurvus* used a mixture of *Tulasnella* 8 and *Tulasnella* 4B. In this case, the microhabitat differences were obvious, with *C. despectans* found on mossy patches on and between the dunes, while *C. recurvus* is found in the peppermint tree (*Agonis flexuosa*) thickets behind the dunes. At Sandy Creek in South Australia, most taxa (*C. despectans*, *C. diemenicus*, and *C. dentatus*) heavily utilized *Tulasnella* 8, but the *C. incurvus* samples were all found to associate with *Tulasnella 4C.* At Scott Conservation Park, all three species (*C. diemenicus*, *C. incurvus*, and *C. dentatus*) primarily used *Tulasnella* 8*,* but a quarter each of the *C. incurvus* and *C. dentatus* specimens sampled were found to associate with *Tul*. 4C instead. This suggests that even when species are growing in quite close proximity to one another, they still tend to have somewhat different suites of associates. In other words, larger scale climatic and substrate factors do

not entirely explain patterns of association. It is uncertain whether the slight tendency to partition fungal resources is tied to genetically based preferences for particular fungi, or to minor differences in microhabitat that impact both seed germination and fungal distributions.

Relationship between fungal specificity and range size

We did not obtain compelling results regarding the potential relationship between fungal specificity and range size. Species with a broader range of associates did not have larger geographical ranges. In non-phylogenetically structured analyses, the trend was always slightly negative but not significant. This was true regardless of whether *C. dentatus* was included as a distinct taxon or not and whether specificity in regards to the full range of fungal associates or *Tulasnella* only was being considered.

Using phylogentically independent contrasts (PIC), we detected a statistically significant inverse relationship between *Tulasnella* specificity and range size. That is, taxa with smaller geographical ranges use a broader suite of *Tulasnella* fungi, as shown in Figure 4B ($r= -0.862$, $p=0.01$). However, PICs may not be the most valid form of comparison. Neither variable showed significant phylogenetic signal, though there did appear to be a slight trend in regards to *Tulasnell*a specificity. Interestingly, using PIC, range size was also positively correlated with Axis 2 score (mostly related to usage of *Tulasnella* 8), though this correlation was not quite significant ($r=0.698$, $p=0.08$).

Together, these correlations suggest that those taxa with larger ranges tended to be *more* specialized, but to specialize on the most common, widespread symbiont.

Discussion:

Fungal associations and specificity

The data presented here on the symbionts of the Corysanthes complex confirm the findings of Warcup (1981). In Australia, *Corybas* is primarily *Tulasnella*-associated, with occasional symbionts in other major clades of mycorrhizal fungi (especially *Sebacina* and *Ceratobasidium*). Very few individuals contained only non-*Tulasnella* fungi, suggesting that these fungi are often secondary colonizers. As reported by Warcup, the *Tulasnella* taxa used by Australian *Corybas* tend not to be members of the common, widespread *T. calospora*, but rather to be unusual, slow-growing *Tulasnella* that are difficult to culture.

We suspect that our *Tulasnella* 8, possibly in combination with members of *Tulasnella* 4, are the slow growing fungi noted by Warcup. Because none of his cultures of these fungi survived to be archived and sequenced, we cannot confirm this. Because these fungi have not been successfully cultured, we also cannot confirm whether these are the same fungi that support germination. Some orchids do completely switch fungal partners between the protocorm and adult stages (McCormick et al., 2004; Ogura-Tsujita et al., 2009). This does not seem particularly common, though certainly different fungi are known to differ in their functional importance (Huynh et al., 2009). Furthermore, because of the clonal nature of *Corybas*, individuals may not be limited to forming

associations with germination-supporting fungi—though populations almost always consist of multiple patches and contain at least some genetic diversity (S. Lyon, unpublished) — so clearly reproduction by seed is occurring as well. The widespread usage of most of these groups (*Tulasnella* 8, 4B, 4C, 5, and to a lesser extent *Tulasnella* 1) throughout populations and species is suggestive of their importance to the orchids, at least in the adult stages. Seed baiting techniques (Phillips et al., 2011; McCormick et al., 2012) would be useful to confirm which fungi support germination and protocorm development.

No members of the Corysanthes group are as specialized in their fungal relationships as several other Australian genera. *Chiloglottis* (Roche et al., 2010), *Drakaea* (Phillips et al., 2011), *Diuris* (Smith et al., 2010), *Caladenia* (Huynh et al., 2009; Swarts et al., 2010; Wright et al., 2010), *Rhizanthella* (Bougoure et al., 2010) associate exclusively with a very narrow group of fungi throughout their entire range. However, the members of the Corysanthes group are not quite as broad in their fungal associations as some other orchids either, for instance *Microtis* (Warcup, 1981; Bonnardeaux et al., 2007; Long et al., 2013). *Corybas* is often found growing together with members of *Cyrtostylis* and *Acianthus*, and is only rarely found using the purported fungi associated with these groups. The fungi primarily utilized by *Corybas* have not commonly been detected in other orchids, especially the most frequently utilized symbionts *Tulasnella* 8 and *Tulasnella* 4. As suggested by the nestedness of interaction networks in *Orchis* (Jacquemyn et al., 2010, 2011), there may be a tradeoff between

specialization on common, widespread fungi species and utilization of a broad suite of less common fungi species.

As in the South African Coryciinae (Waterman et al., 2011), specialization may occur at a broad level. The Corysanthes clade does seem to have a strong preference for the large *Tulasnella* clade containing *T. tomaculum* and relatives, despite occasional association with other fungal groups. The strong degree of specialization in both Australia and South Africa, with particular clades of orchids having a strong preference for particular clades of fungi, may be related to old, weathered soils. While recent climate change has occurred in Australia (drying and cooling in the last few million years), both plant and animal taxa have tended to persist in numerous small pockets within the landscape, rather than dispersing out of a few major refugia (Byrne, 2008). This long-term relative stability, combined with poor soils, may have promoted specialization on particular groups of fungi and allowed for the coexistence of different orchid groups through partitioning of fungal resources.

Phylogeny versus environment

We did not detect strong phylogenetic structure in fungal relationships, nor did we detect more switching of partners than expected at random (over-dispersal). However, the Corysanthes clade has diversified over a very short time span (1-2 million years) At the species level, they often have different sets of fungi associated with different species, including in sister taxa. If we had examined fungal relationships at more of an individual

level within a small subset of species, we may have detected significant phylogenetic structure as did Barrett et al. (2010).

We detected a significant phylogenetic signal only in Axis 1 scores. These scores correlate with the usage of *Tulasnella* 1 and 5. These fungal groups are restricted to coastal dune environments. However, these environments are utilized by *Corybas* only in Western Australia and South Australia, where *C. despectans* and *C. recurvus*—the first two lineages to diverge, and the only two lineages to utilize *Tulasnella* 5—are found. We also detected higher than expected Axis 2 scores, associated with increased usage of *Tulasnella* 8, in the fimbriate margins group. The members of this group are all eastern taxa found in wetter microhabitats (*C. hispidus* to a lesser extent, but it also utilizes *Tulasnella* 8 to a lesser extent). It is not clear that these apparent phylogenetic patterns can be separated from distribution and climatic factors.

Some additional deviations from what might be expected at random were also detected in regards to specificity. The root node was reconstructed as having a broader range of *Tulasnella* associates than expected at random. This suggests a significant shift from less specialized to more specialized at that node. Because specialization was calculated as a weighted average of genetic distances among the fungi used, this was likely driven by usage of *Tulasnella* 1, which is in the other main clade of *Tulasnella* (including *T. calospora*), and frequent usage of *Tulasnella* 5, which is part of the *T. tomaculum* clade but on long branch. In regards to the breadth of fungi as calculated using the combined 5.8 data, there were hints of a phylogenetic signal in regards to two pairs of species: the node connecting *C. diemenicus* and *C. incurvus* was reconstructed as

having higher specificity than expected at random, and *C. hispidus* and *C. fimbriatus* had less variance in their specificity than expected. There was no significant phylogenetic signal in either specificity measure, however.

By contrast, we detected a clear signal of environmental conditions on the fungal communities associated with *Corybas* populations. Numerous climatic and soil variables strongly associated with the both axes in the ordination of fungal communities. The variable correlated with Axis 1, in particular a thick, unstructured A horizon with low clay content, low nutrient content, and high water conductivity, tending to overlay bedrock with a different weathering history, describes the sand dune habitats where populations utilizing *Tulasnella* 1 and 5 live. Axis 1 was also strongly correlated with precipitation seasonality and longitude. At least along the southern coast of Australia, these two variables are highly correlated. To a lesser extent, Axis 1 also correlates with increased annual temperature and decreased temperature seasonality. Axis 2 was most strongly correlated with altitude, temperature seasonality, and decreased mean annual temperature. Other researchers have detected significant effects of soil moisture, organic content, and pH on orchid mycorrhizal communities (Diez, 2007; McCormick et al., 2012) and habitat type (Long et al., 2013; Pandey et al., 2013). Our findings are consistent with these results.

Individual species in the Corysanthes complex are not limited to individual fungi, nor are the fungal communities associated with different species mutually exclusive, nor are fungal associations independent of environmental factors. However, orchid species as a factor had the highest R^2 value in the ordination of fungal communities. Moreover,

there was at least a tendency for different species in the Corysanthes clade to use different suites of fungi when growing in close proximity. Even in these cases however, we cannot completely rule out microhabitat differences, unrelated to fungal availability, which may be necessary for orchid seed germination.

Relationship to range size

To the extent that we detected a significant relationship between range size and breadth of fungal association, it was the reverse of what we initially expected based on the idea that specialists would have greater limitations on dispersal. We only detected this relationship using PICs, which assume Brownian motion-like evolution that may not be appropriate in this case, and only when considering the *Tulasnella*-only data set. Still, this is at least suggestive of a more complicated relationship between specificity and range, and fits with the emerging literature on this topic (Bailarote et al., 2012; Pandey et al., 2013). Why might there be an inverse relationship, or at least a lack of correlation of range size and breadth of fungal association? The work of Jacquemyn et al. (2010, 2011) offers a partial explanation. In the system they studied, orchids that were highly specialized in their fungal preferences used fungi that were associated with many other orchid taxa and had geographically wide ranges, while those fungi that were less common were used only by generalist orchids. This may be the result of natural selection against relationships where both partners are highly specialized. In the one clear demonstration of an orchid specialized on a very rare fungal strain, this orchid species was also rare and threatened (Swarts et al., 2010).

There are both advantages and disadvantages of being either a specialist or a generalist. Usage of a wider range of fungi may maximize nutritional uptake (Jacquemyn et al., 2010) which may be particularly important in marginal habitats, such as the calcareous dune environments where many *C. despectans* populations are found. In these habitats, there may be less competition for fungal resources from other species of orchid, though even in the swales between dunes there are other orchid genera such as *Cyrtostylis*. Having the ability to employ a variety of fungal partners might also help to reduce intraspecific competition for resources (Rasmussen, 2002). In the case of *Corybas despectans*, and likely other orchids as well, the marginal habitats that they are adapted to may have narrow geographical ranges.

However, if a lineage of orchid is able to specialize on a particular fungal group, this may promote more effective germination and more efficient nutrient uptake (Bonnardeaux et al., 2007) assuming that the fungus is sufficiently widespread and abundant in the environment. This type of specialization may also promote the coexistence of many different lineages of orchids in a single area, which is likely to be more important in habitats with greater resource availability. Thus in the case of *C. diemenicus* or *C. fimbriatus*, specialization on *Tulasnella* 8, which appears to be found throughout the continent, though not necessarily in every habitat, may be a strategy for efficient colonization and growth in richer, moister environments where interspecific competition is more of an issue.

At a broader scale, the apparent flexibility in fungal associations may be tied to the dispersal success of the genus *Corybas* as a whole. Within the diurids, the only other

genera with similarly large ranges are *Stigmatodactylus*, *Cryptostylis*, and *Microtis* (represented in northern areas only by a single species). There are few studies of the fungal relationships in *Cryptostylis* and *Stigmatodactylus*. *Microtis*, however, is known to be quite promiscuous in its fungal relationships (Warcup, 1981; Bonnardeaux et al., 2007; Long et al., 2013). The potential for *Corybas* as a whole to form relationships with other groups of fungi (in particular members of the *T. calospora* group and the *Sebacina* "B" clade which includes *Sebacina vermifera*), even if particular lineages show more specialized preferences, may have allowed it to disperse outside of Australia while groups such as *Chiloglottis* were more limited by the availability of fungal associates.

Literature Cited

ALTSCHUL, S.F., W. GISH, W. MILLER, E.W. MYERS, AND D.J. LIPMAN. 1990. Basic local alignment search tool. *Journal of molecular biology* 215: 403–410.

ASRIS. 2011. ASRIS - Australian Soil Resource Information System. Available at: http://www.asris.csiro.au [Accessed December 11, 2012].

AVH. 2014. Australia's Virtual Herbarium, Council of Heads of Australasian Herbaria. Available at: http://avh.chah.org.au [Accessed January 2, 2014].

BAILAROTE, B.C., B. LIEVENS, AND H. JACQUEMYN. 2012. Does mycorrhizal specificity affect orchid decline and rarity? *American Journal of Botany* 99: 1655–1665.

BARRETT, C.F., J.V. FREUDENSTEIN, D.L. TAYLOR, AND U. KOLJALG. 2010. Rangewide analysis of fungal associations in the fully mycoheterotrophic Corallorhiza striata complex (Orchidaceae) reveals extreme specificity on ectomycorrhizal Tomentella (Thelephoraceae) across North America. *American Journal of Botany* 97: 628–643.

BATES, R. 2011. South Australia's Native Orchids. Australian Orchid Foundation.

BIDARTONDO, M.I., B. BURGHARDT, G. GEBAUER, T.D. BRUNS, AND D.J. READ. 2004. Changing partners in the dark: isotopic and molecular evidence of ectomycorrhizal liaisons between forest orchids and trees. *Proceedings of the Royal Society B: Biological Sciences* 271: 1799–1806.

BONNARDEAUX, Y., M.C. BRUNDRETT, A. BATTY, K. DIXON, J. KOCH, AND K. SIVASITHAMPARAM. 2007. Diversity of mycorrhizal fungi of terrestrial orchids: compatibility webs, brief encounters, lasting relationships and alien invasions. *Mycological Research* 111: 51–61.

BOUGOURE, J.J., M. LUDWIG, M.C. BRUNDRETT, AND P.F. GRIERSON. 2010. Identity and specificity of the fungi forming mycorrhizas with the rare mycoheterotrophic orchid Rhizanthella gardneri. *Mycological Research* 113: 1097–1106.

BROWN, A., P. DUNDAS, K. DIXON, AND S. HOPPER. 2008. Orchids of Western Australia. University of Western Australia Press.

BYRNE, M. 2008. Evidence for multiple refugia at different time scales during Pleistocene climatic oscillations in southern Australia inferred from phylogeography. *Quaternary Science Reviews* 27: 2576–2585.

CAMERON, D.D., I. JOHNSON, J.R. LEAKE, AND D.J. READ. 2007. Mycorrhizal acquisition of inorganic phosphorus by the green-leaved terrestrial orchid Goodyera repens. *Annals of Botany* 99: 831–834.

CAMERON, D.D., I. JOHNSON, D.J. READ, AND J.R. LEAKE. 2008. Giving and receiving: measuring the carbon cost of mycorrhizas in the green orchid,Goodyera repens. *New Phytologist* 180: 176–184.

CHAMBERS, S.M., C.J. HITCHCOCK, AND J.W.G. CAIRNEY. 2005. Ectomycorrhizal mycobionts of Pisonia grandis on coral cays in the Capricorn-Bunker group, Great Barrier Reef, Australia. *Mycological Research* 109: 1105–1111.

CLEMENTS, M.A., H. MUIR, AND P.J. CRIBB. 1986. A preliminary report on the symbiotic germination of European terrestrial orchids. *Kew Bulletin* 41: 437–445.

CLEMENTS, M.A., J.T. OTERO, AND J.T. MILLER. 2011. Phylogenetic relationships in Pterostylidinae (Cranichideae: Orchidaceae): combined evidence from nuclear ribomsomal and plastid DNA sequences. *Australian Journal of Botany* 59: 99–117.

DIEZ, J.M. 2007. Hierarchical patterns of symbiotic orchid germination linked to adult proximity and environmental gradients. *Journal of Ecology* 95: 159–170.

EDGAR, R.C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* 32: 1792–1797.

GIRLANDA, M., M.A. SELOSSE, D. CAFASSO, F. BRILLI, S. DELFINE, R. FABBIAN, S. GHIGNONE, ET AL. 2006. Inefficient photosynthesis in the Mediterranean orchid Limodorum abortivum is mirrored by specific association to ectomycorrhizal Russulaceae. *Molecular Ecology* 15: 491–504.

HIJMANS, R.J., S.E. CAMERON, J.L. PARRA, P.G. JONES, AND A. JARVIS. 2005. Very high resolution interpolated climate surfaces for global land areas. *International Journal of Climatology* 25: 1965–1978.

HUYNH, T.T., R. THOMSON, C.B. MCLEAN, AND A.C. LAWRIE. 2009. Functional and genetic diversity of mycorrhizal fungi from single plants of Caladenia formosa (Orchidaceae). *Annals of Botany* 104: 757–765.

JACQUEMYN, H., O. HONNAY, B.P.A. CAMMUE, R. BRYS, AND B. LIEVENS. 2010. Low specificity and nested subset structure characterize mycorrhizal associations in five closely related species of the genus Orchis. *Molecular Ecology* 19: 4086–4095.

JACQUEMYN, H., V. MERCKX, R. BRYS, D. TYTECA, B.P.A. CAMMUE, O. HONNAY, AND B. LIEVENS. 2011. Analysis of network architecture reveals phylogenetic constraints on mycorrhizal specificity in the genus Orchis (Orchidaceae). *New Phytologist* 192: 518–528.

JEANES, J., AND G.N. BACKHOUSE. 2006. Wild Orchids of Victoria, Australia. Aquatic Photographics.

JONES, D.L. 1993. Corybas. *In* Flora of New South Wales, 212–215. UNSW Press.

JONES, D.L. 1973. Corybas hispidus. *Victorian Naturalist* 90: 96.

JONES, D.L. 2008. Corysanthes grumula. *The Orchadian* 15: 550.

KÕLJALG, U., R.H. NILSSON, K. ABARENKOV, L. TEDERSOO, A.F.S. TAYLOR, M. BAHRAM, S.T. BATES, ET AL. 2013. Towards a unified paradigm for sequence-based identification of fungi. *Molecular Ecology* 22: 5271–5277.

LARKIN, M.A., G. BLACKSHIELDS, N.P. BROWN, R. CHENNA, P.A. MCGETTIGAN, H. MCWILLIAM, F. VALENTIN, ET AL. 2007. Clustal W and Clustal X version 2.0. *Bioinformatics* 23: 2947–2948.

LILLESKOV, E.A., AND T.D. BRUNS. 2005. Spore dispersal of a resupinate ectomycorrhizal fungus, Tomentella sublilacina, via soil food webs. *Mycologia* 97: 762–769.

LINDE, C.C., R.D. PHILLIPS, M.D. CRISP, AND R. PEAKALL. 2014. Congruent species delineation of Tulasnella using multiple loci and methods. *New Phytologist* 201: 6–12.

LONG, J.R.D., N.D. SWARTS, K.W. DIXON, AND L.M. EGERTON-WARBURTON. 2013. Mycorrhizal preference promotes habitat invasion by a native Australian orchid: Microtis media. *Annals of Botany* 111: 409–418.

MADDISON, W.P., AND D.R. MADDISON. 2011. Mesquite: a modular system for evolutionary analysis. Available at: http://mesquiteproject.org.

MARTOS, F., F. MUNOZ, T. PAILLER, I. KOTTKE, C. GONNEAU, AND M.-A. SELOSSE. 2012. The role of epiphytism in architecture and evolutionary constraint within mycorrhizal networks of tropical orchids. *Molecular Ecology* 21: 5098–5109.

MATSUDA, Y., A. AMIYA, AND S.-I. ITO. 2009. Colonization patterns of mycorrhizal fungi associated with two rare orchids, Cephalanthera falcata and C. erecta. *Ecological Research* 24: 1023–1031.

MCCORMICK, M.K., D. LEE TAYLOR, K. JUHASZOVA, R.K. BURNETT, D.F. WHIGHAM, AND J.P. O'NEILL. 2012. Limitations on orchid recruitment: not a simple picture. *Molecular Ecology* 21: 1511–1523.

MCCORMICK, M.K., D.F. WHIGHAM, AND J.P. O'NEILL. 2004. Mycorrhizal diversity in photosynthetic terrestrial orchids. *New Phytologist* 163: 425–438.

MIDGLEY, D.J., J.A. SALEEBA, M.I. STEWART, A.E. SIMPSON, AND P.A. MCGEE. 2007. Molecular diversity of soil basidiomycete communities in northern-central New South Wales, Australia. *Mycological Research* 111: 370–378.

NILSSON, R.H., E. KRISTIANSSON, M. RYBERG, N. HALLENBERG, AND K.-H. LARSSON. 2008. Intraspecific ITS Variability in the Kingdom Fungi as Expressed in the International Sequence Databases and Its Implications for Molecular Species Identification. *Evolutionary Bioinformatics Online* 4: 193–201.

OGURA-TSUJITA, Y., G. GEBAUER, T. HASHIMOTO, H. UMATA, AND T. YUKAWA. 2009. Evidence for novel and specialized mycorrhizal parasitism: the orchid Gastrodia confusa gains carbon from saprotrophic Mycena. *Proceedings of the Royal Society B: Biological Sciences* 276: 761–767.

OGURA-TSUJITA, Y., J. YOKOYAMA, K. MIYOSHI, AND T. YUKAWA. 2012. Shifts in mycorrhizal fungi during the evolution of autotrophy to mycoheterotrophy in Cymbidium (Orchidaceae). *American Journal of Botany* 99: 1158–1176.

OKSANEN, J., F.G. BLANCHET, R. KINDT, P. LEGENDRE, P.R. MINCHIN, R.B. O'HARA, G.L. SIMPSON, ET AL. 2013. vegan: Community Ecology Package. Available at: http://cran.r-project.org/web/packages/vegan/index.html [Accessed April 5, 2014].

PANDEY, M., J. SHARMA, D.L. TAYLOR, AND V.L. YADON. 2013. A narrowly endemic photosynthetic orchid is non-specific in its mycorrhizal associations. *Molecular Ecology* 22: 2341–2354.

PHILLIPS, R.D., M.D. BARRETT, K.W. DIXON, AND S.D. HOPPER. 2011. Do mycorrhizal symbioses cause rarity in orchids? *Journal of Ecology* 99: 858–869.

POSADA, D. 2008. jModelTest: Phylogenetic Model Averaging. *Molecular Biology and Evolution* 25: 1253–1256.

QGIS DEVELOPMENT TEAM. 2013. QGIS Geographic Information System. Open Source Geospatial Foundation Project. Available at: http://qgis.osgeo.org.

RASMUSSEN, H.N. 2002. Recent developments in the study of orchid mycorrhiza. *Plant and Soil* 244: 149–163.

RASMUSSEN, H.N., AND F.N. RASMUSSEN. 2009. Orchid mycorrhiza: implications of a mycophagous life style. *Oikos* 118: 334–345.

ROCHE, S.A., R.J. CARTER, R. PEAKALL, L.M. SMITH, M.R. WHITEHEAD, AND C.C. LINDE. 2010. A narrow group of monophyletic Tulasnella (Tulasnellaceae) symbiont lineages are associated with multiple species of Chiloglottis (Orchidaceae): Implications for orchid diversity. *American Journal of Botany* 97: 1313.

SCHOCH, C.L., K.A. SEIFERT, S. HUHNDORF, V. ROBERT, J.L. SPOUGE, C.A. LEVESQUE, W. CHEN, ET AL. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy of Sciences* 109: 6241–6246.

SCHOENER, T.W. 1970. Nonsynchronous diet overlap of lizards in patchy habitats. *Ecology* 51: 408–418.

SHEFFERSON, R.P., C.C. COWDEN, M.K. MCCORMICK, T. YUKAWA, Y. OGURA-TSUJITA, AND T. HASHIMOTO. 2010. Evolution of host breadth in broad interactions: mycorrhizal specificity in East Asian and North American rattlesnake plantains (Goodyera spp.) and their fungal hosts. *Molecular Ecology* 19: 3008–3017.

SHEFFERSON, R.P., D.L. TAYLOR, M. WEISS, S. GARNICA, M.K. MCCORMICK, S. ADAMS, H.M. GRAY, ET AL. 2007. The evolutionary history of mycorrhizal specificity among lady's slipper orchids. *Evolution* 61: 1380–1390.

SMITH, Z.., E.. JAMES, AND C.. MCLEAN. 2010. Mycorrhizal specificity of Diuris fragrantissima (Orchidaceae) and persistence in a reintroduced population. *Australian Journal of Botany* 58: 97–106.

SUÁREZ, J.P., M. WEIß, A. ABELE, S. GARNICA, F. OBERWINKLER, AND I. KOTTKE. 2006. Diverse tulasnelloid fungi form mycorrhizas with epiphytic orchids in an Andean cloud forest. *Mycological Research* 110: 1257–1270.

SWARTS, N.D., E.A. SINCLAIR, A. FRANCIS, AND K.W. DIXON. 2010. Ecological specialization in mycorrhizal symbiosis leads to rarity in an endangered orchid. *Molecular Ecology* 19: 3226–3242.

SWOFFORD, D. 2003. PAUP*: phylogenetic analysis using parsimony, version 4.0b10.

TAYLOR, D.L., T.D. BRUNS, AND S.A. HODGES. 2004. Evidence for mycorrhizal races in a cheating orchid. *Proceedings of the Royal Society B: Biological Sciences* 271: 35–43.

TAYLOR, D.L., AND M.K. MCCORMICK. 2008. Internal transcribed spacer primers and sequences for improved characterization of basidiomycetous orchid mycorrhizas. *New Phytologist* 177: 1020–1033.

THOMPSON, J.N. 1994. The Coevolutionary Process. University of Chicago Press.

TREMBLAY, R.L., J.D. ACKERMAN, J.K. ZIMMERMAN, AND R.N. CALVO. 2004. Variation in sexual reproduction in orchids and its evolutionary consequences: a spasmodic journey to diversification. *Botanical Journal of the Linnean Society* 84: 1–54.

WARCUP, J.H. 1981. The mycorrhizal relationships of Australian orchids. *New Phytologist* 87: 371–381.

WATERMAN, R.J., AND M.I. BIDARTONDO. 2008. Deception above, deception below: linking pollination and mycorrhizal biology of orchids. *Journal of Experimental Botany* 59: 1085.

WATERMAN, R.J., M.I. BIDARTONDO, J. STOFBERG, J.K. COMBS, G. GEBAUER, V. SAVOLAINEN, T.G. BARRACLOUGH, AND A. PAUW. 2011. The Effects of Above- and Belowground Mutualisms on Orchid Speciation and Coexistence. *American Naturalist* 177: E54–E68.

WATKINS, R.L.S. 2012. The biogeography, ecology and endophyte mycorrhiza of the New Zealand Corybas alliance (Orchidaceae) : specifically, Nematoceras iridescens (Irwin et Molloy) Molloy, D.L.Jones & M.A.Clem. (species) : a thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Plant Biology at Massey University, Palmerston North, New Zealand. Thesis. Available at: http://mro.massey.ac.nz/handle/10179/4063 [Accessed March 14, 2014].

WEBB, C.O., D.D. ACKERLY, AND S.W. KEMBEL. 2008. Phylocom: software for the analysis of phylogenetic community structure and trait evolution. *Bioinformatics* 24: 2098–2100.

WRIGHT, M.M., R. CROSS, R.D. COUSENS, T.W. MAY, AND C.B. MCLEAN. 2010. Taxonomic and functional characterisation of fungi from the Sebacina vermifera complex from common and rare orchids in the genus Caladenia. *Mycorrhiza* 20: 375–390.

ZHANG, L., J. CHEN, Y. LV, C. GAO, AND S. GUO. 2012. Mycena sp., a mycorrhizal fungus of the orchid Dendrobium officinale. *Mycological Progress* 11: 395–401.

ZWICKL, D.J. 2006. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. Ph.D. dissertation. The University of Texas at Austin.

Figures and Tables

Figure 1. Elevation map showing mycorrhizal fungi sampling locations on the Australian continent.

Table 1. Collecting locations, *Corybas* species present, and the number of individuals for each taxon that successful yielded mycorrhizal fungi sequences.

Figure 2. Maximum likelihood phylogenies for both members of the Corysanthes clade and their associated fungi. Support values are based on 100 bootstrap replicates. The width of lines connecting plant species to fungi OTUs vary according to the percentage of individuals of the plant species found associating with the particular fungal OTU.

Figure 3. Ancestral character state reconstructions for: A. *Tulasnella* specificity; B. Mycorrhizal fungus specificity calculated using an alignment of the 5.8S region only; C. Fungal community ordination Axis 1 score; and D. Fungal community ordination Axis 1 score. Nodes where character state or variance in that character state in descendent nodes differed significantly from random are marked with the appropriate statistic A, A var, T, T var (see text) as well as the direct in which they differ from random.

Figure 4. Regressions of: A. Niche breadth (based on frequency of usage of different fungal OTUs) as calculated using the Schoener index again mean phylogenetic distances among plant taxa; B. Phylogenetic independent contrasts of specificity on *Tulasnell*a OTUs against phylogenetic independent contrasts of range size; C. B. Phylogenetic independent contrasts of Axis 2 scores against phylogenetic independent contrasts of range size.

Figure 5. Plots of fungal communities ordinated with NMDS. A. Vectors for significant fungal OTUs. B. Vectors for environmental variables significantly correlated with ordination axes. C. Centroids for plant species associated with fungal communities.

Table 2. Correlation coefficients of each of the environmental variables significantly associated with NMDS axes, their R^2 scores, and p-values.

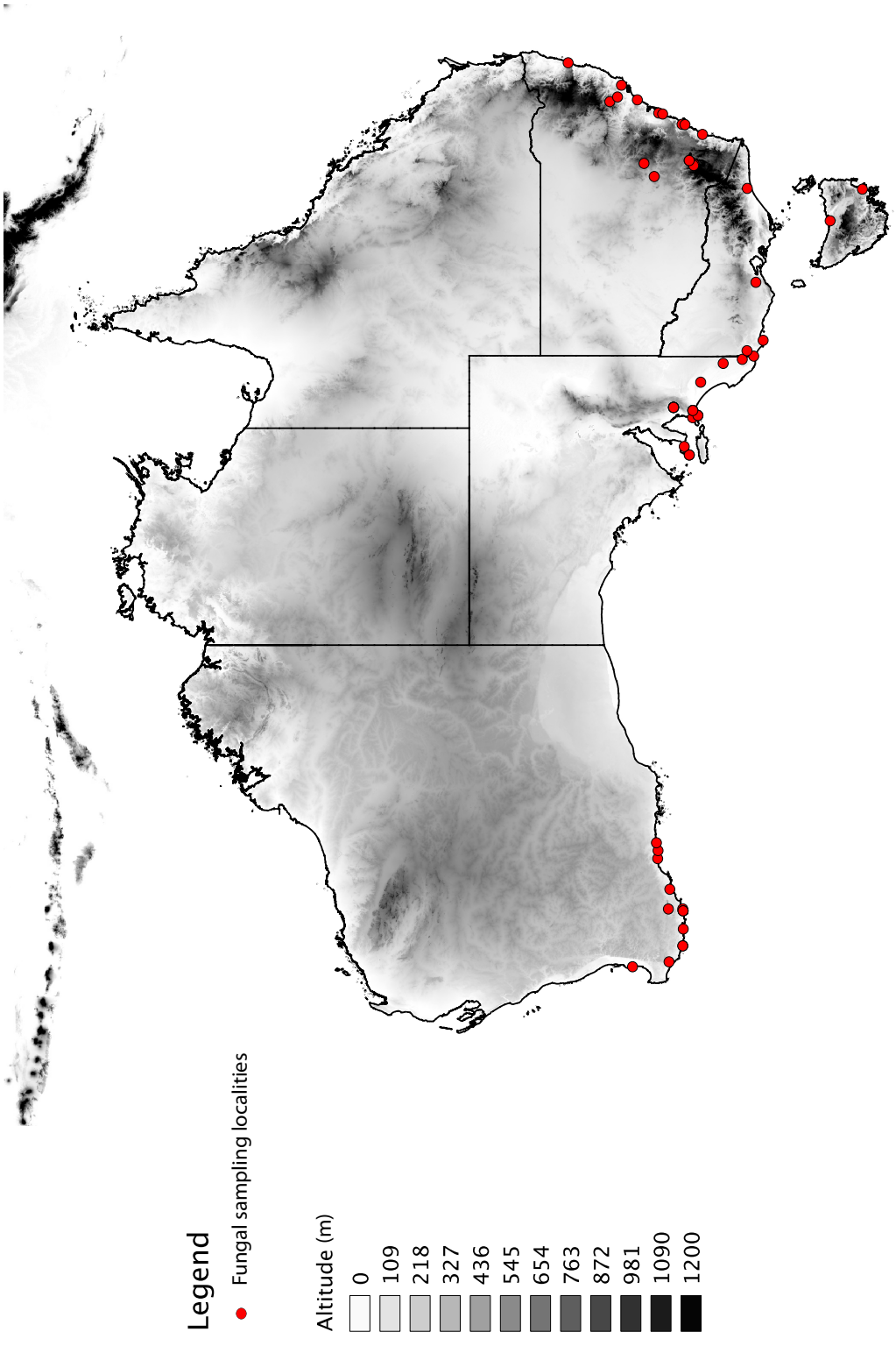
Legend
Fungal sampling localities
Altitude (m)
0
109
218
327
436
545
654
763
872
981
1090
1200

Figure 1.

Table 1.

State	Site	Species
ACT	Black Mt	*hispidus (5), incurvus (4)*
	Gibralter Falls	*hispidus (5)*
	Tidbinbilla	*hispidus (6)*
NSW	Barrington Tops	*diemenicus (4), fimbriatus (5)*
	Bateman's Bay	*fimbriatus (4)*
	Bulahdelah	*pruinosus (6)*
	Conimbla NP	*incurvus (5)*
	Falls Creek	*pruinosus (5)*
	* L Macquarie SCA	*pruinosus (1)*
	Mt Canobolas	*hispidus (6)*
	Oatley Park	*fimbriatus (6)*
	* Orara E SF	*fimbriatus (1)*
	Pelican Pt	*pruinosus (6)*
	Royal NP	*pruinosus (4)*
SA	Coonalpyn	*despectans (4)*
	Frome Rd	*dentatus (2)*
	Innes NP	*expansus/despectans (9)*
	Nangawarry	*dentatus (6)*
	Newland Hd CP	*despectans/expansus (6)*
	* Nixon-Skinner CP	*diemenicus (1)*
	Sandy Creek	*dentatus (4), despectans/expansus (7), incurvus (2), dilatatus (5)*
	Scott CP	*dentatus (4), dilatatus (4), incurvus (4)*
	* Stenhouse Bay	*despectans/expansus (1)*
	* The Gap	*incurvus (1)*
Tas	* Harford	*incurvus (1)*
	Orford	*diemenicus (2)*
Vic	* 6 mile Rd	*fimbriatus (1)*
	Inverleigh	*diemenicus (6)*
	Lower Glenelg NP	*diemenicus (2)*
	Point Danger	*diemenicus (3)*
	Wilkin FFR	*diemenicus (2)*
WA	Beedelup NP	*recurvus (4)*
	D'Entrecasteau NP	*despectans (2)*
	Gull Rock	*despectans/limpidus (3), recurvus (5)*
	Hopetoun	*despectans/limpidus (2)*
	Mason Bay	*despectans/limpidus (5)*
	Millers Point	*despectans/limpidus (2)*
	Munglinup Beach	*despectans/limpidus (4)*
	Nanarup Beach	*despectans (2)*
	Stirling Range NP	*recurvus (5)*
	Williams Bay	*recurvus (5)*
	Yalgorup NP	*recurvus (4)*

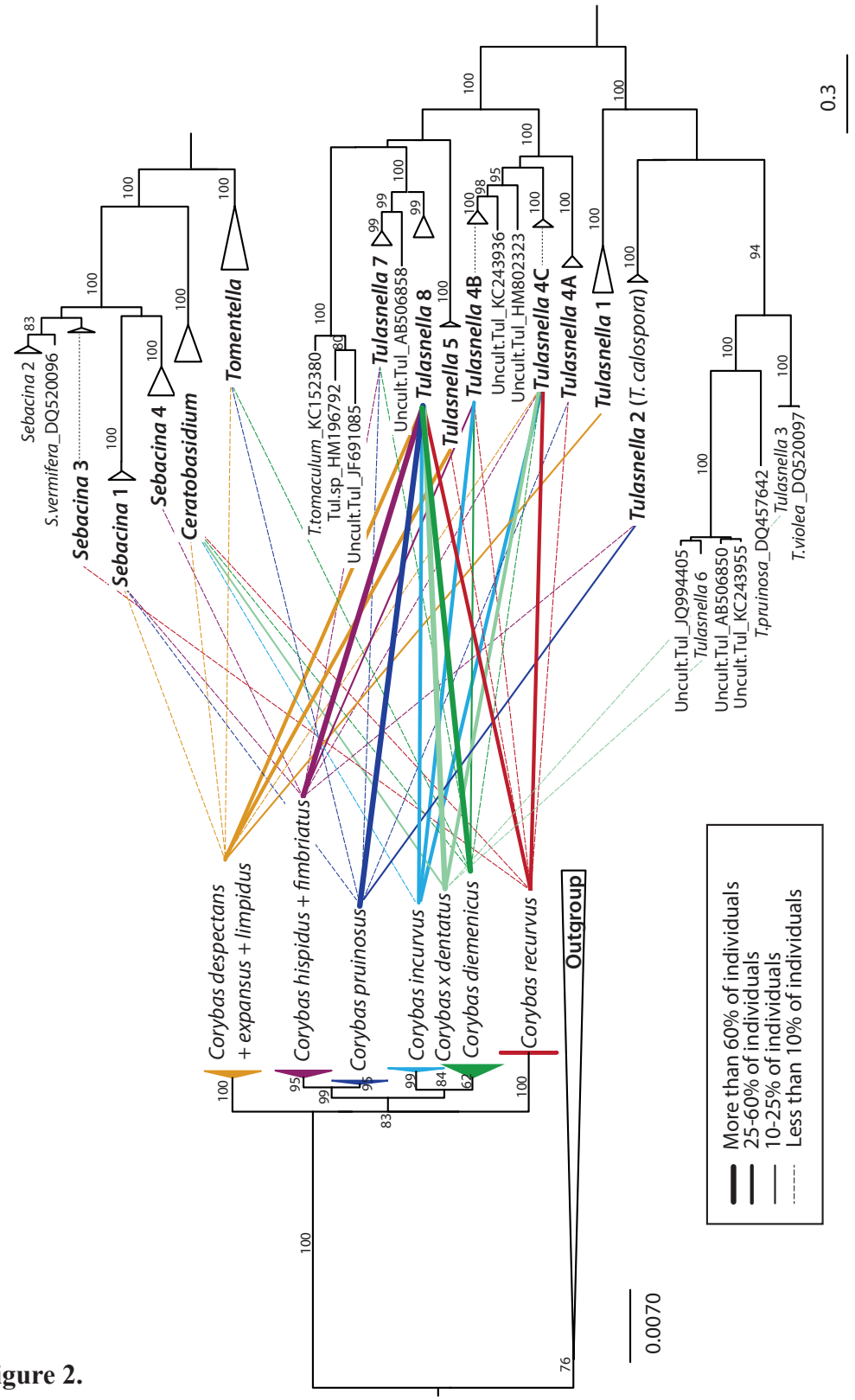
Sebacina 2
S.vermifera_DQ520096
Sebacina 3
Sebacina 1
Sebacina 4
Ceratobasidium
Tomentella
T.tomaculum_KC152380
Tul.sp_HM196792
Uncult.Tul_JF691085
Tulasnella 7
Uncult.Tul_AB506858
Tulasnella 8
Tulasnella 5
Tulasnella 4B
Uncult.Tul_KC243936
Uncult.Tul_HM802323
Tulasnella 4C
Tulasnella 4A
Tulasnella 1
Tulasnella 2 (T. calospora)
Uncult.Tul_JQ994405
Tulasnella 6
Uncult.Tul_AB506850
Uncult.Tul_KC243955
T.pruinosa_DQ457642
Tulasnella 3
T.violea _DQ520097
Corybas despectans + expansus + limpidus
Corybas hispidus + fimbriatus
Corybas pruinosus
Corybas incurvus
Corybas x dentatus
Corybas diemenicus
Corybas recurvus
Outgroup
0.3
0.0070
More than 60% of individuals
25-60% of individuals
10-25% of individuals
Less than 10% of individuals

Figure 2.

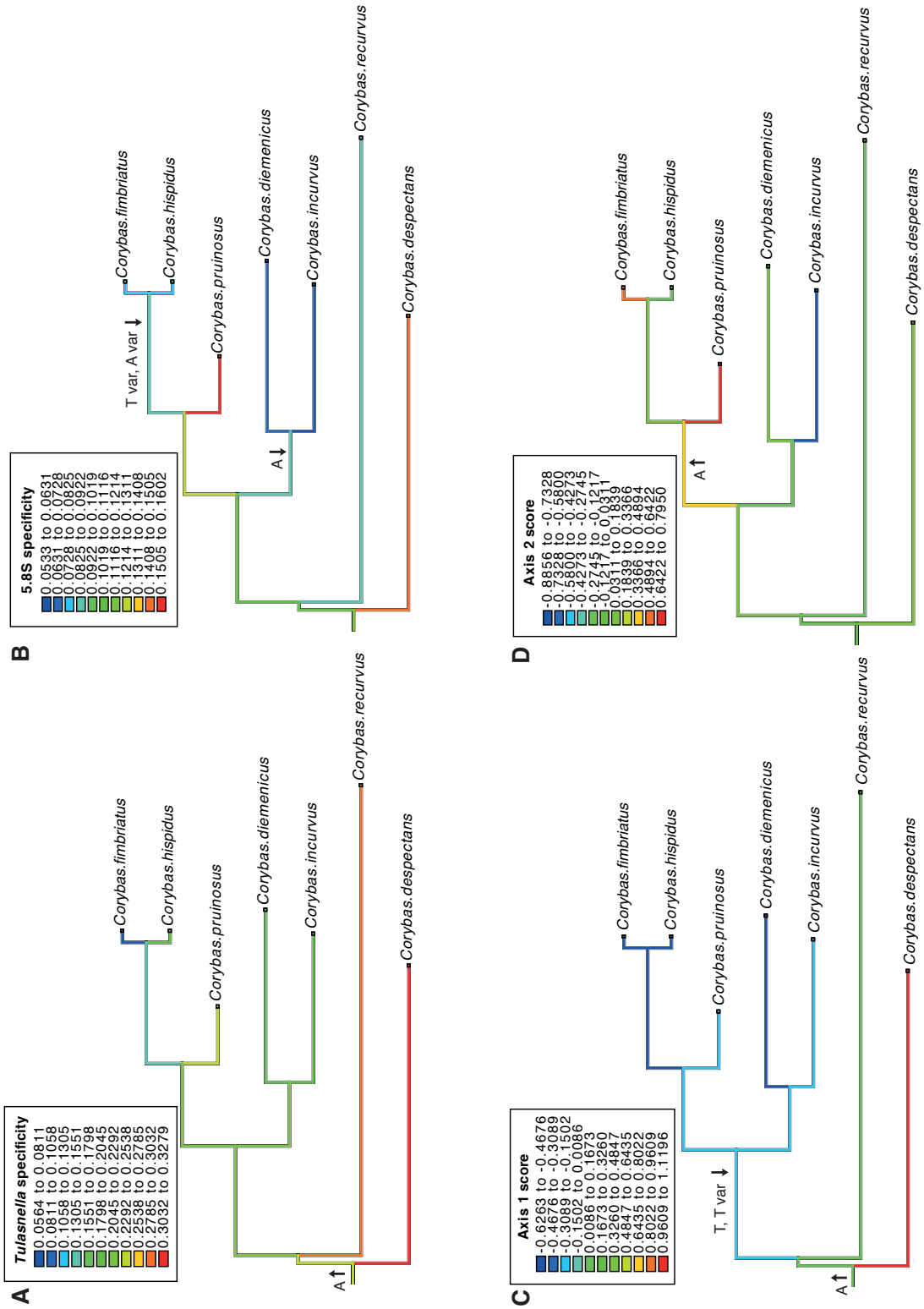
A
Tulasnella specificity
0.0564 to 0.0811
0.0811 to 0.1058
0.1058 to 0.1305
0.1305 to 0.1551
0.1551 to 0.1798
0.1798 to 0.2045
0.2045 to 0.2292
0.2292 to 0.2538
0.2538 to 0.2785
0.2785 to 0.3032
0.3032 to 0.3279
Corybas.fimbriatus
Corybas.hispidus
Corybas.pruinosus
Corybas.diemenicus
Corybas.incurvus
Corybas.recurvus
Corybas.despectans
A
B
5.8S specificity
0.0533 to 0.0631
0.0631 to 0.0728
0.0728 to 0.0825
0.0825 to 0.0922
0.0922 to 0.1019
0.1019 to 0.1116
0.1116 to 0.1214
0.1214 to 0.1311
0.1311 to 0.1408
0.1408 to 0.1505
0.1505 to 0.1602
T var, A var
A
C
Axis 1 score
-0.6263 to -0.4676
-0.4676 to -0.3089
-0.3089 to -0.1502
-0.1502 to 0.0086
0.0086 to 0.1673
0.1673 to 0.3260
0.3260 to 0.4847
0.4847 to 0.6435
0.6435 to 0.8022
0.8022 to 0.9609
0.9609 to 1.1196
T, T var
A
D
Axis 2 score
-0.8856 to -0.7328
-0.7328 to -0.5800
-0.5800 to -0.4273
-0.4273 to -0.2745
-0.2745 to -0.1217
-0.1217 to 0.0311
0.0311 to 0.1839
0.1839 to 0.3366
0.3366 to 0.4894
0.4894 to 0.6422
0.6422 to 0.7950
A
* Significant phylogenetic signal, p = 0.038

Figure 3.

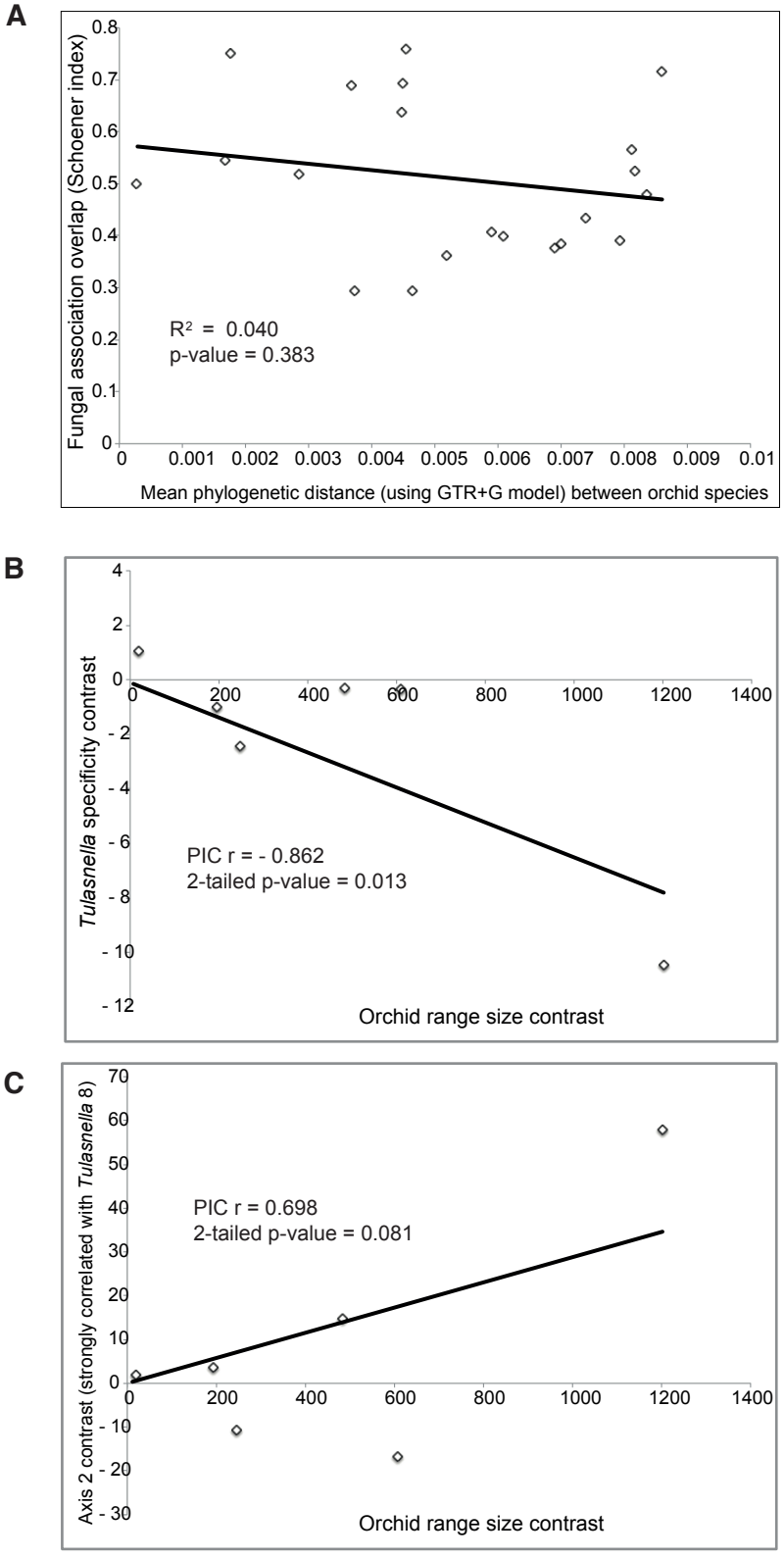
A
Fungal association overlap (Schoener index)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
R² = 0.040
p-value = 0.383
0
0.001
0.002
0.003
0.004
0.005
0.006
0.007
0.008
0.009
0.01
Mean phylogenetic distance (using GTR+G model) between orchid species
B
Tulasnella specificity contrast
4
2
0
- 2
- 4
- 6
- 8
- 10
- 12
0
200
400
600
800
1000
1200
1400
PIC r = - 0.862
2-tailed p-value = 0.013
Orchid range size contrast
C
Axis 2 contrast (strongly correlated with Tulasnella 8)
70
60
50
40
30
20
10
0
- 10
- 20
- 30
0
200
400
600
800
1000
1200
1400
PIC r = 0.698
2-tailed p-value = 0.081
Orchid range size contrast

Figure 4.

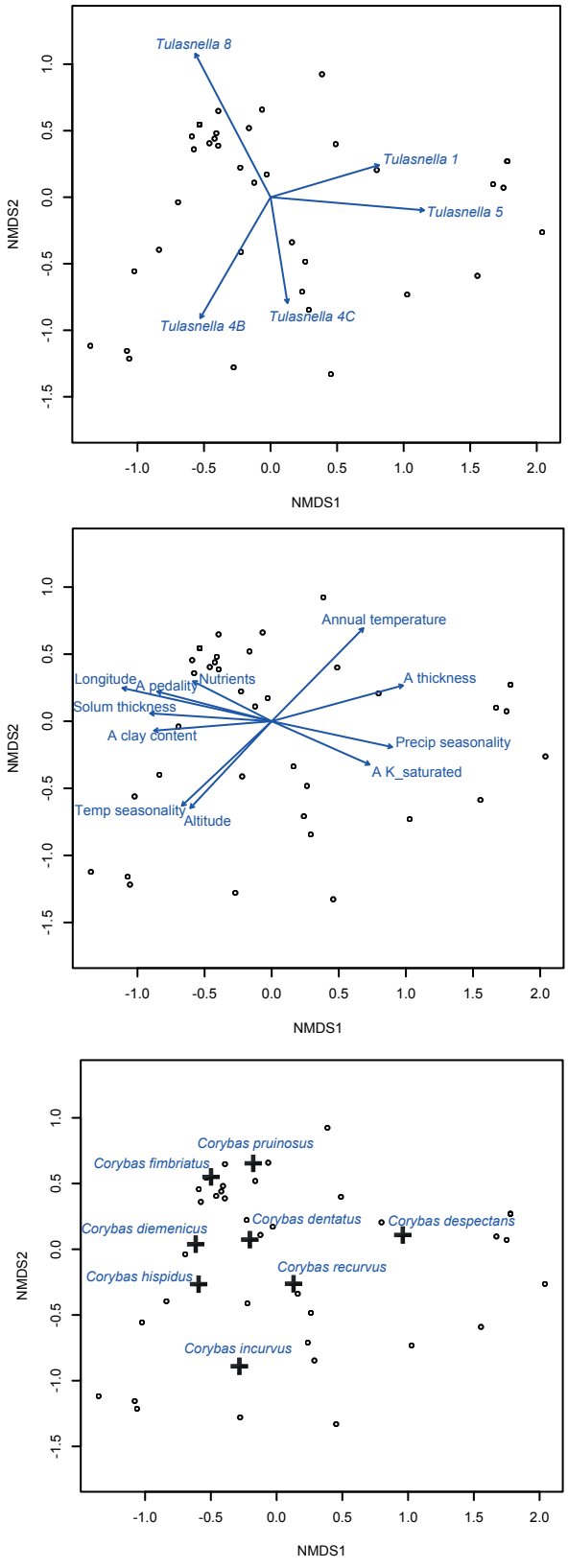
Tulasnella 8
Tulasnella 1
Tulasnella 5
Tulasnella 4B
Tulasnella 4C
NMDS1
NMDS2
Annual temperature
Longitude
A pedality
Nutrients
A thickness
Solum thickness
A clay content
Precip seasonality
A K_saturated
Temp seasonality
Altitude
Corybas pruinosus
Corybas fimbriatus
Corybas diemenicus
Corybas dentatus
Corybas despectans
Corybas hispidus
Corybas recurvus
Corybas incurvus

Figure 5.

Table 2.

Species/Variable	Axis 1 Corr.	Axis 2 Corr.	R^2	p-value	
Tulasnella 1	0.95877	0.28418	0.4064	0.002	**
Tulasnella 5	0.99639	-0.08489	0.7541	0.001	***
Tulasnella 8	-0.46418	0.88574	0.836	0.001	***
Tulasnella 4B	-0.50253	-0.86456	0.6217	0.001	***
Tulasnella 4C	0.15769	-0.98749	0.3653	0.001	***
Temp. seasonality	-0.7306	-0.6828	0.2747	0.001	***
Mean annual temp.	0.70377	0.71043	0.3085	0.001	***
Longitude	-0.97607	0.21745	0.4224	0.001	***
A horiz. thickness	0.96574	0.25952	0.3346	0.001	***
Precip. seasonality	0.97662	-0.21495	0.2741	0.002	**
A horiz. pedality	-0.96675	0.25572	0.2529	0.002	**
Solum thickness	-0.99758	0.06949	0.2683	0.003	**
Altitude	-0.68645	-0.72718	0.2559	0.005	**
A horiz. clay content	-0.99721	-0.0746	0.2518	0.005	**
A horiz. Ksat	0.9133	-0.40729	0.2067	0.013	*
Nutrients	-0.88936	0.45722	0.1408	0.044	*
Orchid species	-	-	0.4608	0.001	***