Investigating the Effects of Domestication on Crop Toxicity: Analyses in Manioc (*Manihot esculenta*) and Oca (*Oxalis tuberosa*)

By

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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: 11/12/13

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Acknowledgements & Dedication

First, I would like to dedicate this body of work to my husband, Samuel Mook, and to my mother, Dr. Kirsten Bradbury. To my mother for always inspiring me to reach for my best and to my husband for unwaveringly supporting me on my way. Without these two individuals in my life, several times over this research would never have happened.

Additionally, there are so many wonderful, dedicated, supportive people who have contributed to this body of work in a plethora of diverse ways that I do not even know where to begin. Certainly, this work could not have been accomplished without funding, for which I thank Sigma Delta Epsilon—Graduate Women in Science for the Adele Lewis Grant Research Fellowship and the Ruth Dickie Scholarship; the UW-Madison Botany Department for the Eldon Newcomb Summer Research Fellowship, the Judy Croxdale Award for Women in Science, and the Davis Research Grant; the UW-Madison for the Vilas Travel Grant and the Tinker-Nave Travel Grant; and the NSF Franco-American Cultural Exchange (NSF OISE-0623583).

Many thanks must also go to the people who consistently helped me overcome funding limitations, primarily my international collaborators. Dr. Carlos Arbizu, curator of the International Potato Center (CIP) oca germplasm collection, graciously granted me access to the germplasm tubers in the collection. Dr. David Tay and Dr. Stef de Haan have both served as Director of the Plant Genetic Resources and Conservation Division at CIP and provided me incredible access to the facilities, services, labor, and invaluable help wrangling the bureaucracies of a foreign country. Without them, I would never have begun to search the CIP data base for samples, much less grown out my experimental plot on CIP land, or used the facilities at CIP's labs. This leads me to thank Dra. Gabriella Burgos of CIP for generously letting me use her nutritional biochemistry lab to conduct my preliminary analyses of oxalic acid

in oca tubers and Dr. Ivan Manrique for helping me acquire all of the necessary reagents and chemicals for my work. Similarly, I must thank Ivan for helping me with my collection permits and Material Transfer Agreement and for collecting leaf tissue from my growing plants, which I used to extract the DNA genotyped in Chapter 4.

Of course I must thank Sr. Francisco Vivanco for being a dedicated collaborator and more. Without Francisco, my common garden plot would never have survived. Additionally, he was one of the most warm and welcoming friends to me when I was alone in a very foreign place for the first time in my life. The memories of dining with him and his family are some of my most warming. Our philosophical dialogues made routine and monotonous field-work stimulating and engaging and he has always been the most patient and bemused Spanish tutor. I particularly thank him for being such a help to my undergraduate student, Rollin, who conducted oxalic acid extractions for me the summer of 2010 when the birth of my daughter prevented me from traveling to Peru.

Additionally, I must most sincerely thank Dr. Raul Blas, of the Universidad Nacional de la Agraria in La Molina, Peru, for being the most enthusiastic and welcoming collaborator even when I so unexpectedly contacted him on my quest to confirm the existence of tetraploid ocas. Even now he is working to help me get the last little bits of data that I can to strengthen my story, along with his dedicated students. One of his students in particular, Daniel Huaman, has been my compatriot in the field for two seasons and I owe him my eternal thanks for his logistical organization of our field trips, his hard labor during collecting trips, and for so generously opening his family's home to me during my trip there this past April. Our meal of *cuy* and *choclo* and *papas nativas* during that cold April rain will never be forgotten and is one of the moments during my research that ameliorated so many other frustrations.

Yet, my international collaborations crossed many borders, and I must thank my collaborators on Chapter 1 for all of their dedication and hardwork to our manioc project. Many thanks to Dr. Joe Manu and Dra. Alexandra Trujillo, who, though we have never met, entrusted me with their collections of manioc leaf tissue to be included for analysis in Chapter 1. Additionally, I extend the warmest thanks to Dr. Doyle McKey for coordinating my exchange and allowing me to benefit from his extensive network of manioc researchers. Similarly, I could not have accomplished this research without several of Doyle's students, including Dr. Marc Delêtre, who was always a supportive and encouraging resource during data analysis and writing, Dr. Anne Duputié for her rigor, funny jokes, and the beer in Austin, and Dr. Caroline Roullier for being a really great friend.

Speaking of friends, I could not have accomplished this work without an amazing and supportive learning community of colleagues and friends. First, Lauren Moscoe, is the best surprise of my graduate career. I never thought we would get a chance to work together, but without her I would not have been able to complete Chapter 4 or 5. Her willingness to share her work and successes with me enabled me to take my research where I did not think I would have the opportunity to explore. Additionally, she is one of the most inspirational people to me on a personal level, and her positive outlook on life helped me through a fundamental shift in personal paradigm. The last two years of my life have enabled me to grow more than I thought possible, and this would not have happened without Lauren.

Additionally, I must acknowledge several other graduate cohorts of mine, in particular Dr. Emily Sessa, Katie Konchar, and Dr. Andy Gardner. Emily and Katie provided me strength in sisterhood during the ups and downs of graduate school and were always good for a much-needed escape, whether to grab a cider on the Terrace or to explore the Sheep and Wool Festival.

And, as for Andy, what can I say? Andy and I arrived in the Emshwiller lab together and in step we weathered the storm. Andy and Sasha are invaluable lifetime friends of ours and words cannot express all of the love and support they have given us. In many ways, they are more like family than friends.

Which, of course, brings me to my family. Certainly, I must thank my parents, Dr. Kirsten Bradbury and Jim Flores. My mother taught me what it means to be a fighter and how to dig deep and never give up. My dad taught me what it means to commit yourself to someone and the power of personal choice in action. Together, they taught me to value intellect, to strive for knowledge, and to seek to better myself through education. Without their love and dedication, coaching through countless tearful phone calls about research tragedies, and repeated financial bailouts of international research adventures gone awry, I would certainly never have finished this thesis (and may still be stuck in Aguas Calientes).

Additionally, my mother- and father-in-law Dr. Linda Mook and Wes Mook deserve acknowledgement for their incredible generosity of spirit and the support they have given me during my graduate career. In particular, they have always come to Madison to help us when we most needed it, and without Linda so generously coming to stay with my husband and daughter during my last two field trips, I would never have been able to go and confirm the existence of tetraploid oca. Those two field trips were transformational experiences for me and I am a different, and fuller, person now for having gone.

How could I thank my in-laws without thanking my husband? How can I even begin to thank my husband for everything he has done for me over the last seven years? His support for me and my career and my dreams has never wavered, from when he first moved to Madison with me so that I could start my graduate career, to this very night that he took off of work to be able to put our daughter to bed while I finish writing my thesis. I have known for a decade that I could not live without him and I cannot wait to start our life together "post Ph.D." I could not ask for a more loving and uplifting husband to me and father to Elle. And, thanks must go to Elle for being the brightest light on the darkest days. Her sweet smile and rambunctious greetings improve even the dreariest of days.

Lastly, but certainly not least, I must acknowledge the many wonderful mentors who have guided me through this process. My committee, Drs. David Baum, Don Waller, Bret Larget, and Jiwan Palta have been an incredible resource to me throughout this process. Special thanks must go to Bret Larget for his dedication in helping me devise an analytical framework for Chapter 4, and on terribly short notice, too! Additionally, I would like to thank Dr. Cecile Ané for being a talented and effective professor of my statistics course, which laid the foundation for a greater understanding and fluency in statistics than I ever thought possible. Many thanks to Cecile, also, for so generously and warmly mentoring me in many of the statistics used in this thesis, especially Chapter 1 and Chapter 3.

And, of course, to my adviser, Dr. Eve Emshwiller, I can only say *muchísimas gracias*. I feel like Eve and I embarked upon this cosmic journey together, arriving at a new school, in a new place, and a new metaphysical space in life all at the same time. Eve has been incredibly dedicated to me and to my project, always willing to squeeze in a late-night Skype across thousands of miles to resolve the most detailed research question. Additionally, she has served as an invaluable mentor in ethnobotany. Investigating the patterns that emerge from the complex intersections of culture and biology has fundamentally altered the way I approach life, and I feel my understanding of the meaning of my life is greatly enhanced by the discipline in which Eve has mentored me. But, perhaps most of all, Eve has always supported *me.* There have been many times during my graduate career when I made decisions that were unconventional, but Eve never wavered in her support of my choices. I have always been able to trust that she most wants what is best for me and having that foundation of support enabled me to take risks that turned out to be incredibly rewarding. So, Eve, thank you, for everything, for all of this, and everything it took to get here. I couldn’t have done it without you.

Abstract

Though domestication is traditionally considered to reduce plant chemical defenses, a minority group of crops appears to have retained toxicity in domesticated forms. The most well-known example is manioc (*Manihot esculenta* Crantz). Manioc varieties are divided into two distinct "use-categories", those that must be processed prior to consumption to remove toxic levels of cyanide ("bitter") and varieties that do not produce toxic quantities of cyanide and may be eaten without processing ("sweet"). Other crops, though less well-studied, appear to present a similar syndrome of domestication, with two distinct cultural use-categories separating varieties reserved for processing and those varieties that do not need to be processed prior to consumption. Oca (*Oxalis tuberosa* Molina), a high-altitude Andean tuber crop, is one example of a lesser-known crop that fits this description. The goal of this dissertation is to investigate what molecular bases may exist for cultural use-categories in manioc and oca. In manioc, we seek to investigate what genetic structuring may be observed in manioc populations from South America and Africa and how this genetic structuring may correlate with manioc toxicity. In oca, we seek to identify what underlying biochemistry may correlate with cultural use-categories and how this biochemistry may also relate to population genetic structuring and evolutionary history. We found evidence to suggest that although these crops may have superficially similar cultural and culinary use-categories, the evolutionary histories of the crops are quite different. Analyses in manioc demonstrate that population genetic structuring based on toxicity is entirely dependent upon strict maintenance of separation of bitter and sweet varieties from one another during cultivation. In areas of introduction of the crop in Africa, where South American traditional agricultural techniques were not employed, genetic mixing of bitter and sweet manioc is common and cyanide-related diseases are on the rise due to inconsistent classifications of toxic varieties as “bitter”. In contrast, though cultural use-categories in oca appear to loosely correlate with tuber oxalic acid content, high-acid (“sour”) oca cultivars form a genetically distinct lineage of ocas defined by low DNA-ploidy in the crop, suggesting a complicated evolutionary history involving multiple polyploidization events.

Introduction

The advent of agriculture and the resulting domestication of many plant and animal species is arguably the single most important "event" in human history. I use the term "event" loosely, of course, because the processes of agricultural development and domestication are continuous and spanned hundreds of human generations, occurring multiple times throughout the world. Nonetheless, agriculture and domestication serve as the foundation for the development of complex human societies and centers of civilization and represent a fundamental era in human history.

However, in spite of this, in modern American culture, the farm-to-table system is largely ignored by the majority of the population and the evolutionary processes of domestication are even more remote. Yet, we are currently facing one of the greatest challenges to our agricultural system since, perhaps, the mini "Ice Age" in Northern Europe that ushered in approximately three hundred years of crop failure, famine, and the bubonic plague (Fagan, 2000). The reality of a quickly changing climate is already stressing our agricultural system (Alexandratos, 1999; Easterling, 2007; Tubiello et al., 2007; Nabhan, 2011) and the projected climactic change would cause catastrophic drops in yield (Alexandratos, 1999; Schmidhauer & Tubiello, 2007). Interestingly, some estimates suggest that the negative impacts of climate change are more drastic in larger-scale agriculture than in smaller-scale operations (Bakker et al., 2005).

When looking ahead to the climatological disaster that looms, as a scientific community we need to explore every possible option for adaptation of our crop systems to quickly-changing pressures. While we must work to find applied solutions, there are also many areas of our basic understanding of mechanisms of crop domestication that need significant improvement (Gepts, 2002). Additionally, the scientific community continues to discover new pockets of crop genetic diversity (Brush, 2004).

We must continue to expand our ability to understand how our crops and crop diversity originated and how crop diversity interacts with different environmental conditions and agricultural systems. Conservation of rare crop genetic resources becomes critical. Answering the question, “What do people want from their food, and how do they get it?” becomes paramount. In this respect, much knowledge can be gained from studying traditional agricultural systems. It is the high-input, low-diversity industrialized agriculture that is most dependent on resource availability and most susceptible to failure from resource limitations caused by a shifting climate (Bakker et al., 2005; Nabhan, 2011). When balancing competing anthropogenic and ecosystemic selective forces, the industrialized strategy is primarily to eliminate the impact of ecosystemic selection, e.g. irrigation to combat water limitation, nutrient supplementation to replenish depleted soils, and pesticide use to conquer invading predators. However, all of these strategies depend on intense resource use, an approach that is proving to be less and less viable as these resources diminish (Nabhan, 2011). So, the question becomes, what do we do? How do we balance the needs of a growing global and globalized population (anthropogenic pressures) and the ever-increasing ecosystemic pressures being placed on agricultural systems by a rapidly changing climate? *What do we want from our food and how can we get it?*

When asking myself these questions, I realized that we are now facing a biological scenario very similar to the scenario faced by our ancestors during the primary era of crop domestication—a scenario that is fundamentally evolutionary and involves understanding multiple layers of selective forces and how these forces interact to enhance or compete with one another. For these reasons, study of the mechanisms of domestication can provide important insights into the questions posed above. In particular, today, we are seeing increasing conflict between anthropogenic pressures in the agricultural system (the desire for high-input, high-yield, genetically uniform crops) and ecosystemic realities (diminishing resources, decreasing suitability of current crop genetics to abiotic and biotic pressures). Studying the mechanisms of domestication in traditional agricultural systems can help us understand the interactions of selective forces in systems without intensive resource input (and, therefore, more similar to agricultural systems of both the evolutionary past and future). In particular, I have chosen to focus on crops that retain toxicity in their domesticated forms because these crops present a clearly observable conflict between human needs (low toxin) and plant survival (high toxin, implying high defense). These crops are additionally interesting because toxicity is rare in domesticated plants and reducing crop toxicity is often cited as one of the common elements of domestication. This suggests to me that toxic crops address an uncommon combination of human and ecosystemic needs, and that their evolutionary mechanisms and associated cultural food practices could hold valuable insights for understanding agricultural scenarios very different from the industrialized agricultural systems so prevalent today.

For my study organisms I have chosen two crops that retain toxicity in their domesticated forms, manioc (*Manihot esculenta* Crantz) and oca (*Oxalis tuberosa* Molina). Both crops display a similar “cultural syndrome”—both are distinguished into two cultural use-categories: varieties that are consumed only after laborious processing and varieties that are cooked and consumed without such processing. In manioc it is well-known that processing of “bitter” cultivars is necessary to eliminate elevated levels of cyanide, which in some varieties can be so high as to cause acute toxicity if consumed without processing. However, in oca, little research has been done to investigate a biochemical basis for the cultural traditions of food processing.

My broader goals for these crop systems are: 1) to investigate genetic mechanisms of domestication in *Manihot esculenta* and *Oxalis tuberosa*, in particular focusing on the origins of “processed” vs. “unprocessed” cultivars; 2) to determine what, if any, biochemical difference exists between “processed” and “unprocessed” cultivars of oca and if any observable biochemical difference relates to toxicity for humans; 3) to explore the underlying reasons, both cultural and biological, for continued cultivation of toxic domesticates.

My goal for this collection of work was to begin exploring these broader concepts relating to the evolutionary histories and cultural patterns associated with these crops by filling in some of the gaps in the existing knowledge. I began by conducting a study of the patterns of genetic differentiation in bitter and sweet cassava as a way to assess the extant strength of evolutionary signals (Chapter 1). I was able to address the second goal most fully, with my investigations of oxalic acid concentration in oca tubers from different use-categories (Chapters 2 and 3). Additionally, based on the patterns observed in the oxalic acid data, I used genotype analyses and flow cytometry analyses to lay the foundation for demonstrating the role of polyploidization in oca tuber biochemistry and relating cultural food processes (Chapters 4 and 5). For both crops, I investigated to the extent available what the underlying biological benefits for continued cultivation of toxic domesticates may be (Chapters 1, 2, and 3).

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CHAPTER 1

Geographic differences in patterns of genetic differentiation among bitter and sweet manioc (*Manihot esculenta* ssp. *esculenta*: Euphorbiaceae)[1]

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[1] Published in the *American Journal of Botany* 100 (5): 857 – 866. 2013.

Acknowledgements

The authors extend deepest thanks to J. Salick, Missouri Botanical Gardens, for her generous contribution of dried leaf material to this project, though we were unable to extract usable DNA from the material; also L. Benoit and M.P. Dubois at Centre d'Écologie Fonctionnelle et Évolutive (CEFE), Montpellier, France for all of their help in the laboratory; F. Cerqueira for her help with the ABI 3130 sequencer; and S. Friedrichs, University of Wisconsin-Madison Botany Department Graphics Lab, for her help with our figures. Genotyping was performed using the technical facilities of the IFR-119 "Montpellier Environnement Biodiversité." This research was funded by NSF Franco-American Cultural Exchange (NSF OISE-0623583) and by the program 'Ecosystèmes Tropicaux' of the Ministry of Ecology and Sustainable Development, France. AD was funded by European Commission's FP7 Marie Curie IOF grant TRECC-2009-237228. MD received a grant (RS/2005/44) from The Irish Research Council for Science, Engineering and Technology (IRCSET, funded under the National Development Plan).

Abstract

Premise of the study

Manioc (*Manihot esculenta* ssp. *esculenta* Crantz), one of the most important tropical food crops, is commonly divided according to cyanide content into two use-categories, “sweet” and “bitter.” While bitter and sweet varieties are genetically differentiated at the local scale, whether this differentiation is consistent across continents is yet unknown.

Methods

Using eight microsatellite loci, we genotyped 522 manioc samples (135 bitter and 387 sweet) from Ecuador, French Guiana, Cameroon, Gabon, Ghana, and Vanuatu. Genetic differentiation between use-categories was assessed using Double Principal Coordinate Analyses (DPCoA) with Multivariate Analysis of Variance (MANOVA) and Jost’s measure of estimated differentiation (D_{est}). Genetic structure was analyzed using Bayesian clustering analysis.

Key Results

Manioc neutral genetic diversity was high in all sampled regions. Sweet and bitter manioc landraces are differentiated in South America but not in Africa. Correspondingly, bitter and sweet manioc samples share a higher proportion of neutral alleles in Africa than in South America. We found seven clones classified by some farmers as “sweet” and by others as “bitter.”

Conclusions

Lack of differentiation in Africa is most likely due to post-introduction hybridization between bitter and sweet manioc. Inconsistent transfer from South America to Africa of ethnobotanical knowledge surrounding use-category management may contribute to increased hybridization in Africa. Investigating this issue requires more data on the variation in cyanogenesis in roots within and among manioc populations and how manioc diversity is managed on farm.

Key words

Cassava; crop management; crop migration; cyanogenesis; domestication; microsatellite loci; population structure; small-holder agriculture

Introduction

Manioc (*Manihot esculenta* ssp. *esculenta* Crantz), also known as “cassava,” “mandioca,” “tapioca,” and “yuca,” is one of the most important subsistence and economic crops of the tropics. Originally domesticated along the southwestern rim of Amazonia in South America (Allem, 1994; Olsen & Schaal, 1999, 2001), manioc is now grown throughout tropical regions. Manioc was first introduced into Africa from Brazil in the 16th century (Jones, 1959), with multiple subsequent introductions until the 1800s (Fregene et al., 2003). Its introduction into Oceania was much later; its presence in Vanuatu, for example, dates from around 1850 (Weightman, 1989; Sardos et al., 2008). Cultivated for its starchy roots, manioc is estimated to be the second most harvested crop in least developed countries and the fourth most harvested starch crop in the world (FAO STAT, 2010).

Manioc is also known for its extensive varietal diversity. Hershey (1994) estimated total manioc diversity to be over 7,000 varieties, cultivars, and landraces. However, this figure should be considered an underestimate, since even though manioc is usually clonally propagated, plants are still capable of sexual reproduction, and the incorporation, conscious or accidental, of volunteer seedlings into clonally propagated stock is continuously generating new genotypes (Salick et al., 1997; Elias et al., 2000; Pujol et al., 2005; Pujol et al., 2007). In this paper, we use the term “landrace” to refer to a set of clones identified by farmers under a single name. Landraces are separated into two primary categories based on traditional folk culinary use-categories: “bitter” (also called *brava* in Portuguese, *amarga* in Spanish) and “sweet” (*aipim* or *macaxeira* in Portuguese and *dulce* in Spanish).

Classification of manioc into use-categories is based on the taste of the uncooked roots, which depends largely on the levels of cyanogenic glucosides in the plant tissue (Dufour, 1988; McKey & Beckerman, 1993; Chiwona-Karltun et al., 2004). Tissue damage brings the cyanogenic glucosides into contact with the plant's endogenous glucosidases, releasing free hydrogen cyanide (HCN; de Bruijn, 1973; Hösel, 1981; Kakes, 1990). Generally, "bitter" manioc landraces produce over 100 mg/kg fresh weight (FW) free HCN when macerated (Dufour, 1988; McKey et al., 2010). Bitter manioc can be toxic to humans if chronically consumed without proper removal of the cyanide through a labor-intensive pre-cooking process (Dufour, 1988; McKey & Beckerman, 1993). Some bitter manioc landraces can even cause acute toxicity, especially in children and populations with low dietary availability of sulfur-rich proteins (McKey et al., 2010). Landraces containing less than 100 mg/kg FW of cyanide are considered non-toxic and can be eaten without pre-treatment (Lancaster et al., 1982; Dufour, 1988; Mowat, 1989).

However, evidence suggests that the bitter-sweet division is often a false dichotomy and that, in fact, HCN production in manioc can show continuous variation (Rogers, 1965). The primary method of landrace classification to a use-category is based on how the roots taste to the farmers, allowing for a large area of subjectivity in classification. Indeed, what tastes "bitter" to one farmer may not be "bitter" to another, for cultural reasons (Chiwona-Karltun et al., 1998) and owing to genetically based variation in taste perception (Soranzo et al., 2005). Because HCN content in manioc roots is highly influenced by environmental conditions (de Bruijn, 1973; Prinz, 1988; Bokanga et al., 1994), even the same genotype may taste "bitter" in some environments and "sweet" in others. Though the perception of bitterness by farmers affects a wide range of agricultural and culinary decisions, from where, when, and how to grow a landrace to how to prepare it for consumption, inconsistencies in use-category classification remain under-studied.

Patterns of traditional manioc cultivation throughout South America have resulted in some degree of isolation between the two categories. Many indigenous groups in areas of the Andean foothills of western Amazonia, including Peru (Salick et al., 1997) and Ecuador (Hinostroza, 1991), exclusively cultivate sweet manioc, while in the lower Amazon Basin farmers often grow bitter and sweet manioc with a predilection for the former (Renvoize, 1972). Large-scale geographical segregation of sweet and bitter landraces can be reinforced at the local level by small-scale segregation. When both bitter and sweet manioc are cultivated, farmers in South America often deliberately grow sweet and bitter varieties in separate fields or in distinct monovarietal patches within the same field (Elias et al., 2004; A. Duputié, pers. obs.).

In contrast, bitter and sweet manioc landraces are usually mixed in Africa, although a wide variation has been observed in manioc cultivation in Africa, ranging from bitter and sweet manioc being grown in complete sympatry (Jones, 1959; Chiwona-Karltun et al., 1998; Mkumbira et al., 2003; Delêtre, 2010), to sweet manioc being grown in homegardens and bitter manioc in the fields, mirroring practices of Amerindian farmers (Cock, 1985; McKey et al., 2010). In some parts of eastern Africa, farmers grow exclusively sweet manioc (Jones, 1959). In areas of the South Pacific, in particular in Vanuatu, only sweet manioc is grown (Weightman, 1989). Such geographical variation in on-farm management of bitter and sweet manioc raises many questions regarding the management of manioc genetic diversity in the different systems. Studies have shown that bitter and sweet varieties are differentiated for neutral genetic markers at the village scale in Guyana (Elias et al., 2004) and in Malawi (Mkumbira et al., 2003). The same pattern holds across Brazil (Mühlen et al., 2000; 2010); however, whether this pattern is consistent across larger geographical scales is unknown.

This study is an initial comparative examination of the differentiation between sweet and bitter manioc in parts of the crop's native range and in four countries in two areas of introduction. Manioc samples were collected in two South American countries, Ecuador and French Guiana; three central and western African countries, Cameroon, Gabon, and Ghana; and the South Pacific nation Vanuatu. Our specific research questions are: 1) Are manioc collections genetically structured by use-category, geography, or neither? 2) Are there consistent patterns in genetic differentiation among bitter and sweet manioc across multiple, geographically distant collections?

MATERIALS AND METHODS

Sampling**—**We analyzed 522 manioc samples (135 from "bitter" landraces and 387 "sweet") from six countries: Cameroon, Ecuador, French Guiana, Gabon, Ghana, and Vanuatu (Table 1; for more detailed sampling information, see Appendix S1 in Supplemental Data with the online version of this article)**.** Although African manioc samples were collected from central and western Africa only, for brevity we refer to these samples as from "Africa" throughout the manuscript. Manioc sampling was conducted by different investigators. Although sampling strategies varied, samples were generally collected with the aim of obtaining a representative sample of the diversity present at the local scale as perceived and managed by farmers (e.g., Duputié et al., 2007; Duputié et al., 2009; Delêtre, 2010). For a more comprehensive description of sampling strategies, see Appendix S2 in Supplemental Data with the online version of this article. In order to detect the underlying pattern of manioc diversity resulting from original dispersal and traditional management, we specifically asked for local landraces in the attempt to avoid “improved” cultivars disseminated by breeding programs. Determination of use-categories relied upon farmer categorization. This method of classification reflects whether farmers detoxify roots prior to consuming them, but does not rely upon quantification of HCN production.

DNA extraction and microsatellite genotyping—DNA was extracted using Qiagen® (Venlo, Netherlands) DNeasy™ Plant 96-well extraction kits. Each sample was genotyped using eight microsatellite loci (GA12, GA21, GAGG5, GA126, GA127, and GA134 [Chavarriaga-Aguirre et al., 1998] and SSR55 and SSR68 [Mba et al., 2001]). All loci were amplified jointly using the Qiagen® Multiplex PCR Kit™ following the manufacturer’s recommendations, in a final volume of 10 µL with 1 µL of undiluted DNA extraction product. Amplification was conducted after an initial denaturation phase of 15 min. at 95°C, with 30 cycles of 30 s denaturation at 94°C, 30 s annealing at 57°C, and 60 s elongation at 72°C, followed by a final elongation phase of 30 min at 60°C. Genotyping was performed on an ABI 3130 Genetic Analyzer (Applied Biosystems™, Foster City, CA, USA). Alleles were scored using GENEMAPPER® 3.0 (Applied Biosystems™) and visually confirmed. Two negative control wells containing 1 µL of ddH$_2$O in lieu of DNA were included in each 96-well reaction plate, all of which returned negative. Typing error rate was assessed by genotyping 100 samples two independent times, resulting in no genotype discrepancies.

Statistical analysis—Duplicate clones were removed such that there was only one clone of each multi-locus genotype per use-category per village before statistical analyses were conducted. Indeed, the lack of random sampling prevents extrapolation of “clonal frequency” from our data set, which was aimed instead at collecting the most diverse sample, i.e. only one or a few individuals per landrace per field or village. Samples were categorized into five sample groups: French Guiana bitter, South America sweet, Africa bitter, Africa sweet, and Vanuatu (sweet). Genetic differentiation among sample groups was assessed in two ways: 1) Jost's measure of estimated differentiation (D_{est}, Jost, 2008) with the program SMOGD 1.2.5 (Crawford, 2010); 2) Double Principal Coordinate Analysis (DPCoA), with significance of differentiation assessed by Multivariate Analysis of Variance (MANOVA), using the program R 2.13 (R Development Core Team, 2011).

Worldwide genetic structure of the sample was explored using the model-based Bayesian clustering analysis implemented in STRUCTURE 2.2 (Pritchard et al., 2000). The program was run five times using the admixture model and assuming correlated allelic frequencies with 110,000 Markov-Chain Monte Carlo iterations (the first 10,000 were discarded as burn-in and were always sufficient to achieve convergence) and values of the number of clusters (*K*) ranging from *K* = 1 to 8, with no prior information regarding the geographic origin or toxicity levels of the landraces. The most likely number of clusters was determined as the number that maximized the second-order rate of change in posterior likelihood of the data given the model (Evanno et al., 2005).

Allele counts (A), number of private alleles (PA), and observed and expected heterozygosity (H_o and H_e) were computed using the program GENEPOP 3.4 (Raymond & Rousset, 1995). Rarified allelic richness (AR) and deficit of heterozygotes (*f*; Weir & Cockerham, 1984) were calculated using the program FSTAT 2.9.3.2 (Goudet, 2002). The significance of differences in AR was assessed with one-sided Wilcoxon signed-rank tests on locus-specific allelic richness using R.

RESULTS

Allelic comparisons of collections on the global level—We identified 188 unique clones of manioc across all collections (Table 1). The majority of these clones (98.3%) were only represented in a single collection locality. However, one well-traveled sweet clone was found in every sweet collection except Ecuador. Remarkably, seven genotypes (six from Africa, one from French Guiana) were inconsistently classified by farmers, with some individuals of the genotype classified as "bitter" and some individuals classified as "sweet." We refer to these clones as "double-classified" clones.

Sweet manioc landraces showed higher allelic richness in South America than in both areas of introduction (Table 2; one-sided Wilcoxon signed rank tests on locus-specific rarefied allelic richness, South America vs. Vanuatu, $V = 0$, $n = 8$ loci, $P = 0.01$ and South America vs. Africa, $V = 0$, $n = 8$ loci, $P = 0.02$). This was not the case for bitter manioc ($V = 4$, $n = 8$ loci, $P = 0.86$).

The eight SSR loci returned 48 total alleles (Table 3). Of these, 21 alleles were present in all groups (Table 3). The remaining 27 alleles were absent in at least one group; we refer to these as "informative." Nine of them (33%) were private to one group. Strikingly, nine others (33%) were shared by South American sweet manioc and African bitter and sweet manioc, but were not detected in South American bitter manioc. Similarly, bitter and sweet manioc shared a much greater proportion of alleles in Africa (70.6% of the 17 informative alleles present in Africa) than they did in South America (21.7% of the 23 informative alleles present in South America). For detailed presence/absence information for each allele and each sample group, see Appendix S3 in Supplemental Data with the online version of this article.

Differentiation between manioc groups—Overall, pairwise differentiation between groups is low (Table 4). Sweet and bitter manioc showed much stronger differentiation in South America ($D_{\text{est}} = 0.106$) than in Africa ($D_{\text{est}} = 0.003$). This pattern is further supported by the Double Principal Coordinate Analyses (DPCoA; Figures 1 and 2), which showed that the French

Guiana bitter landraces were significantly differentiated not only from the South American sweet landraces ($F_{2,50}$ =37.715, $P < 0.001$; Figure 2.1) but also from all landraces, sweet and bitter combined, from western and central Africa and Vanuatu ($F_{4,\,192}$ = 53.386, P < 0.001; Figure 1). Contrastingly, in Africa no significant differentiation was observed among any of the sample sets ($F_{25,\,400}$ = 1.531, P = 0.12; Figure 2.2).

These results are further consistent with genetic structuring inferred by STRUCTURE (Figure 3). The most likely clustering was obtained for K = 3, with one cluster formed of French Guiana bitter landraces, and two clusters gathering all other landraces (i.e. African bitter plus all sweet landraces). Forcing STRUCTURE to form K = 2 clusters did not result in distinct global "bitter" and "sweet" clusters, but rather maintained French Guianan bitter landraces as distinct from all others, including bitter landraces from west and central Africa. Graphs of log posterior likelihood and delta K as a function of K are shown in Appendix S4 (see Supplemental Data with the online version of this article).

DISCUSSION

Do manioc samples exhibit differentiation based on use-category, geography, or both, and do we see consistent patterns across sampling locations?—Genetic differentiation between bitter and sweet manioc does not show consistent patterns across continents. In South America, sweet and bitter landraces are strongly differentiated, which is consistent with the previously observed patterns at the local and regional levels in Amazonia (Mühlen et al*.,* 2000, 2010; Elias et al., 2004; Peroni et al., 2007). Although it could be argued that bitter-sweet differentiation might be a small-scale pattern nested within larger geographical differentiation, we postulate that hybridization between bitter and sweet manioc in South America is limited by farmers' distinct management of the two types separately in regions where both are grown, and by the persistence of regions where only one of the two types is grown (e.g., sweet manioc in Peru and Ecuador).

In contrast, African manioc landraces showed no genetic differentiation based either on use-category or geography. STRUCTURE analyses (Figure 3) and allele presence/absence tracking (Table 3) suggest that lack of differentiation in Africa is due primarily to allele sharing among African bitter manioc and global sweet manioc. Our results contrast to those of Mkumbira and colleagues (2003), who did find bitter-sweet differentiation among ten landraces in Malawi. This could be due to the differences in geographical origin of our samples, as manioc was introduced independently in western and eastern Africa (Jones, 1959). Further sampling from other regions in Africa along these two main waves of introduction would help test this hypothesis. Many possible scenarios could have resulted in the observed lack of differentiation between sweet and bitter manioc in western and central Africa (e.g., loss via genetic drift in African bitter varieties of alleles characteristic of South American bitter manioc, secondary selection for bitter landraces from within an original sweet collection, with or without previous introgression of alleles influencing cyanogenic potential in sweet clones in Africa, or hybridization in Africa between sweet and bitter manioc). We begin our discussion with the third, most plausible, scenario of hybridization between use-categories in Africa. The improbability of the other two scenarios is discussed under the section "*Alternative hypotheses.*"

Manioc introduction into Africa and subsequent crop management—Manioc was first brought to Africa in the 1550s by Portuguese traders who valued manioc flour as a provision for slave ships (Jones, 1959; Ross, 1975; Carter et al., 1992; Hillocks et al., 2002). Manioc flour is almost always produced from bitter manioc, suggesting that the first manioc landraces introduced in western Africa would mostly have been Brazilian bitter landraces (Jones, 1957;

Nweke, 1994; Chiwona-Karltun et al., 1998; H. Ceballos, CIAT, pers. comm.). Though our sampling did not include Brazilian manioc landraces, Elias and colleagues (2004) found little genetic differentiation between (primarily bitter) manioc from Brazil and from the Guianas. Furthermore, the lack of differentiation observed in our African data contrasts sharply with the results obtained by Mühlen and colleagues (2010), who demonstrated bitter-sweet differentiation among 263 bitter and 302 sweet landraces from throughout Brazil.

Though no precise date of introduction is known for sweet manioc, it is believed to have reached Africa much later than bitter manioc (Jones, 1959). Though it may have reached the coastal plains of Ghana in the 18th century (Manu-Aduening et al., 2005), it did not spread inland in Ghana until as late as the early 1980s (Korang-Amoako et al., 1987). Similarly, historical and genetic analyses of manioc in Gabon suggest a smaller and more recent introduction of sweet manioc (Delêtre, 2010).

The patchy and inconsistent pattern of manioc introduction to Africa is matched by a similarly inconsistent traditional knowledge associated with manioc cultivation and detoxification (McKey et al., 2010; Delêtre, 2010). The lack of consistent transfer of traditional knowledge, coupled with environmental and socio-political constraints to traditional lifestyles, has led to numerous outbreaks of cyanide-related diseases in Africa over the last three decades (Ministry of Health, Mozambique, 1984; Cliff et al., 1997; Nhassico et al., 2008), whereas similar problems have not been reported in South America (for a comprehensive discussion see: McKey et al., 2010).

Although in some parts of eastern Africa only sweet manioc is grown (Jones, 1959), both use-categories are cultivated in the western and central African countries we studied, with no consistent pattern of small-scale segregation (Carter, 1992; Delêtre, 2010). Communities relying upon manioc for subsistence generally prefer bitter manioc (Wilson & Dufour, 2002), though not exclusively (Chiwona-Karltun et al., 1998, 2004). In Gabon, Delêtre (2010) observed considerable variation in crop management, including bitter and sweet landraces planted together in the same field, in distinct monovarietal stands, and separated, with bitter and sweet in distinct locations, similar to a pattern frequent in South America (McKey & Beckerman, 1993; Wilson, 2002). Mixed fields of bitter and sweet manioc were observed in Ghana, although 76% of farmers cultivated only one or two landraces, effectively creating patchworks of monovarietal stands (Manu-Aduening, 2005). In Cameroon, various planting strategies are employed that are largely driven by pest control (Poubom et al., 2005). Poubom and colleagues (2005) observed completely mixed farming stands (usually defended from predating monkeys by wind-powered noise-making contraptions), monovarietal stands of bitter manioc, and fields with sweet landraces surrounded by a wide swath of bitter landraces to provide protection for the sweet landraces from monkeys and other herbivores, a pattern that has also been reported in South America (McKey & Beckerman, 1993).

Admixture after introduction to Africa?—The farming practices documented on African manioc farms offer opportunities for substantial hybridization between sweet and bitter varieties that, when followed by incorporation, exchange, and diffusion of hybrid volunteer seedlings, could have obscured genetic differentiation between sweet and bitter varieties. However, gene mixing between bitter and sweet categories is seemingly asymmetrical, with greater flow from sweet to bitter manioc: while eight SSR alleles typical of sweet manioc ("sweet-type" alleles) were found in bitter manioc from western and central Africa, only one neutral allele typical of bitter manioc ("bitter-type" allele) was present in sweet manioc from the same region (Table 3). Furthermore, Bayesian clustering analyses formed one cluster comprising French Guiana bitter landraces and two clusters grouping all other landraces, including African bitter and all sweet manioc (Figure 3). These last two clusters were unrelated to either taste or geography and were present even in areas where bitter manioc is not grown, suggesting that the subdivision of sweet manioc into two distinct clusters is not due to hybridization between sweet and bitter landraces. Interestingly, Mühlen and colleagues (2010) identified two groups of sweet manioc in Brazil. In conjunction with our data, future research comparing this subdivision within sweet manioc in Africa and Brazil is certainly warranted. Additionally, the larger number of "double-classified" clones in Africa (six) compared with South America (one) suggests that there may be clones in Africa that have levels of cyanogenesis intermediate between bitter and sweet, and thus may be considered alternately bitter or sweet depending on environmental influences and differences in individual taste. These clones may be the product of bitter-sweet crosses.

Although our sampling was not designed to assess the relative frequency of sexual reproduction in African and South American systems, our results suggest that there may be higher rates of cross mating between bitter and sweet manioc in Africa than in South America. Indeed, given the pattern of allele sharing in Africa and the impact that bitter-sweet mixing could have on reliable detoxification of manioc landraces, future research assessing frequency of bitter-sweet crosses in African farming systems could be critical to our understanding of when and how often farmers incorporate volunteer manioc seedlings into their stock of propagules, as well as how they classify these new clones as "bitter" or "sweet."

***Determinism of cyanogenesis in manioc*—**Evidence suggests, however, that toxicity in manioc roots is highly labile and ranges along a biochemical continuum (Rogers, 1965; de Bruijn, 1973; Nye, 1991; Bokanga, 1994). Cyanogenesis in manioc seems to be primarily controlled by additive effects of a complex of recessive minor QTLs (Hahn, 1973; Mahungu, 1994; Sayre et al., 2003; Whankaew et al., 2011), with broad-sense heritability around 43% (Kizito et al., 2007). This leads to cases where offspring of sweet-by-sweet crosses can produce sufficient HCN to be classified as "bitter," or vice-versa (Valle et al., 2004; Kawuki et al., 2011).

Additionally, cyanogenic potential of a particular genotype is highly dependent upon environmental factors. Drought and elevated soil nitrogen concentrations (de Bruijn, 1973; Bokanga et al., 1994), as well as the plant's increasing age (Prinz, 1988), tend to increase the content of cyanogenic glucosides. Thus, the combination of inheritance mechanisms of cyanogenesis in manioc, environmental effects on HCN production, and the broad range of cyanogenesis levels encompassed by the category "bitter," could easily allow for a scenario whereby most offspring of bitter-by-sweet crosses carry sufficient cyanogenesis-conferring alleles to be categorized by farmers as "bitter" regardless of their neutral genotype. This may explain why we did not find any sweet manioc showing "bitter-type" alleles: hybrids of parents from the two categories are more likely to be classified as "bitter" than as "sweet."

Alternative hypotheses**—**The weak founder effect indicated by our rarified allelic richness assessments makes genetic drift unlikely as a single explanation for the patterns we observed, though drift may have compounded the effect of admixture by causing the loss of bitter-type alleles in African bitter manioc. A scenario involving a secondary selection of bitter manioc from within a sweet collection (introgressed with additional cyanogenesis-conferring alleles or otherwise) is less parsimonious than the admixture scenario, given the widely-accepted inference of the early presence of bitter manioc in Africa (Jones, 1957; Nweke, 1994; Chiwona-Karltun et al., 1998; H. Ceballos, CIAT, pers. comm.).

Conclusions**—**The complicated history of manioc introduction into and dispersal within Africa, along with the dramatic shift in cultivation techniques from South American

Amerindians to African smallholders, likely combined to create a situation favorable to hybridization between sweet and bitter manioc. Our data support this hypothesis, revealing significantly less differentiation between bitter and sweet manioc in our central and western African samples. Additionally, our results suggest that use-categories are less well-defined in Africa, both genetically (in terms of differentiation) and ethnobotanically (classification by farmers). Our data align with public health data noting the persistence of cyanide-related diseases in Africa, which could partially result from incorrect classification of bitter manioc by farmers. Systematic ethnographic studies of manioc farming systems in Africa focusing on management of bitter and sweet manioc, processing techniques, and management of manioc volunteer seedlings, will be necessary to test whether or not variation in crop management strategies and use-category classification contributes to bitter-sweet hybridization in Africa. Our results also stress the need to quantify the natural distribution of cyanogenesis in manioc use-categories across native and introduced regions. These investigations would improve our understanding of manioc genetic diversity and the biological and cultural bases of manioc toxicity.

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Table 1. Manioc (*Manihot esculenta* ssp. *esculenta*) sampling by country and use-category showing number of samples analyzed and number of genotypes obtained. Note that "Bitter" and "Sweet" columns do not sum to "Total" column because duplicated genotypes across countries and use-categories were counted independently for each locality and use-category. Samples provided by [1]Doyle McKey, [2]Alexandra Narváez Trujillo, [3] Marc Delêtre, [4]Joseph A. Manu-Aduening, [5]Anne Duputié, [6]Caroline Roullier.

Country	Number of Samples	Bitter: Number of Samples: **Number of Genotypes**	Sweet: Number of Samples: **Number of Genotypes**
Cameroon[1]	44	19:**7**	25:**9**
Ecuador[2]	24	0	24:**17**
Gabon[3]	147	73:**34**	74:**28**
Ghana[4]	12	2:**2**	10:**6**
French Guiana[5]	64	41:**27**	23:**8**
Vanuatu[6]	231	0	231:**58**
Total	522: **188**	135: **70**	387: **125**

Table 2. Summary of allelic data for eight microsatellite loci in five groups of manioc (*Manihot esculenta* ssp. *esculenta*). Sample groups are "Africa bitter" (bitter manioc from central and western Africa), "Africa sweet" (sweet manioc from central and western Africa), "French Guiana bitter" (bitter manioc from French Guiana), "South America sweet" (sweet manioc from French Guiana and Ecuador), and "Vanuatu (sweet)" (manioc from Vanuatu, all sweet). Number of genotypes (n), number of alleles per locus (A), expected (H_e) and observed (H_o) heterozygosity, deficit of heterozygotes (*f*), number of private alleles (PA), and rarefied allelic richness (AR) are listed for each collection and locus.

Sample Group		**GA12[2]**	**GA21[1]**	**GA57[1]**	**GA126[1]**	**GA127[1]**	**GAGG5[1]**	**SSR168[3]**	**SSR55[2]**	**Total**
Africa bitter n = 43	**A**	3	5	3	7	4	2	8	5	37
	PA	0	0	0	2	0	0	0	1	3
	AR	3	4.22	3	6.57	3.99	2	7.21	4.86	4.36
	He	0.64	0.57	0.47	0.8	0.62	0.5	0.8	0.69	0.64
	Ho	0.76	0.5	0.6	0.93	0.69	0.57	0.86	0.79	0.71
	f	-0.17	0.11	-0.24	-0.14	-0.1	-0.11	-0.12	-0.09	-0.11
Africa sweet n = 43	**A**	3	3	3	6	5	2	7	6	35
	PA	0	0	0	0	0	0	0	0	0
	AR	3	2.93	3	5.99	4.57	2	6.39	5.87	4.22
	He	0.46	0.53	0.58	0.8	0.64	0.49	0.73	0.64	0.61
	Ho	0.58	0.53	0.73	1	0.71	0.58	0.8	0.69	0.7
	f	-0.22	0.02	-0.22	-0.24	-0.1	-0.12	-0.09	-0.13	-0.14
Africa, total	**AR**	3	4.24	3	6.62	4.62	2	7.48	5.94	4.61
French Guiana bitter n = 27	**A**	3	3	3	5	5	2	7	5	33
	PA	0	0	1	1	1	0	0	1	4
	AR	3	2.93	3	5	4.99	2	6.93	5	4.11
	He	0.65	0.51	0.43	0.74	0.66	0.5	0.78	0.74	0.63
	Ho	0.68	0.54	0.36	0.75	0.64	0.32	0.71	0.82	0.6
	f	-0.12	-0.1	0.18	-0.01	-0.04	0.37	-0.14	0.16	0.02
South America sweet n = 25	**A**	3	4	3	6	4	2	8	6	36
	PA	0	0	0	1	0	0	0	1	2
	AR	3	4	3	6	4	2	8	6	4.5
	He	0.48	0.49	0.54	0.75	0.65	0.49	0.82	0.78	0.63

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[2] Chavarriaga-Aguirre et al., 1998

[3] Mba et al., 2001

	Ho	0.46	0.54	0.65	0.81	0.73	0.38	0.65	0.77	0.62
	f	0.22	0.03	-0.3	-0.04	-0.04	0.15	0.05	0.14	0.02
South America, total	**AR**	3	5	4	7	6	2	10	7	5.5
Vanuatu (sweet) n = 58	**A**	2	3	3	5	5	2	5	6	31
	PA	0	0	0	0	0	0	0	0	0
	AR	2	2.91	3	4.91	4.45	2	4.99	5.36	3.7
	He	0.39	0.52	0.63	0.77	0.67	0.47	0.73	0.71	0.61
	Ho	0.53	0.55	0.67	0.81	0.72	0.47	0.83	0.71	0.66
	f	-0.36	-0.06	-0.06	-0.05	-0.07	0.03	0.01	-0.12	-0.07

Table 3. Allele counts in sample groups of manioc (*Manihiot esculenta* ssp. *esculenta*) samples. Numbers for each category represent the number of alleles across all eight loci that are present in each category. Total category count is found along the bottom row of the table. Total count of alleles present in each sample group is found in the last column. All alleles sum to 48 total alleles identified across eight loci. The category " 'sweet-type' alleles" refers to alleles appearing in African and South American sweet manioc but only one, not both, bitter groups. " 'Bitter-type' alleles" were defined as those found in both bitter groups but only one sweet group. Bitter manioc samples from French Guiana are abbreviated "F. G. Bitter"; South American sweet manioc samples are abbreviated "S. Am. sweet"; samples from central and western Africa are abbreviated "Afr. bitter" and "Afr. sweet" for bitter and sweet manioc, respectively; and samples from Vanuatu, consisting entirely of sweet manioc, are abbreviated "Vanuatu (sw)."

		F.G. Bitter	S. Am. Sweet	W. Af. Bitter	W. Af. Sweet	Vanuatu (Sw)	**Count**
	Alleles present in all groups	21					**21**
Geography x Use-category	Alleles private to one group	4	2	3	0		**9**
Geography	Alleles in all groups except Vanuatu	2				0	**2**
	Alleles private to S. America	2		0			**2**
	Alleles private to Africa	0		1		0	**1**
Use-Category	Alleles private to one use-category	1	0	1	0		**1**
	"Sweet-type" alleles	1	9	8	9	8	**9**
	"Bitter-type" alleles	1	0	1		0	**1**
	Alleles presenting heterogeneous patterns	2	0		1		**2**
	Total	**34**	**11**	**14**	**11**	**9**	**48**

Table 4. D_{est} (Jost, 2008) between pairs of manioc (*Manihot esculenta* ssp. *esculenta*) groups; values greater than 0.05 are in bold face. Samples from central and western Africa are denoted as "Africa."

Africa	**Cameroon Sweet**	**Gabon Bitter**	**Gabon Sweet**	**Ghana Bitter**	**Ghana Sweet**
Cameroon Bitter	0.0002	< 0.0001	0.005	0.0002	0.0002
Cameroon Sweet	-	**0.0605**	0.0136	< 0.0001	0.0152
Gabon Bitter	-	-	< 0.0001	0.0032	0.0132
Gabon Sweet	-	-	-	0.0031	0.0003
Ghana Bitter	-	-	-	-	0.0225

South America	**French Guiana Bitter**	**French Guiana Sweet**
Ecuador	**0.1893**	**0.0547**
French Guiana Bitter	-	0.0037

Global	**Africa Sweet**	**French Guiana Bitter**	**South America Sweet**	**Vanuatu**
Africa Bitter	0.003	**0.0572**	0.0348	0.0372
Africa Sweet	-	**0.1175**	0.046	0.0253
French Guiana Bitter	-	-	**0.1057**	**0.1289**
South America Sweet	-	-	-	0.0346

Figure 1. Double Principal Coordinate Analysis of genetic differentiation among all manioc (*Manihot esculenta* ssp. *esculenta*) samples. Groups are coded at the continental scale, bitter samples in yellow, sweet samples in blue. "Double-classified" clones are coded split yellow-blue. Ellipses around manioc groups represent 66% of sample set diversity. Samples from central and western Africa are denoted as "African sweet" and "African bitter." Bitter samples from French Guiana are significantly differentiated from all other groups of samples (MANOVA: $F_{4, 192} = 53.386$, $P = 2.2 \times 10^{-6}$).

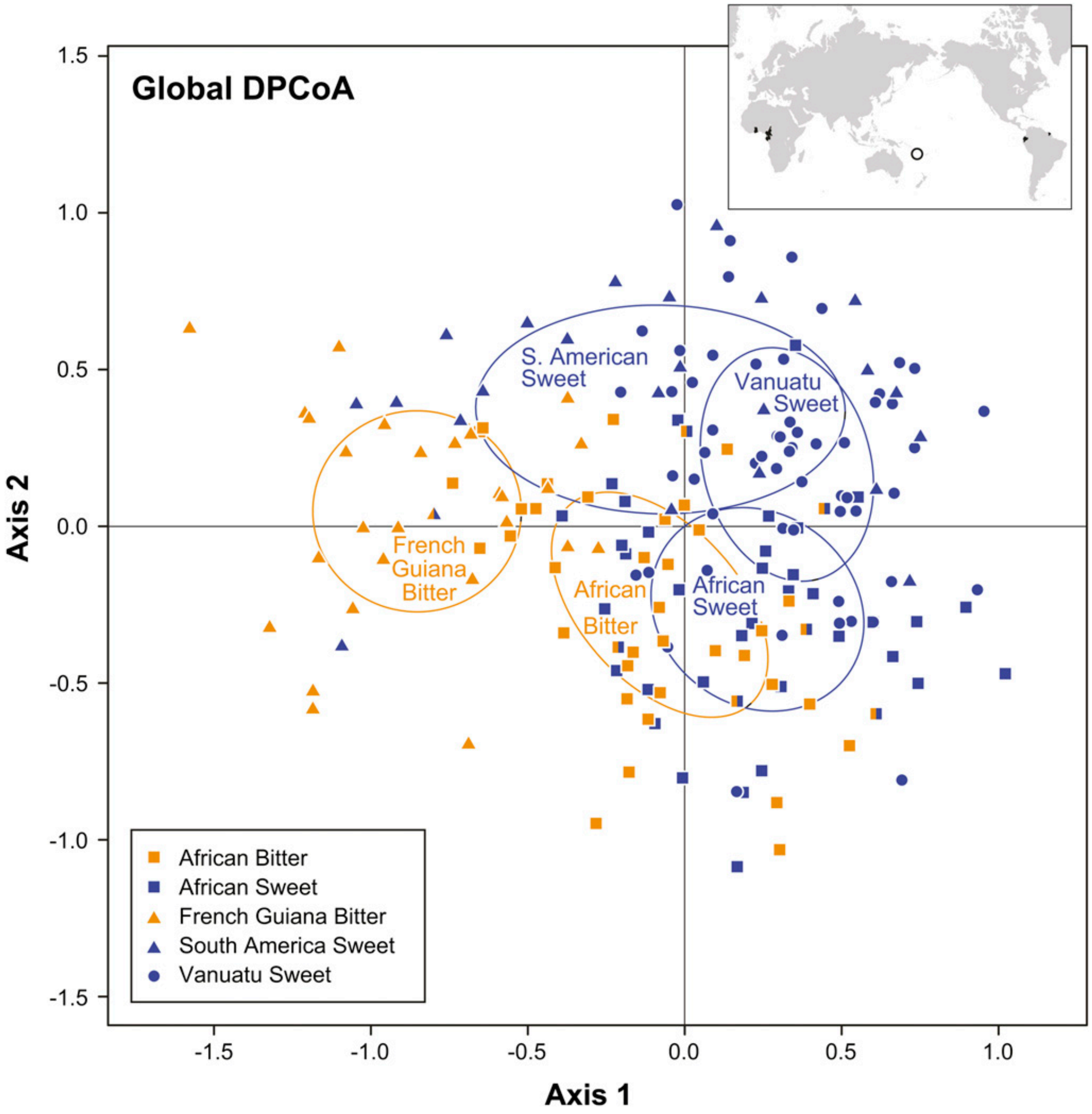


Figure 2. Double Principal Coordinate Analysis of genetic differentiation among bitter and sweet manioc (*Manihot esculenta* ssp. *esculenta*) in A) South America, B) central and western Africa (denoted as “Africa”). Groups are coded at the national scale, bitter samples in yellow, sweet samples in blue. Legend as in Figure 1. In South America, bitter samples from French Guiana are significantly differentiated from all sweet samples (MANOVA: $F_{2,50}$ =37.715, P = 0.0002). All samples from French Guiana are significantly differentiated from Ecuadorian samples (MANOVA: $F_{2,\,50}$ =53.214, $P = 2.6 \times 10^{-5}$). In Africa, no significant differentiation is observed (MANOVA: $F_{25,\,400}$ = 1.531, P = 0.12).

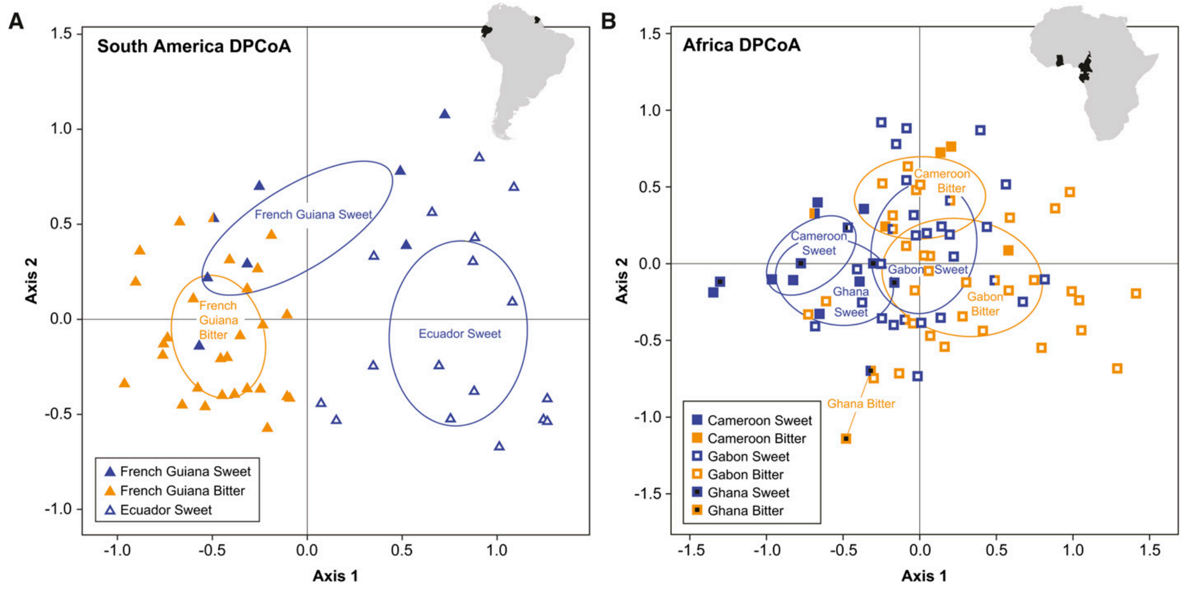
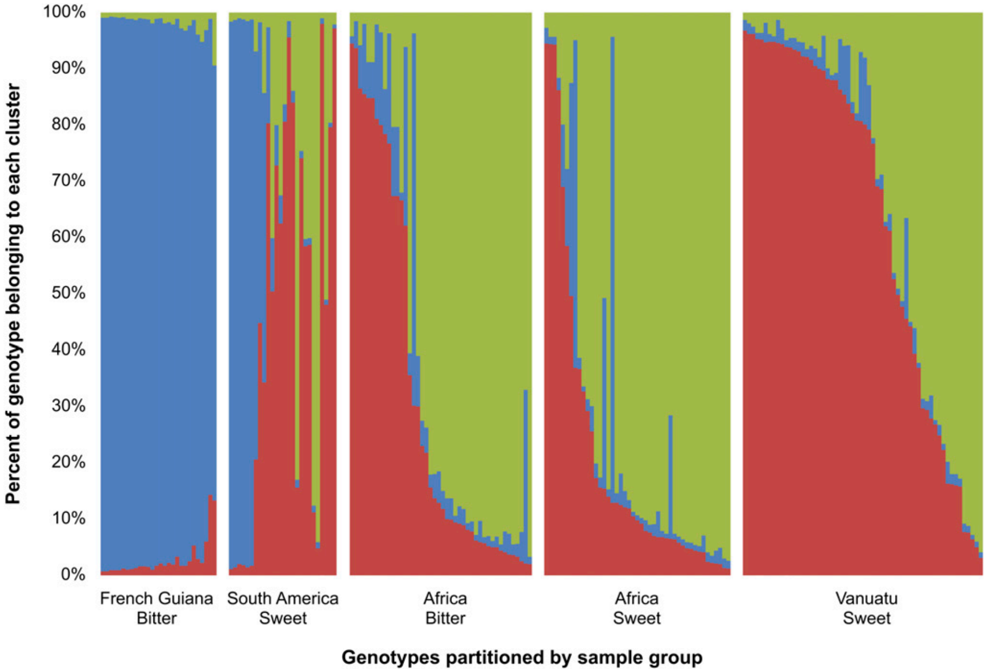


Figure 3. STRUCTURE output of manioc (*Manihot esculenta* ssp. *esculenta*) sample groups sorted by use-category and geography for most likely number of clusters *K* = 3. Each genotype is represented by a vertical bar colored proportionately to amount of genotype belonging to each cluster. Genotypes are arranged by sample group. Samples from central and western Africa are denoted as "Africa." This clustering shows a fairly strong French Guiana bitter cluster (blue), with two admixed clusters grouping all sweet samples and bitter African samples (red and green).



CHAPTER 2.

The Role of Organic Acids in the Domestication of *Oxalis tuberosa*: a new model for studying domestication resulting in opposing crop phenotypes

E. Jane Bradbury and Eve Emshwiller

Abstract

Though few crops display directly opposing domesticated phenotypes, these crops may be the key to understanding domestication processes that address conflicting selective pressures in the agricultural ecosystem. Two relatively well-known examples are cassava (*Manihot esculenta* Crantz), which has high-cyanide and low-cyanide varieties, and potato (*Solanum* section *Petota*), among which several species, including common potato (*Solanum tuberosum* L.), have low levels of glycoalkaloids and other species of "bitter potato" have elevated levels of glycoalkaloids. We propose that *Oxalis tuberosa* Molina, "oca", may represent a third example of such a crop system, with opposing high organic acid and low organic acid cultivars. Each cultivar set has different cultural food preparation practices ("use-categories"), similar to the "use-categories" that have been described for potatoes in the Andes (Brush *et al.*, 1981; Zimmerer 1991). Our initial analyses suggest that organic acids in tubers may be an important biochemical difference between use-categories, based on both oxalic acid and pH data. Here, we review our understanding of organic acids in oca tubers, while highlighting areas that merit further investigation.

Introduction

The evolutionary history of crops in traditional agricultural systems remains an area of research critical to our understanding of the processes and mechanisms of domestication

(Doebley, 2006; Gavrilenko, *et al.*, 2007; Doebley, 2009; Gregory, 2009). By corollary, we must also understand the changes—morphological, biochemical, ecological—that plant species undergo during domestication, changes broadly grouped as the "adaptive syndrome of domestication" (de Wet & Harlan, 1975). Often, the domestication syndrome of crops is discussed as a force of directional selection towards a set of phenotypic traits, such as larger fruits, population-level synchrony in flowering and fruiting, reduced seed coat thickness, increase or decrease of targeted chemical compounds, or a non-shattering rachis (Zohary, 1969, 1971; de Wet & Harlan, 1975; Gepts, 2004). Populations of domesticated plants can also undergo disruptive selection (also known as diversifying selection), whereby two or more phenotypes are preferred over an intermediate phenotype (Futuyma, 1998). Interestingly, we often see disruptive selection in crop plants across multiple traits, e.g. in the wide diversity of enlarged edible structures in broccoli, cabbage, kohlrabi, Brussels sprouts, and other domesticated forms of *Brassica oleracea* L. Rarely do we find crops that are the product of disruptive selection across a single trait, resulting in only two strongly contrasting phenotypes. One example of this type of disruptive selection is cassava, *Manihot esculenta*, which has both low-cyanide and high-cyanide strains, although there is growing evidence that cyanogenesis in cassava may be more continuous rather than discrete (McKey & Beckerman, 1993). A second example is bitter potato (several species in *Solanum* section *Petota*, including *S.* × *curtilobum* Juz. & Bukasov and *S.* × *juzepczukii* Bukasov), which has elevated levels of glycoalkaloids compared with the common potato, *Solanum tuberosum* L. (Johns, 1996). These crops present a unique opportunity to understand domestication strategies used to address situations when disruptive selection may be favorable in the agricultural ecosystem, e.g. when there are conflicting challenges presented by the ecosystem, when there are diverse human uses for a crop, or when artificial and natural selective pressures conflict.

However, a very small number of model crops exist that could provide a basis for understanding how domesticates with opposing biochemical phenotypes may address conflicting selective pressures within the agricultural ecosystem. To fulfill the need for additional model species that present similar dichotomies, we believe that the traditional Andean tuber crop oca (*Oxalis tuberosa*) could serve as an important new study organism for understanding crops that have been domesticated towards two distinct, opposing phenotypic ends. The purposes of this paper are: 1) to review our current knowledge of the evolutionary history and biochemistry of oca as it relates to the establishment of two separate oca use-categories; 2) to add new data on oxalic acid and other organic acids in oca tubers; and 3) to highlight the areas of research still necessary to understand how organic acid content in oca tubers might address conflicting selective pressures in the agricultural ecosystem.

II. *Oxalis tuberosa* Use-categories

Oca, a tuber crop native to the Andes, is an important staple food for native Andean peoples as a high altitude subsistence crop, grown both for dehydration and storage and for fresh consumption. Oca plays a critical role for food security for rural highland communities and is a key crop in the traditional crop rotation system (National Research Council (NRC), 1989; Trivelli, 1996). Oca tubers are colorful, with firm flesh ranging from white to peach and shiny skins ranging from white, yellow, red, pink, and even deep purple (NRC, 1989; Flores *et al.*, 2003; personal observation). Just as there are two separate uses for oca, there are two separate "use-categories" of oca tubers—those that are reserved exclusively for dehydration into a storable product called *khaya*, and those that are used primarily for fresh consumption, usually cooked after being set out in sunlight for a few days to “sweeten” (Hermann & Erazo, 2000; Emshwiller, 2006). *Khaya* is often compared to the dried potato product *chuño*, as both are formed by extensive soaking, squeezing, and freezing and drying treatments to form an essentially indefinitely storable food product (Johns, 1996; Hermann & Erazo, 2000; Emshwiller, 2006).

Cultivated most abundantly in central to southern Peru and Bolivia, oca is grown primarily by traditional Quechua- and Aymara-speaking agriculturists. Interestingly, both languages have names for the two use-categories. In Quechua, the ocas for drying are either *p'osqo* (literally meaning “fermented” or “sour”) or *khaya* (the name of the dried product) (Hermann & Erazo, 2000; Emshwiller, 2006; Carlos Arbizu, International Potato Center (CIP), personal communication). Ocas that are consumed fresh belong to the category *wayk'u* (“boiling”) or *misk'i* (“sweet”, “delicious”). Aymara speakers refer to ocas for processing as “*luk'i”* and those for fresh consumption as “*q'eni*”. No research to date has addressed a) the potential underlying biochemical differences between the use categories or b) whether or not any references to perceived biochemical differences are consistent across linguistic schemes. Similarly, the potential benefits that the ocas used for processing may present in the agricultural system are unstudied. However, before we can research which different selective pressures may have contributed to these opposing phenotypes, we must first demonstrate that these phenotypes are biologically different, as opposed to being separated solely based on cultural practice.

III. The Evolutionary History of *Oxalis tuberosa*

The approximately 500 to 800 species of the genus *Oxalis* have base chromosome numbers ranging from $x = 5$ to 12. The “*Oxalis tuberosa* alliance” is an informal group of several dozen species from the Central and Northern Andes, first named by de Azkue and

Martínez (1990) based on their shared base chromosome number of $x = 8$, which is not found in other members of the genus. Cultivated *O. tuberosa* is the only octoploid ($2n = 8x = 64$) (de Azkue & Martínez, 1990) in the alliance, whereas the majority of alliance species are diploid. Nuclear DNA sequence data confirmed the "*O. tuberosa* alliance" as a monophyletic clade within the genus *Oxalis* and also confirmed the origins of oca from within that clade (Emshwiller & Doyle, 2002).

In addition to oca, four wild members of the *O. tuberosa* alliance clade produce tubers: *O. chicligastensis* R. Knuth, *O. picchensis* R. Knuth, and two as-yet un-named taxa, one from central Peru and one from northern Bolivia. Ongoing research is addressing which of these may be oca's progenitor(s) (Emshwiller & Doyle, 2002; Emshwiller *et al.*, 2009). AFLP analyses suggest that the two use-categories discussed above are molecularly distinct from one another (Emshwiller, 2006; Emshwiller *et al.*, 2009) and initial flow cytometry data suggest that some accessions of *p'osqo* ocas may be tetraploid instead of octoploid (Kelly Vivanco, CIP, unpublished data). This suggests an underlying genetic difference between these two use-categories and we are currently studying how their evolutionary histories may differ.

IV. Oxalic Acid and Other Organic Acids in Oca Tubers

Six organic acids accumulate in oca tubers: ascorbic, malic, tartaric, succinic, glutaric, and oxalic acids (Ross *et al.*, 1999; Hermann & Erazo, 2000). Of these, oxalic acid is the most studied, due to its potentially deleterious effects on human health (Ross, *et al.*, 1999; Albihn & Savage, 2001a, 2001b; Sangketkit, *et al.*, 2001). Following usage by these and other authors, we will use "oxalate" as shorthand for both oxalic acid and oxalate ion, unless specified. As in more commonly consumed plant foods, oxalic acid content in raw *O. tuberosa* tubers shows a wide range of published values, despite limited sampling of oca's diversity (**Table 1**). The lowest reported values are 20.3 – 50.3 mg/100 g fresh weight (FW) (King, 1988). Ross *et al.* (1999) report values of 92 – 213 mg/100 g FW and attribute the discrepancy with King's data to the unreliability of King's wet titration method as opposed to HPLC quantification and to the fact that King's samples were stored prior to extraction in ethanol, which is known to be an effective oxalate solvent. Sangketkit and colleagues (2001) also found more than two-fold differences in oxalate content among 14 oca cultivars, ranging from 80 to 194 mg/100g FW. Hermann and Erazo (2000) reported the highest values, finding 306-539 mg/100g FW. Hermann and Erazo (2000) attribute their elevated oxalate values to the fact that these ocas were grown in greenhouses and treated with chemical fertilizers, which are known to increase oxalate levels. Although some of the samples used by Ross *et al.* (1999) and Sangketkit *et al.* (2001) were New Zealand market samples, presumably grown using conventional Western agricultural methods including chemical fertilizers, several came from traditional Bolivian and Peruvian markets.

Other possible explanations for the discrepancies in oxalate values discussed above include: a) differences in oxalate concentrations throughout the tuber; b) oca use-category; and c) potential effects of processing on oxalate solubility. Albihn and Savage (2001a) reported appreciable differences in oxalate concentrations in different areas of the tuber, with the highest concentration in the skins as compared with the outer and inner fleshes of the raw tubers. They demonstrated four-to-ten-fold greater oxalate concentrations in the skin as compared with the other areas of the tuber, reporting values of 4.82 to 10.88 g/kg FW for the skin and 1.59 to 1.64 g/kg FW in the outer flesh and slightly lower but comparable values for the inner flesh. This corresponds to 482 to 1088 mg/100g FW and 159 to 164 mg/100g FW, respectively.

In none of the publications on oxalate values have any of the authors stated to which use-category their oca samples belong. We suspect the vast majority of the ocas used were ocas traditionally not processed, particularly samples either collected in Andean markets (ocas for preserving rarely come to market in fresh form) and for samples from New Zealand where no *khaya* is produced (Morgan, 2005).

Despite the wide range of oxalate values, the published literature is consistent in the observation that only soluble oxalate is present in oca tubers in the samples used (King, 1988; Ross *et al.*, 1999; Hermann & Erazo, 2000; Sangketkit *et al.*, 2001). King (1988) and Hermann and Erazo (2000) both used light microscopy to search for the presence of calcium oxalate crystals and found none. Ross (1999) and Sangketkit (2001) and their colleagues performed both hot 2M HCl acid extractions for total oxalate content and water extractions for soluble oxalate content and found no significant difference ($p < 0.05$) between the methods, suggesting that all oxalate in oca is of the water-soluble type. However, as discussed above, it is unlikely that any of these studies included the varieties traditionally used for making *khaya*. In addition, traditional exposure of oca tubers to sunlight prior to fresh consumption greatly contributes to organic acid breakdown (Hermann & Erazo, 2000). Since many of the tubers included in these studies came from native markets, they were probably exposed to sunlight prior to sale. This could explain both the absence of insoluble oxalate crystals and the wide differences in oxalate content in tubers.

Anti-nutritional Effects and Toxicity of Oxalates

Oxalate is not believed to play any important physiological function in the human body and, in fact, has anti-nutritional and even toxic effects. Though normally excreted in the urine as a waste product, oxalate can form deleterious crystals in the kidneys and bladder (“kidney stones”) when present in high concentrations or when improperly metabolized by the renal system (Hatch & Freel, 1995). Additionally, soluble oxalate is generally believed to reduce the intestinal absorption of calcium due to the formation of insoluble calcium oxalate salts, potentially leading to calcium deficiency (Lovelace *et al*., 1950; Murillo *et al*., 1972; Hodgkinson, 1977; Kelsay, 1985; Hermann & Erazo, 2000; Albihn & Savage, 2001a, 2001b; Massey, 2007). Moreover, there is some evidence that soluble oxalate could impair the availability of magnesium, iron, and other trace minerals that are also known to form insoluble oxalate salts (Peters *et al*., 1971; Singh, 1973).

Beyond the nutritional concerns, the toxic effects of oxalic acid in mammals have been recognized since the early nineteenth century (reviewed in Hodgkinson, 1977 Chapter 1). Cases of both acute and chronic poisoning in humans have been described, from as little as 2 g to as much as 30 g of ingested oxalate. It is generally believed that acute toxicity is the result of cellular death in the nervous system caused by calcium ion deficiency, which results in tetany, muscle cramps, lowered blood coagulability, cardiovascular collapse, and other related symptoms (Hodgkinson, 1977; Libert & Franceschi, 1987).

V. Oxalates and the Ethnobotany of Oca

Effects of oxalates on oca domestication

Very little research has addressed the potential role of organic acids in the domestication and utilization of *O. tuberosa* as a crop plant. Some evidence suggests that humans have selected for changes in secondary chemistry during the domestication of oca (Johns, 1996). Thus, a prerequisite to understanding the potential role of oxalates in the domestication of oca is knowing how crop diversity is named and classified by farmers in order to determine "how this diversity is perceived and valued by farmers" (Elias *et al.,* 2001) and therefore to "understanding behavioral patterns that affect crop evolution" (Quiros *et al*., 1990).

Folk Classification of Oca

As previously mentioned, farmers classify oca tubers into two use-categories. Intuitively, it seems likely that the different naming schemes must also reflect the levels of oxalic acid in the different varieties. However, there has never been a large-scale comparison of *p'osqo* and *wayk'u* ocas. We conducted two pilot studies investigating the potential differences in organic acid content in different categories of oca: one smaller study in which oxalic acid content was quantified in a small sample of oca tubers and one wild tuber-bearing relative (an un-named taxon from the Lima department of Peru) and a second study using pH as a proxy for overall acid content in a larger sample of oca tubers. In both studies, the International Potato Center (CIP) in Peru kindly provided oca tubers for analysis. In our first study, we would have liked to include samples from all four wild tuber-bearing relatives, but unfortunately at the time material was only available from one taxon. All tubers were grown in the CIP fields in the Junín Department of Peru and were treated only moderately with chemical fertilizers and pesticides (Francisco Vivanco, CIP, personal communication).

Oxalic acid extraction methods followed Ross *et al.* (1999). We used Roche Biochemical® Oxalic Acid Enzymatic Assay Kits to quantify total oxalate content in nine oca accessions (two *wayk'u* accessions and seven *p'osqo* accessions) and one sample of the unnamed wild tuber-bearing *Oxalis* taxon from central Peru. Even with the small sample size, differences in oxalate content between the *p'osqo* and *wayk'u* ocas were statistically highly significant ($p = 0.000006084$), in a Welch's two-sample t-test with unequal variance **(Figure 1)**. The values for oxalate content in the *wayk'u* varieties were 147 mg/100g FW and 183 mg/100g FW, values that fall within the range reported by both Ross et al. (1999) and Sangketkit *et al*. (2001). Even the *p'osqo* ocas, however, contained lower oxalate levels (273 mg/100g FW – 298 mg/100g FW) than the values reported by Hermann and Erazo (2000). The wild tuber-bearing taxon from Lima fell within the range of the *wayk'u* samples. Though no statistical comparisons could be done due to the wild tuber-bearing taxon sample size of one, it remains interesting that there is not initial evidence for a decrease in oxalate production from the wild taxon sampled to the domesticated accessions.

Subsequently, a larger study was conducted involving all 473 oca accessions in the CIP germplasm collection, with 14 accessions of *p'osqo* ocas and 459 accessions of *wayk'u* ocas. In this study we used pH as a proxy for total organic acid content in tubers. Two tubers from each accession of oca were measured. The mean pH values range from 4.615 to 5.025 in *p'osqo* tubers and 5.155 to 6 for *wayk'u* tubers **(Figures 2, 3)**, a highly significant difference ($p = 0.00000008245$) in a Welch's two-sample t-test with unequal variance. As a logarithmic measurement, these pH differences between oca use-categories reflect approximately a 10-fold difference in acid content. Ongoing research is addressing whether the observed difference in pH is due to oxalic acid, any of the other five acids present in the tubers, or a combination of all acids.

Traditional Use and Treatment of Oca and Effects on Acid Content

No research has been conducted to analyze the organic acid contents of oca before and after *khaya* processing (Hermann & Erazo, 2000). However, during *khaya* processing the "bitterness disappears to leave bland-tasting products that can be stored without refrigeration" (NRC, 1989). The drastic change in taste suggests that acid content is greatly decreased. Given that the oxalic acid present in oca is entirely soluble in water, the *khaya* process, which involves nearly a month of soaking followed by squeezing water out of the tubers, should significantly decrease oxalate content in oca tubers. Whether the other organic acids that are present in the tubers are also leached out during khaya processing is as yet unknown.

In contrast, research has addressed the effects of the sunning process on organic acid content in oca tubers. Hermann and Erazo (2000) compared quantities of oxalic, malic, tartaric, succinic, ascorbic, and glutaric acids in five accessions of *wayk'u* oca before and after sunning. Levels of glutarate and malate were the most greatly reduced by sunning, with an average reduction to 15.6% and 31.2% of original levels, respectively. Oxalate was only reduced on a dry matter basis to 47-95% of the pre-treatment levels, with a mean value of reduction to 74% of original levels. Levels of succinate also changed only slightly, with an average of 82.8% of the original content remaining after sunning. Tartarate did not change significantly, and on average showed a slight increase in acid levels due to sunning (a mean of 110% of original levels). These data overall "confirm that sunning…leads to considerable compositional changes, which improve the eating quality of oca" (Hermann & Erazo, 2000). However, these data may also suggest that the reported change in taste of oca due to post-harvest sunning may be due more to glutarate and malate levels than to oxalate. This highlights the importance of quantifying all five organic acids in our continuing studies.

Why Are Oxalate-Rich Varieties Still Grown?

First, the value of creating *khaya* for storage and flour purposes should not be underestimated. This allows families to stretch their oca crops across the winter months and guard against crop failure. However, *wayk'u* varieties can also be transformed into *khaya* for storage and flour, particularly some of the varieties with firmer flesh, so food storage alone cannot explain the maintenance of the varieties that must be processed prior to consumption.

There are a number of possible reasons for maintaining high-toxicity varieties in the crop system, including pest resistance, crop productivity, and adaptation to other challenges such as calcium-deficient soils, frost tolerance, and high-altitude environments. Elevated oxalate levels have been associated with pest inhibition (Yoshihara *et al*., 1979; Yoshihara *et al*., 1980; Franceschi & Nakata, 2005). Libert and Franceschi (1987) suggest that soluble oxalate inhibits microorganisms and sucking insects. In the Andes, the principal oca insect pest is a weevil whose larvae bore through the oca tubers (NRC, 1989). There has been no research evaluating the effect of oxalic or other organic acids on oca weevil larvae, although farmers say that ocas for processing are more resistant (Carlos Arbizu [CIP], unpublished data; E. Emshwiller, unpublished data).

VI. The Potential for Divergent Domestication: the need for data

Molecular data reveal a division between the two use categories of oca (Emshwiller, 2006; Emshwiller *et al.*, 2009), but it remains unknown whether this separation is due to disruptive selection or to separate origins of domestication. Traditional classification of the crop revolves around the use of the tubers and organic acid content seems to significantly affect the uses of different varieties. Whereas preliminary data strongly suggest that there is a difference in organic acid accumulation between *p'osqo* and *wayk'u* ocas, it is yet unknown if this pattern is consistent across different linguistic schemes, or which organic acid(s) may be responsible for the differences observed. Only by analyzing clonal replicates will it be possible to assert that clones consistently produce particular quantities of organic acids, and therefore, whether or not different organic acid profiles can be maintained (or removed) in the agricultural ecosystem (through selection by farmers).

Moreover, it is not understood which environmental variables influence the production and accumulation of organic acids in oca. Understanding the consistency of organic acid production is critical to designing future experiments that will address potential gene-by-environment interactions that could stimulate production and accumulation of acids in the plant.

Given that higher-acid varieties might have a different ploidy level than lower-acid accessions, oca could prove to be an interesting model crop for understanding the complicated effects of polyploidy.

Additionally, the underlying evolutionary mechanism that could be responsible for the divergent phenotypes we are observing remains unknown. There are two important evolutionary scenarios that should be evaluated: 1) disruptive (diversifying) selection and 2) multiple instances of domestication from separate progenitor species or populations. Though there is a binary distinction between *khaya* and *wayk'u* ocas, it is unknown if this linguistic distinction correlates with the distribution of the underlying biochemical trait (acid content). Additionally, multiple instances of domestication could create a similar domestication syndrome to that caused by disruptive selection.

As reviewed above, surprisingly little research exists to address these questions. We hope to provide future insight into the role of organic acids in oca's domestication. Similarly, we believe these questions may play an important role in understanding the lesser-told story of the few crops that display opposing domesticated phenotypes. Examining opposing crop phenotypes as a response to divergent selection pressures could help address such problems as the consequences of a changing climate, food productivity pressures, sustainable pest management in large-scale agriculture, and palatability.

Acknowledgements

The authors thank David Tay, Carlos Arbizu, and Francisco Vivanco for providing oca tubers for testing and for granting access to CIP's germplasm collection and trial fields; Cécile Ané for her indispensable help with statistical analyses; and Kandis Elliot for her help with our figures.

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Tables and Figures

Table 1. The Oxalate Content of Individual Foods (mg/kg of fresh weight).

Food	**Oxalic Acid Content**
Cabbage	3 to 35[a], 0 to 125[b]
Onion	1[a]
Potato	15[a], 20 to 141[b]
Rhubarb	440 to 1100[a], 460[b]
Beetroot	40.4[a], 121 to 450[b]
Spinach	500 to 9390[a], 320 to 1260[b]
Orange	8.7[a]
Banana	2.2 to 524[a]
Tomato	3.6 to 26.3[a], 5 to 35[b]
Tea (leaf)	219.2 to 1000[a], 300 to 2000[b]
Coffee (brewed)	154[a], 50 to 150[b]
Oca	**20.3 to 50.3[c], 92 to 213[d], 80 to 194[e], 306 to 539[f]**

[a]Duke, 1992; [b]Noonan & Savage, 1999; [c]King, 1988; [d]Ross, *et al.*, 1999; [e]Sangketkit, *et al.*, 2001; [f]Hermann & Erazo, 2000.

Figure 1. Total oxalate content in oca tubers, mg/100g FW. The x-axis represents the category of ocas. The y-axis represents concentration of oxalate in mg/100g FW. Group 1: ocas processed before consumption. Group 2: ocas traditionally not processed prior to cooking. Group 3: published data for ocas not processed prior to cooking (Ross, *et al.*, 1999). Group 4: one sample of a wild tuber-bearing taxon from central Peru.

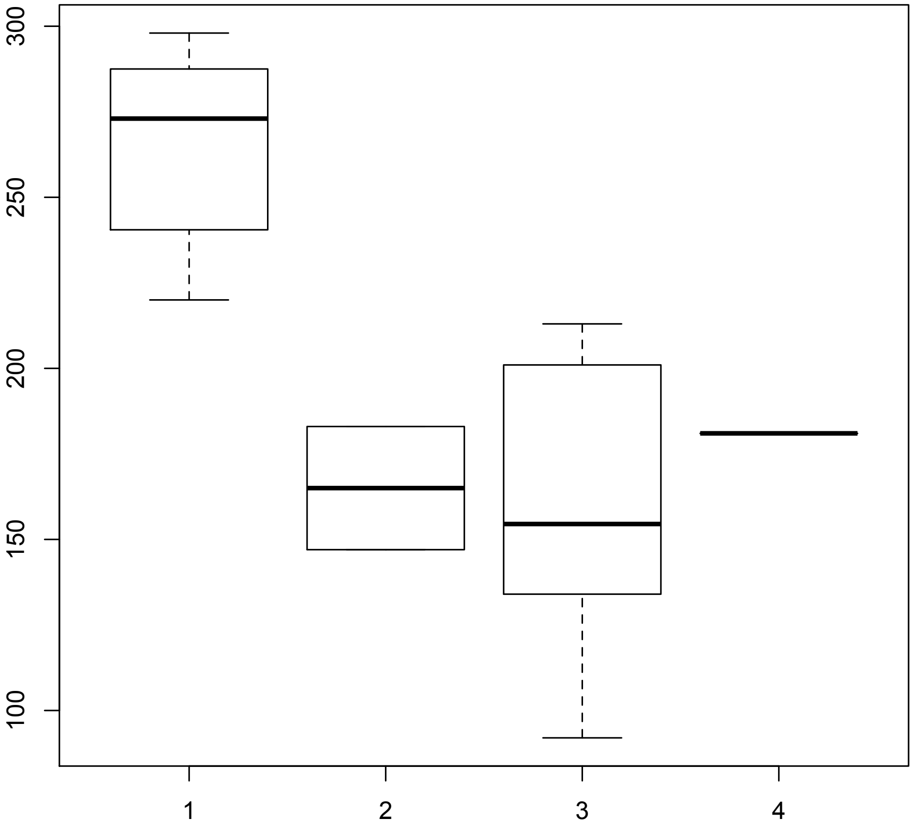


Figure 2. pH of Two Oca Use Categories. The x-axis represents the category of ocas. The y-axis represents pH. Group 1 consists of ocas traditionally processed before consumption. Group 2 contains ocas traditionally eaten without processing before cooking. Each data point represents the average of measurements of two tubers per plant.

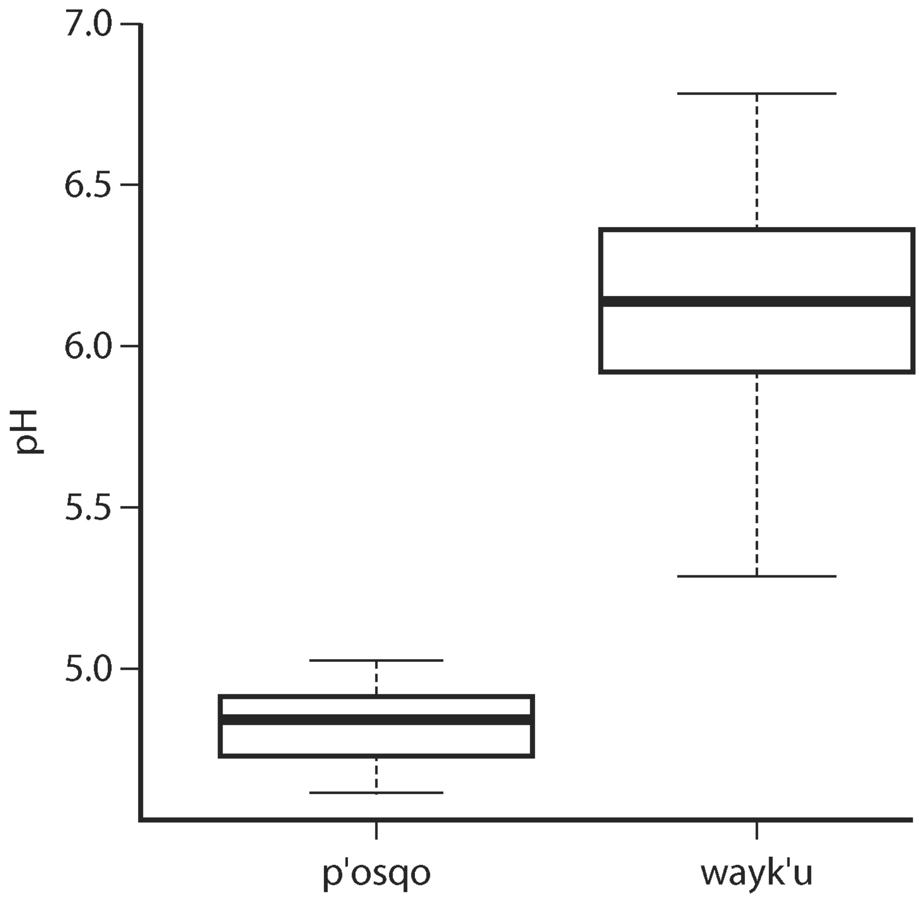
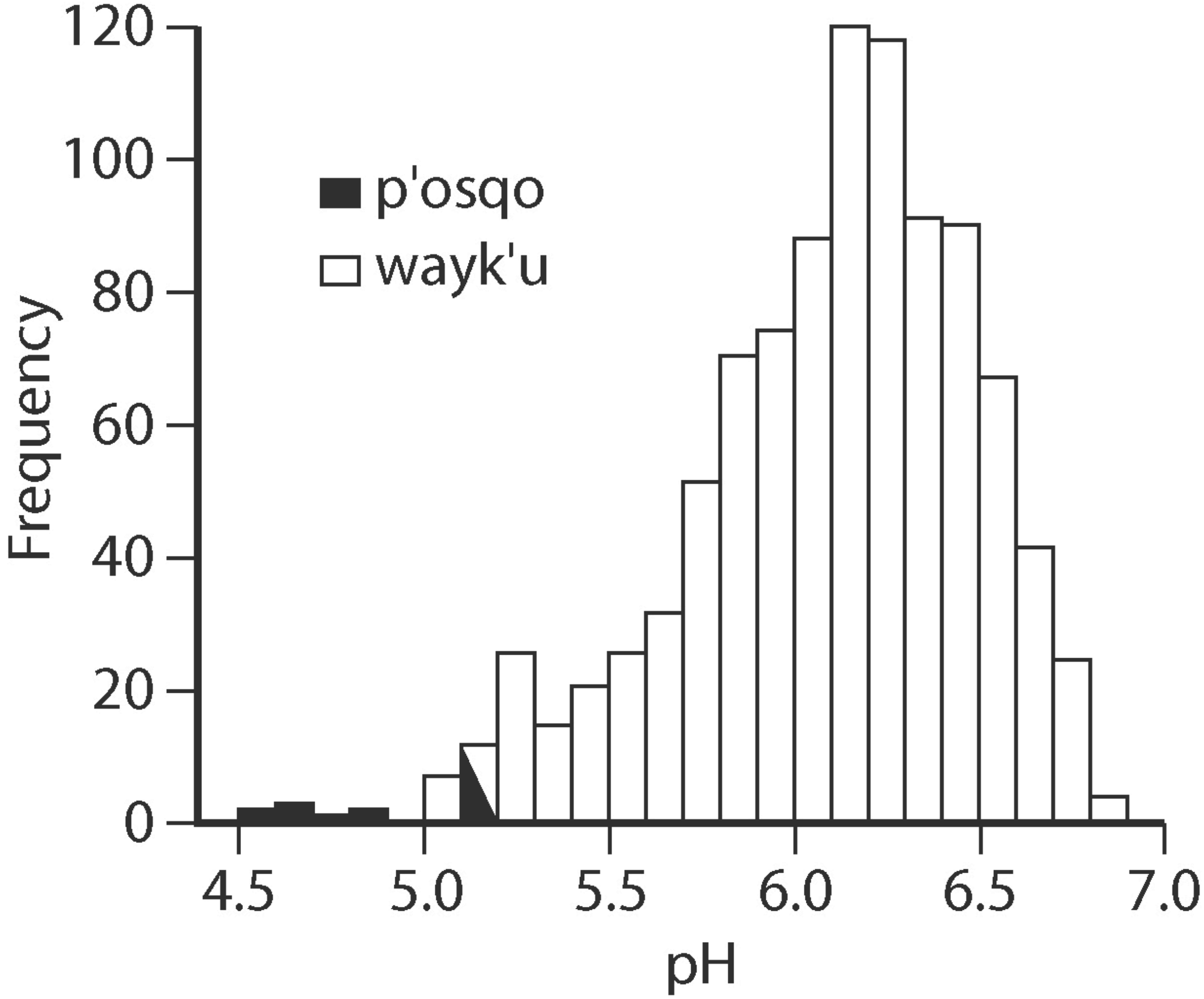


Figure 3. pH Distribution Among 473 Oca Accessions. The x-axis represents the pH range. The y-axis represents numbers of ocas in each pH range. Black bars represent ocas traditionally processed before consumption. White bars represent ocas traditionally eaten cooked without prior processing. Each data point represents pH measurement from a single tuber.



CHAPTER 3.

Bimodal Distribution of Oxalic Acid Production in Oca (*Oxalis tuberosa* Molina) Tubers

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Key words: traditional agriculture, use-categories, *khaya*, Quechua, Aymara

Abstract

Oca (*Oxalis tuberosa* Molina) tubers are known for their relatively high levels of oxalic acid, however quantities reported in the literature vary greatly, from 20.3 – 539 mg/100 g FW. We seek to resolve the inconsistencies in reported oxalic acid quantities in oca tubers by analyzing a broad sampling that represents both ocas that are processed before consumption and those that are cooked fresh. We grew 48 oca cultivars in 6 replicates in a randomized block design common garden experiment. Tuber oxalic acid content was quantified via HPLC analysis. Our results indicate a bimodal distribution of tuber oxalic acid content of 28.48 – 377.03 mg/100 g FW and 421.38 – 618.75 mg/100 g FW. The high-acid oca cultivars represent only 0.08% of our total sample and only 16.7% of ocas for processing, suggesting the need for more extensive field work to determine the range and dietary impact of the high-acid clones.

Introduction

Oxalis tuberosa Molina (Oxalidaceae), commonly known as “oca”, is an under-studied tuber crop native to the Andean highlands (de Azkue & Martínez, 1990; Emshwiller & Doyle, 2002; Flores et al., 2003). Oca is cultivated clonally via tuber plantings, though sexual reproduction remains possible (Trognitz et al., 1998). While generally low in market value, oca retains economic significance as an important rotation crop in the traditional potato cultivation system and as an important subsistence crop for rural farmers (National Research Council [NRC], 1989; Trivelli, 1996; Pastor et al., 2008). Additionally, some oca tubers are reserved for traditional processing into a dehydrated storable product called *khaya* (Hermann & Erazo, 2000; Emshwiller, 2006). Even though oca is cultivated by both Quechua- and Aymara-speaking agriculturalists, the separation of oca into two distinct use-categories (those for processing into *khaya* and those for cooking fresh) is consistent across cultures. *Khaya* is traditionally described as being made by soaking oca tubers in running water for a multiple weeks and freeze-drying them by alternating exposure to highland freezing night air and hot daytime sun (Johns, 1996), however variations in this technique have been reported, including using standing water instead of running water (Johns, 1996), which results in a fermentation process, or, particularly among Aymara people, the differentiation between *khaya* made without soaking in water and *khaya* made with soaking ("*uma-khaya"*) (E. Emshwiller, pers. obs.). In Quechua, the ocas for processing are called either *p'osqo* ("fermented" or "sour") or *khaya* while the ocas that are not processed are belong to the category *wayk'u* ("boiling") (Emshwiller, 2006). In Aymara, ocas for processing are called "*luk'i*" and those for fresh consumption "*q'eni*" ocas (Bradbury & Emshwiller, 2011).

Though no research has directly addressed the topic, it is often assumed that the ocas that are reserved for processing are less palatable than the ocas that are not made into *khaya* (Johns, 1990), in large part due to the meaning of the word *p'osqo* and due to field accounts whereby farmers identify *p'osqo* ocas by tasting them and describing them as sour (E. Emshwiller, pers. obs.). Hodge (1951) attributed the differences between traditional use-categories to oxalic acid concentrations in the tubers. Additionally, farmers often report that *p'osqo* ocas "grow better" and are more resistant to the common oca weevil (*Adioristidius tuberculatus* Voss) (E. Emshwiller, pers. obs., C. Arbizu, pers. comm.).

Recently, oca has garnered attention among nutritional chemists for the quantities of soluble oxalic acid in oca tubers, due to the potentially deleterious health effects resulting from elevated oxalic acid consumption (Ross et al., 1999; Albihn & Savage 2001a, b; Sangketkit et al., 2001, Bradbury & Emshwiller, 2011). However, reported quantities of oxalic acid in oca tubers vary greatly among authors, from 20.3 – 50.3 mg/100 g FW (King, 1988) to 306 – 539 mg/100 g FW (Hermann & Erazo, 2000), with the most widely-accepted quantities being 92 – 213 mg/100 g FW (Ross et al., 1999). The discrepancies among reported quantities of oxalic acid in oca tubers are of some concern due to the difficulty of inferring potential dietary consequences—is oca more similar in oxalic acid composition to cabbage and potato (0 – 125 mg/100 g FW and 20 – 141 mg/100 g FW, respectively) or spinach and rhubarb (320 – 1260 mg/100 g FW and 260 – 460 mg/100 g FW, respectively; Noonan & Savage, 1999). The measurements reported by Ross and colleagues (1999) are the most widely accepted because other sets of measurements are regarded as less accurate due to concerns about quantification methods (King, 1988; Ross et al., 1999) or cultivation techniques (Hermann & Erazo, 2000). However, these discrepancies could also be attributed to low samples sizes (n = 5 and 14 in Hermann & Erazo, 2000 and Ross et al, 1999, respectively), lack of measurement of clonal consistency in acid production, or non-representative sampling of cultivars among use-categories (e.g. lack of inclusion of *khaya* ocas). Indeed, it is likely that none of the tubers used by Ross and colleagues (1999) were from varieties traditionally processed into *khaya*, as all of their samples were gathered either at traditional Andean markets (where tubers for *khaya* are rarely sold prior to processing) or from New Zealand, where no *khaya* ocas are grown (Morgan, 2005).

Previously, we have shown a difference in pH among Quechua ocas from the two use-categories (Bradbury & Emshwiller, 2011). However, the underlying biochemistry responsible for the observed difference and whether or not the pattern is consistent across Quechua and Aymara folk classification systems remains unknown. Hermann and Erazo (2000) identified the presence of six organic acids in oca tubers: ascorbate, glutarate, malate, succinate, tartarate, and oxalate, all of which could contribute to overall pH difference. Additionally, the effect of processing on tuber acid content has not been studied, though Hermann and Erazo (2000) did show that post-harvest exposure of the tubers to sunlight results in significant decrease in glutarate (to 15.6% of pre-sunning levels) and malate (to 31.2% of pre-sunning levels) with less dramatic decreases in succinate and oxalate (to 82.8% and 74.4% of pre-sunning levels respectively) and a slight increase in tartarate (110% of pre-sunning levels).

We designed this study to address the inconsistencies in reported tuber oxalic acid quantities with respect to the following questions: 1) Are there different levels of oxalic acid in tubers from the two use-categories and, if yes, are these differences consistent across Quechua and Aymara cultures? 2) What is the level of clonal consistency of acid production? 3) To what extent does tuber oxalic acid content affect oca weevil infestations? 4) What quantities of oxalic acids are found in the dried product *khaya*?

Methods

Plant material

Tubers were obtained from the germplasm collections of El Centro Internacional de la Papa (International Potato Center; CIP) and the Peruvian Instituto Nacional de Innovación Agraria (National Institute of Agrarian Innovation; INIA). Samples were chosen to represent each of five use-cagetories: *p'osqo* (Quechua, processed), *khaya* (Quechua, processed), *luq'i*

(Aymara processed), *keni* (Aymara, not processed), and *wayk'u* (Quechua, not processed). Within each sample category, accessions were chosen to represent the range of recorded pH measurements, when available (Bradbury & Emshwiller, 2011). In total we grew 48 genotypes in 6 replicates (Table 1).

Accessions were planted September 2009 in 6 randomized blocks in a common garden field at the International Potato Center germplasm field in La Libertad, Junín Department, Peru at 3500 m elevation (Figure 1). The field was a southward-facing slope with an approximately 15° grade. Each randomized block contained five rows of eleven accessions, with each accession represented by one tuber during planting. Accessions were spaced approximately 1 m apart during planting. A single mixed manure application at planting was the only external nutrient supplementation applied during the course of the experiment. No pesticides were applied to the field. Tubers were harvested the first week of June 2010 and stored in paper bags in a cool, dark root cellar during the following six weeks during which the organic acid extractions were performed. Tubers from clonal replicates were stored and analyzed separately. Harvesting and extraction was done by field blocks in the following chronological order: Block 1A was harvested and extracted first, followed by Block 2B, Block 3A, Block 1B, Block 2A, and Block 3B. Harvesting and extraction followed this sequence to allow for statistical analyses to detect various potential effects on tuber acid content (described in greater detail below).

To quantify organic acids in dried *khaya* we used 5 whole dried *khaya* tubers from EE's collection. *Khaya* was purchased by E. Emshwiller in 1993 as part of her teaching collection from a local market in Bolivia.

Sample preparation

For each acid extraction, five tubers from each individual plant were chopped and mixed together to diminish the possibility that tuber-to-tuber differences in acid contents from the same plant could skew results. Acid extraction approximately followed the protocol outlined by Ross and colleagues (1999), but using 15 g of tuber in a final quantitative volume of 300 mL of 2M HCl to accommodate blending with a KitchenAid® handheld immersion blender instead of a top-drive homogenizer. Additionally, instead of filtering samples, we spun 2 mL aliquots at 13,000 rpms for 15 minutes before pipetting off 500 µL for analysis.

Acid extractions were shipped from Lima, Peru to Madison, WI under Material Transfer Agreement 0027-2011-INIA-SUDIRGEB-DIA/J and Phytosanitary Statement PS-2011-19.

Chemical analysis

We analyzed all extracted oca samples with high-pressure liquid chromatography (HPLC), using a Hewlett Packard 1090 HPLC equipped with a diode array detector set at 210 nm. We separated compounds with a Bio-Rad Aminex HPX-87H, 300 x 7.8 mm column (BioRad, Hercules, CA) held at 30°C using a mobile phase of 0.0175 M sulfuric acid run isocratically at 0.6 ml min^{-1}. with 20 µl injections. During the entire 20 min runs of both standards and samples we also collected UV spectral data from 190 to 400 nm. We quantified the amounts of the organic acids in samples with external standards of commercially available compounds (Sigma-Aldrich, St. Louis, MO) prepared to a concentration of 1 mg/µl. Our optimal standard concentration was determined via construction of a standard curve. To construct the standard curve, we dissolved 49.5 mg of anhydrous oxalic acid (Sigma-Aldrich) in 50.00 ml of 0.0125 M sulfuric acid (0.99 mg/ml) and further subdiluted to obtain concentrations of 0.0099, 0.0495, 0.099, 0.198, 0.396, and 0.495 mg/ml. We used the integrated peak areas of duplicate injections of all seven concentrations to construct a standard curve. The relationship between the oxalic acid standard concentration and peak area remained linear within the range of dilutions used.

Oca weevil infestation assessment

Oca weevil infestation was assessed at harvest and during sample preparation for organic acid analysis. Tuber weevil damage was scored on the following scale: 0 = no weevil damage visible; 1 = surface damage only; 2 = 1 – 2 weevil burrows; 3 = 3 – 4 weevil burrows; 4 = more than 4 weevil burrows. Each tuber for each plant was scored for damage then averaged to get a "mean damage score" per individual clonal replicate.

Degradation test

Unfortunately, due to unavoidable delays, acid extractions were kept refrigerated in the dark for ten months before HPLC analysis. Due to this, we conducted a separate acid degradation experiment using extractions from 9 tubers (4 yellow and 5 pink) purchased at Central Market grocery store in Austin, TX, USA. According to product labeling, tubers originated in New Zealand. These tubers were processed using the identical acid extraction method used for the Peru grown samples and organic acids were quantified immediately after extraction (Day 0), every week for six weeks, and at one year.

Statistical analysis

All statistical analyses were performed using R (R Core Development Team, 2011). To test for significant differences in oxalic acid production among use-category sample groups, we performed an Analysis of Variance (ANOVA) followed by a pairwise t-test. Additionally, variables such as genotype (clonality), field position (Figure 1), and tuber storage time post harvest prior to laboratory processing could have affected tuber acid content. To assess the effect of clonality and field position on tuber acid content we performed a linear mixed effects (LME) model in R with acid content as response variable, field position (block) as a fixed effect, and clone ID as a random effect. To determine the effect of storage time between harvest and processing on tuber acid content, we performed a linear mixed-effects regression with acid content as response variable, week since harvest as a fixed effect, and clone ID and field position (block) as random effects. To test significance of difference in weevil infestation between high-acid and low-acid ocas we used Welch's two-sample t-test.

Results & Discussion

Acid degradation experiment

The acid degradation experiment shows that whereas oxalic acid concentrations remained relatively stable over the course of one year, all other organic acids originally detected declined to undetectable levels by one year (Figure 2a & 2b). For this reason, we are only able to analyze oxalic acid content in our experimental samples. Interestingly, even though succinic and ascorbic acids have been previously detected in oca tubers (Hermann & Erazo, 2000), we did not detect either of these acids in our store-bought oca tubers (Figure 2a). This suggests that there may be significant inter-varietal differences in acid production of the organic acids that we were unable to quantify and highlights the need for future work in this area. This could also be due to unquantifiable post-harvest acid degradation in the store-bought tubers used for our degradation experiment. Since they originated in New Zealand and were sold in a supermarket in Texas, there is no way to know how much time had elapsed between harvest and initial extraction time.

Are there different levels of oxalic acid in tubers from the two use-categories and, if yes, are these differences consistent across Quechua and Aymara cultures?

Our results of total oxalic acid content for each replicate of each accession indicate a much wider range of values than previously reported, from 28.5 – 618.8 mg/100 g FW (Table 2).

However, the distribution of oxalic acid quantities is strongly bimodal with two non-overlapping ranges of 28.5 – 377.0 mg/100 g FW and 421.4 – 618.8 mg/100 g FW (Figure 3). The high-acid oca cultivars all originate from the Cusco Department, Peru, represent only a very small proportion of our sample set (0.08%, 4 accessions out of 48), and correlate with only one of the 3 "processed" sets of oca cultivars (Figure 4a). Even though only 4 out of 10 *p'osqo* accessions produced elevated quantities of acid, this use-category is significantly different from the others ($p < 0.001$, F = 32.5, df = 4) as assessed with ANOVA and pair-wise t-test (*p'osqo* vs. *keni*, $p <$ 2e-16; *p'osqo* vs. *wayku*, $p < 0.001$, *p'osqo* vs. *luq'i,* $p < 0.001$; *p'osqo* vs. *khaya*, $p < 0.001$). When we ignore cultural use-category designation and simply test between the high-acid and low-acid cultivars, we see a highly significant difference ($p < 0.001$, t = -26.5, df = 24.1) indicative of the bimodal distribution of oxalate production in oca tubers visualized in Figure 3.

Our results do not outline a scenario as clear-cut as we envisioned in our original questions, wherein we imagined that use-category would either be deterministic for oxalic acid content or not. Use-category appears to be a poor predictor for oxalic acid content in oca tubers, except for ocas known as *p'osqo*, of which 40% of the accessions included in this study produce high levels of oxalic acid in the tubers. The extremely low frequency of high-acid ocas (0.083% of our sample and 16.7% of our sample of ocas for processing) raises several questions about the origins of *khaya* production in Quechua and Aymara cultures. Whereas with other crops that display a similar cultural food use pattern, such as bitter manioc (which produces toxic amounts of cyanide; McKey & Beckermann, 1996) and bitter potato (which produces toxic glycoalkaloids; Johns, 1996), in which the varieties reserved for processing consistently produce elevated levels of a secondary toxin, the same cannot be said for oca. Indeed, almost 85% of the ocas included in this study that are used for processing do not "need" to be detoxified. The rare but very consistently high-acid oca clones suggest that oxalate content in oca tubers has played a more complex role in shaping cultural food practices of traditional Andean farmers than a simple one-to-one correlation of use-category with acid production. Certainly the apparent lack of sour ocas from Aymara agriculturalists highlights the need for expanded oca collections from Bolivian Aymara households as well as the need to quantify glutarate, succinate, malate, tartarate, and ascorbate in tubers from the different use-categories. Perhaps one of the most curious results of this study is the overall lack of biological justification for the elaborate "detoxifying" processing of fresh oca into *khaya*.

While our data do not fully resolve questions surrounding the biological basis for oca's cultural use-categories, this study does make strong headway towards resolving the existing confusion over conflicting reports of oxalate content in oca tubers. Notably, there is a significantly larger range of oxalate content in oca tubers than previously reported in a single study. Additionally, the range of oxalate content in our sampling encompass the entirety of the published values by King (1988), Ross and colleagues (1999), and Hermann and Erazo (2000). This demonstrates the value of using an ethnobotanical perspective when sampling cultivated plants, as our wider sampling based on broader ethnographic characteristics has resulted in a significantly more accurate representation of oxalate quantities in oca tubers. This helps resolve nutritional concerns related to oca's oxalate content. While the upper range of the low-acid ocas approaches the lower range of reported levels of oxalate in some high-oxalate foods such as spinach and rhubarb (Noonan & Savage, 1999), the overall mean oxalic acid content for low-acid ocas (162.3 mg/100g FW) is more akin to foods such as cabbage and potato, which are not targeted as significant contributors of oxalate to the diet (Noonan & Savage, 1999). The evidence presented for post-harvest degradation of organic acids during exposure to sunlight by Hermann and Erazo (2000) further lowers the probability of deleterious effects of oxalate resulting from oca consumption among traditional Andean agriculturalists. Interestingly, the glutaric acid, which showed the greatest decline in Hermann and Erazo's study (2000), also showed the greatest decline in our acid degradation trial (Fig 2a). Farmers in New Zealand may consider adopting the practice of "sunning" their ocas to decrease nutritional load of oxalate in New Zealand-grown oca.

What is the level of clonal consistency of acid production?

When analyzed together in our linear mixed-effects model, 85.4% of the variability in tuber oxalic acid content is attributed to genotype (difference between clones) and only 14.6% is attributed to the environment (difference between replicates of each clone) (Figure 4a). However, when we analyze the high-acid and low-acid clones groups separately, we see clonal consistency in acid production sharply decrease, with only 46.5% and of the variability attributed to genotype in the low-acid clones and almost 100% of the variability attributed to the environment in the high-acid clones (Figures 4b & 4c). While it appears that clonal variability is much higher in the high-acid genotypes, with a sample size of only four genotypes, the power of our statistical model is undermined to the point where we cannot draw strong conclusions about the interplay between genotype and environment relative to tuber oxalate content in high-acid ocas. Together, these data suggest a bimodal distribution of oxalic acid production in oca tubers, with high variability among clones within each distribution but consistent limits on oxalate production for each group (high-acid vs. low-acid).

Additionally, field position has a significant effect on tuber oxalic acid content within genotypes, with oxalic acid content significantly higher in clonal replicates grown in blocks 3A and 3B at the bottom of the slope, producing on average 79.3 mg of oxalic acid/100 g FW and

92.14 mg of oxalic acid/100 g FW more than the same clones grown in block 1A, respectively. While this suggests that there are important contributing environmental variables to oxalic acid production in oca tubers, the observed differences between blocks could not account for the observed differences between high-acid and low-acid ocas (difference of means is 380.86 mg oxalic acid/100 g FW). The length of time between tuber harvest and acid extraction also significantly affected tuber oxalic acid content, with oxalic acid content increasing on average by 22.66 mg/100 g FW per week, with a total difference between Week 1 and Week 4 of 90.64 mg/100 g FW.

In relation to the evolution of the crop, the strict divide between sweet clones and sour clones suggests that to some degree oxalate content is a trait for which (or against which) farmers could select. This has important implications for the history of domestication of the crop, which is still unresolved. Currently we believe oca to be an allopolyploid with the possibility of being an auto-allopolyploid (Emshwiller & Doyle, 2002) with multiple polyploidization events in a complicated history. Our data show a phenotypic pattern that tentatively maps onto a genetic one (clonality) that could impact how we understand the progression of this species from wild to domesticated and strongly suggests that oca is another crop in a minority group of crops that has genotypes that are high-toxin and some that are low-toxin, perhaps representing an instance of divergent domestication (Bradbury & Emshwiller, 2011). Further research into underlying genetic patterns which may correlate with the observed phenotypic pattern is certainly warranted.

Additionally, while there were likely differences in nutrient content, water availability, and sunlight across the slope of the mountain where our common garden was planted, by-and-large all of the ocas in this study were grown in a very similar environment. This suggests that micro-differences in environment may strongly contribute to differences in oxalic acid production in oca tubers. Moreover, the physiological function of organic acids in oca tubers, which cumulatively can easily exceed 2 g/100 g of tissue FW, may be highly susceptible to minute variations in soil chemistry. Organic acids have been shown to be critical for detoxification of aluminum ions, both as external rhizophere exudates (Ma, 2000; Ryan et al., 2001) and as metal chelating agents in the cytosol (Ma, 2000). Ma (2000) found that malic, citric, and oxalic acids all function to alleviate aluminum toxicity in acidic soils whereas Ryan and colleagues (2001) demonstrated that succinic acid contributes to root anion exudation. These studies highlight potential soil chemistry variables that should be investigated in relation to tuber organic acid content, particularly nutrient availability (presumably higher at the bottom of the slope as organic matter leeches downhill), temperature differences (potentially warmer at the bottom of the slope), differences in sunlight availability (though with a southward facing slope this may not have been a key factor in our data), and differences in soil chemistry, especially soil aluminum (Al^{3+}) and calcium (Ca^{2+}) content. Moreover, we have no data that address what differences in oxalate quantity may exist among tubers grown from the same individual plant, which would be very important towards helping us assess sources for variability in tuber oxalate content in oca.

To what extent does tuber oxalic acid content affect oca weevil infestations?

Tuber oxalic acid content seems to have no effect on severity of weevil infestation with a mean damage score for all high-acid replicates of 1.327 and a mean damage score for all low-acid replicates of 1.466 (p = 0.283, t = -1.1, df = 17.0). It should be noted, however, that there are certainly other agricultural pathogens, in particular a water mold “Black Rot” (*Lasiodiplodin theobromae*) and a *Nepovirus* “Arracacha virus B”, neither of which were included in this study, and against which oxalic acid might be protective.

What quantities of oxalic acid are found in the dried product khaya*?*

Extracts from the five dried *khaya* samples contained no detectable organic acids. We should note first that our available samples of *khaya*, being approximately 20 years old, cannot be considered fully equivalent to *khaya* that has been freshly-made. However, the complete lack of organic acids present in our *khaya* tubers provides some evidence that *khaya* processing functions to eliminate organic acids, including oxalate, and strongly supports the need for future studies of the biochemical effects of *khaya* processing that directly compare pre-processing and post-processing levels of organic acids. Additionally, further studies of the effects of *khaya* processing are merited given that the mean oxalic acid production for high-acid ocas (543.2 mg/100g FW) exceeds even the quantities of oxalate found in spinach and rhubarb, two foods often targeted for elimination from the diets of patients suffering from chronic kidney stones (Noonan & Savage, 1999). To ensure that the levels of oxalate found in sour ocas do not present a dietary or nutritional concern to traditional Andean agriculturalists we should confirm the efficacy of oxalate removal during *khaya* preparation, though our current results suggest that deleterious effects from oxalate consumption from processed sour ocas are highly unlikely.

However, given the results at hand, the question arises *why* the persistence of *khaya* when we see so little evidence for widespread cultivation of ocas requiring oxalic acid removal and how this processing technique arose in the first place if not driven by the need for detoxification. First, there remains the possibility that sour ocas are significantly more widespread than our sampling suggests, and certainly the discovery of high-acid oca cultivars underscores the importance of greatly expanded field collections of oca. Additionally, it is distinctly possible that the sour ocas were previously more widespread than they are now, which could account for the advent of *khaya* processing. However, there may be an explanation completely unrelated to oxalic acid, and that is the critical importance of food storage for food security in the region. Being able to store *khaya* indefinitely to guard against crop failure, unexpected food loss or spoilage, and to provide storable product to eat during the dry season could certainly be enough to merit making *khaya*. Additionally, since the *khaya* process is almost identical to that used to make detoxified bitter potato (*chuño*), it stands to reason that the process itself could have easily been developed to render potato edible and then also applied to other tuber crops in the interests of food security. Yet, the existence of *some* "toxic" ocas renders this explanation incompletely satisfactory and highlights yet another mystery in the history of oca.

Conclusions

This study serves both to resolve existing conflicts in reported oxalic acid quantities in oca tubers as well as raise additional targeted inquiries into some of the mysteries currently surrounding oca's evolutionary history and associated cultural food practices. Specifically, our results indicate that we do not need to be concerned about deleterious dietary effects arising from oxalate content in low-acid genotypes of oca, particularly given the strongly consistent separation between high-acid and low-acid genotypes, for which clonal replicates always produced oxalic acid contents either above (high-acid) or below (low-acid) a given threshold. Farmers can reliably maintain low-acid oca cultivars without concern that these cultivars could unexpectedly begin producing deleterious amounts of oxalic acid.

Additionally, the bimodal distribution of oxalate production in oca tubers is an important contribution to the ongoing efforts to resolve the evolutionary history of oca domestication, as it both establishes the ability of Andean farmers to select for or against elevated oxalic acid production in oca tubers and provides a phenotypic divide that may correlate with observed genetic divisions among oca varieties (Emshwiller et al., 2009). Yet these data also highlight the many questions that remain regarding possible ecological and physiological functions of organic acids in oca tubers and serve as a strong justification for continued research on the evolutionary history, physiology, and ethnobotany of *Oxalis tuberosa*.

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Figures and Tables

Table 1. Collection information and sampling distribution by use-category.

Accession	Folk name	Use-category	Variable Group	Germ. Coll.	Country	Date	Site	Lat.	Long.
MU 074	Janq'i luq'i	luq'i	Aymara processed	CIP	Peru	7/31/91	Puno Yunguyo Cuturapi	-16.2667	-69.1667
MU 076	Janq'u luq'i	luq'i	Aymara processed	CIP	Peru	7/31/91	Puno Yunguyo Cuturapi	-16.2667	-69.1667
ARV 5356	Qank'o k'eni	k'eni	Aymara not processed	CIP	Peru	8/20/93	Puno Chucuito Apillani	-16.2199	-69.0599
MU 078	Q'illu luq'i	luq'i	Aymara processed	CIP	Peru	6/28/91	Puno Chucuito Chimu Alto Ayriwa	-16.4666	-69.0999
MU 090	Janq'u luq'i	luq'i	Aymara processed	CIP	Peru	7/31/91	Puno P. Yunguyo Chambi Kimsacruz (Yunguyo)	-16.2667	-69.0667
MU 017	Ch'iyara qui–i	k'eni	Aymara not processed	CIP	Peru	7/31/91	Puno P. Yunguyo ? Yunguyo ?	-16.2667	-69.1667
MH 336a	Oca	wayk'u	Quechua not processed	CIP	Bolivia	9/21/89	Potos’ P. Linares Puna	-19.8500	-65.5999
ARB 5235	K'ello panti	wayk'u	Quechua not processed	CIP	Peru	5/27/93	Cusco Calca Amaru	-13.3699	-71.8099
ARV 5350	Qank'o luck'e	luq'i	Aymara processed	CIP	Peru	8/20/93	Puno Chucuito Acari	-16.3833	-69.1999
ARV 5355	Qank'o luck'i	luq'i	Aymara processed	CIP	Peru	8/20/93	Puno Chucuito Acari	-16.3833	-69.1999
AJB 5300	Chaucha amarilla	wayk'u	Quechua not processed	CIP	Peru	7/10/93	Cajamarca Chota Choctapata	-6.5399	-78.5999
ARM 5035	Oca rosada	wayk'u	Quechua not processed	CIP	Peru	5/12/91	Piura Piura Piura	-5.54080	-80.8779
MH 335	Oca	wayk'u	Quechua not processed	CIP	Bolivia	9/20/89	Oruro P. Cercado Oruro	-17.3999	-66.1667
ARB 5052	Blanca amarilo pinto	wayk'u	Quechua not processed	CIP	Peru	6/3/91	Cajamarca Cajamarca Chamis	-7.1300	-78.5500

ARV 5352	Cheara k'eni	k'eni	Aymara not processed	CIP	Peru	8/20/93	Puno Chucuito Acari	-16.3833	-69.1999
ARV 5357	Cheara luck'i	luq'i	Aymara processed	CIP	Peru	8/20/93	Puno Chucuito Unicachi	N/A	N/A
ARB 5234	Kusi pati	wayk'u	Quechua not processed	CIP	Peru	5/27/93	Cusco Calca Amaru	-13.3699	-71.8099
AGM 5090	Oca pi–a	wayk'u	Quechua not processed	CIP	Peru	8/22/92	Cajamarca Chota Rojaspampa	-6.5500	-78.6333
MU 070	Janq'u luq'i	luq'i	Aymara not processed	CIP	Peru	7/31/91	Puno Yunguyo Sanquira	-16.2999	-69.0500
AJA 5252	Blanca	wayk'u	Quechua not processed	CIP	Peru	6/6/93	Cajamarca Cajamarca Puylucana Alta	-7.13000	-78.4400
AGM 5080	Oca mulla grande	wayk'u	Quechua not processed	CIP	Peru	8/22/92	Cajamarca Chota Rojaspampa	-6.5500	-78.6333
O 222 85	Oca q'ene	k'eni	Aymara not processsed	CIP	Peru	7/26/85	Tacna Tarata Tarucachi	-17.5333	-70.0167
MU 069	Wila luq'i	luq'i	Aymara processed	CIP	Peru	7/31/91	Puno Yunguyo Sanquira	-16.2999	-69.0500
0 193 85	Rucky oca	luq'i	Aymara processed	CIP	Peru	7/23/85	Puno Lampa Collana	-15.5832	-70.3666
10-02-06	N/A	khaya	Quechua processed	INIA	Peru	2005	Santa Rosa de Monte Azul, Huancapallac/Kichki, Huánuco, Huánuco	-09.8700	-076.4504
01-01-08	N/A	khaya	Quechua processed	INIA	Peru	2005	Ccochapampa, Laria, Huancavelica, Huancavelica	-12.5496	-075.0535
14-01-33	N/A	k'eni	Aymara not processsed	INIA	Peru	2005	Huisca, Tambo, La Mar, Ayacucho	-12.8354	-073.9365
14-04-16	N/A	k'eni	Aymara not processsed	INIA	Peru	2005	Husmay, Tambo, La Mar, Ayacucho	-12.8819	-073.9134
14-06-21	N/A	k'eni	Aymara not processsed	INIA	Peru	2005	Husmay, Tambo, La Mar, Ayacucho	N/A	N/A
15-01-01	N/A	khaya	Quechua processed	INIA	Peru	2005	Anco, Anco, La Mar, Ayacucho	-13.1166	-073.6898
16-01-22	N/A	k'eni	Aymara not	INIA	Peru	2005	Pacobamba, Anco, La	-13.0751	-073.7859

			processsed				Mar, Ayacucho		
16-02-22	N/A	k'eni	Aymara not processsed	INIA	Peru	2005	Pacobamba, Anco, La Mar, Ayacucho	-13.0751	-073.7859
16-06-12	N/A	k'eni	Aymara not processsed	INIA	Peru	2005	Pacobamba, Anco, La Mar, Ayacucho	N/A	N/A
17-01-16	N/A	k'eni	Aymara not processsed	INIA	Peru	2005	Huayllay, Luricocha, Huanta, Ayacucho	-12.8092	-74.2333
26-01-57	N/A	p'osqo	Quechua processed	INIA	Peru	2005	Patacancha, Ollantaytambo, Urubamba, Cusco	-13.1589	-072.1892
26-01-58	N/A	p'osqo	8x p'osqo	INIA	Peru	2005	Patacancha, Ollantaytambo, Urubamba, Cusco	-13.1589	-072.1892
26-02-42	N/A	p'osqo	Quechua processed	INIA	Peru	2005	Patacancha, Ollantaytambo, Urubamba, Cusco	-13.1585	-072.1895
26-03-05B	N/A	p'osqo	Quechua processed	INIA	Peru	2005	Patacancha, Ollantaytambo, Urubamba, Cusco	-13.1713	-072.1911
26-04-16	N/A	p'osqo	Quechua processed	INIA	Peru	2005	Patacancha, Ollantaytambo, Urubamba, Cusco	-13.1746	-072.1940
26-07-12	N/A	p'osqo	Quechua processed	INIA	Peru	2005	Patacancha, Ollantaytambo, Urubamba, Cusco	-13.1745	-072.1922
26-08-49	N/A	p'osqo	Quechua processed	INIA	Peru	2005	Patacancha, Ollantaytambo, Urubamba, Cusco	-13.1717	-072.1911
26-09-03	N/A	p'osqo	Quechua processed	INIA	Peru	2005	Patacancha, Ollantaytambo, Urubamba, Cusco	-13.1720	-072.1904
32-01-06	N/A	khaya	Quechua processed	INIA	Peru	2005	Pitumarca, Pitumarca, Canchis, Cusco	-13.9746	-071.3810
33-03-05	N/A	p'osqo	Quechua processed	INIA	Peru	2005	Pitumarca, Pitumarca, Canchis, Cusco	-13.9743	-071.3825

34-02-17	N/A	p'osqo	Quechua processed	INIA	Peru	2005	Puma Orqo, Ccatcca (Qatqa), Quispicanchi, Cusco	-13.5739	-071.5983
DP 01 14	N/A	khaya	Quechua processed	CIP	Peru	6/3/91	Cajamarca Cajamarca Chamis	-7.1300	-78.5500
DP 01 36	N/A	khaya	Quechua processed	CIP	Peru	6/3/91	Cajamarca Cajamarca Chamis	-7.1300	-78.5500

Table 2. Oxalic acid (mg/100 g FW) in oca tubers. Measurements for each surviving replicate from each field block. The tubers from Block 2 shaded in dark grey were excluded from the study due to time constraints in the field. Overall mean oxalic acid production for each genotype is reported in the final column.

Oxalic Acid mg/100g FW							
Accession	**Block 1A**	**Block 1B**	**Block 2A**	**Block 2B**	**Block 3A**	**Block 3B**	**Mean**
M 2029	50.40	--	--	--	110.46	--	80.43
26-01-57	91.21	--	--	--	189.09	201.87	160.72
26-02-42	421.38	482.96	--	--	534.42	--	479.58
26-03-05B	376.87	533.36	--		533.42	558.52	500.54
34-02-17	427.57	--	--	--	565.77	608.95	534.10
26-04-16	65.80	202.61	--	--	206.25	225.88	175.14
26-07-12	355.47	487.97	--	--	517.55	602.18	490.79
26-09-03	--	--	--	--	466.46	521.85	494.15
33-03-05	85.93	162.25	--	--	160.59	173.22	145.50
15-01-01	56.16	138.37	--	--	151.57	153.85	124.99
10-02-06	81.28	208.27	--	--	--	282.16	190.57
01-01-08	177.99	268.92	--	--	240.03	244.09	232.76
DP 01 14	104.01	--	--	--	171.98	--	137.99
DP 01 36	161.10	146.14	--	--	154.57	168.95	157.69
14-06-21	66.94	--	--	--	175.12	174.37	138.81
14-04-16	126.69	--	--	--	158.39	146.88	143.99
14-01-33	90.43	--	--	--	185.31	222.54	166.09
16-06-12	48.96	--	--	--	126.76	135.30	103.67
16-01-22	75.50	130.41	--	--	--	157.35	121.09
17-01-16	--	124.41	--	--	--	--	N/A
16-02-22	130.43	--	159.20	--	146.56	215.13	162.83
26-08-49	226.36	244.77	211.22	--	258.25	271.56	242.43
32-01-06	82.09	172.25	125.02	--	166.27	186.67	183.08
26-01-57	166.41	175.46	154.30	--	183.76	329.72	201.93
0 193 85	122.98	--	203.73	103.26	215.12	243.34	177.69
MU 069	100.85	246.39	205.96	141.78	211.26	218.38	187.44
0 222 85	83.36	119.16	172.79	66.32	156.35	122.24	120.04
AGM 5080	80.36	115.40	139.64	118.32	137.23	--	118.19
AJA 5252	167.54	188.06	248.91	--	165.08	--	192.40
MU 070	110.33	114.14	110.85	69.76	118.67	126.02	108.29
AGM	77.13	--	100.69	--	97.90	138.44	103.54

5090							
ARB 5234	145.08	154.79	--	108.09	189.29	168.73	153.19
ARV 5357	208.31	282.54	215.25	299.18	289.60	281.01	262.65
ARV 5352	293.92	322.72	272.02	285.81	299.77	--	294.85
ARB 5052	140.27	--	115.78	110.62	207.92	--	143.65
MH 335	71.32	84.35	--	141.07	143.75	--	110.12
ARM 5035	132.37	--	212.47	--	268.99	197.26	202.77
AJB 5300	125.56	136.15	--	158.65	158.29	166.06	148.94
ARV 5355	61.15	146.16	--	28.48	--	--	78.60
ARV 5350	174.50	249.02	--	296.64	376.82	377.03	294.80
ARB 5235	108.94	240.18	--	197.52	235.55	203.19	197.08
MH 336a	89.72	126.30	--	158.87	187.16	215.40	155.49
MU 017	79.23	91.90	124.87	153.63	--	--	112.41
MU 090	74.65	120.32	158.66	96.56	--	--	112.55
MU 078	59.03	175.00	--	--	260.04	--	164.69
ARV 5356	68.06	149.70	99.49	72.73	204.30	182.42	129.45
MU 076	64.44	123.25	--	163.30	165.71	172.11	137.76
MU 074	249.07	142.87	--	103.44	47.39	154.87	139.53

Figure 1. Schematic of common garden showing randomized block design and approximate slope of hillside.

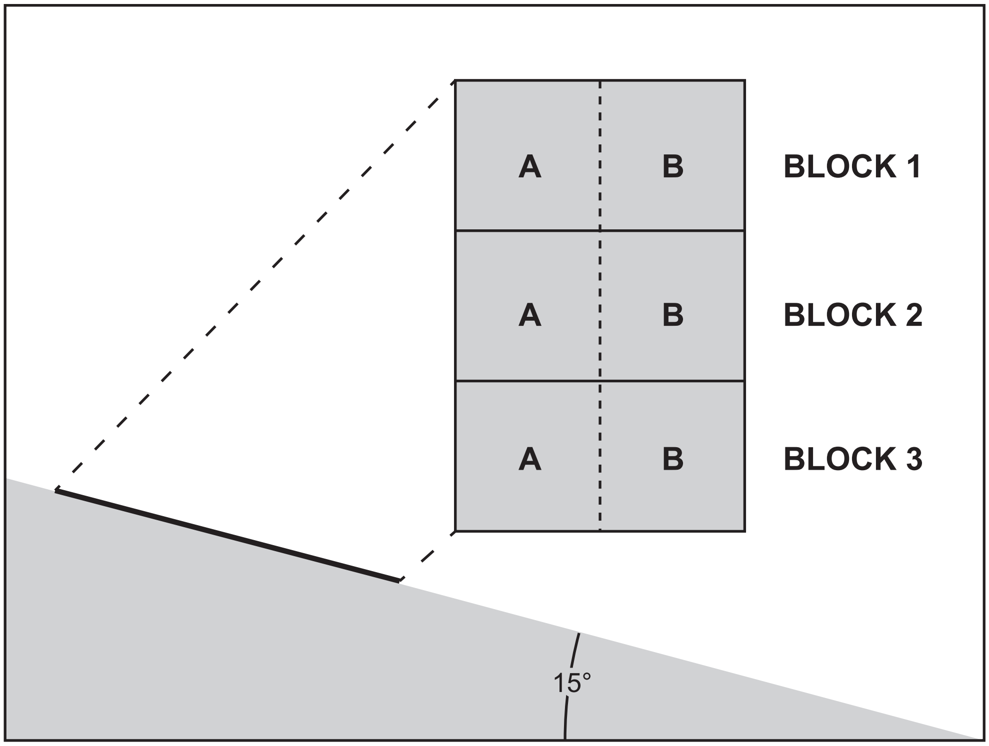


Figure 2a. Mean post-extraction degradation of organic acids in 2M HCl stored at 4º C in the dark. Degradation curves are shown for each of the four organic acids originally detected at Week 0. Each curve represents the mean acid content across all 9 extractions.

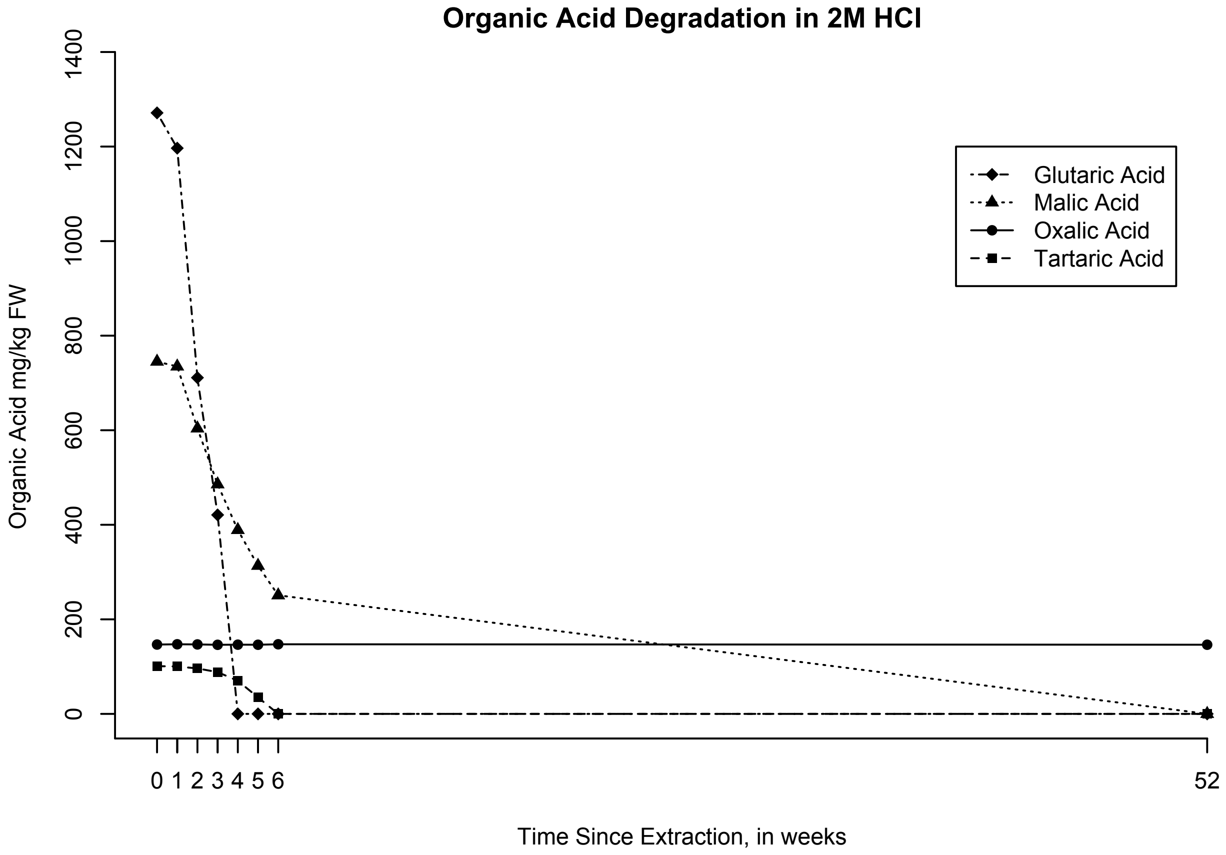


Figure 2b. Post-extraction degradation of oxalic acid in 2M HCl from nine *O. tuberosa* tuber extractions.

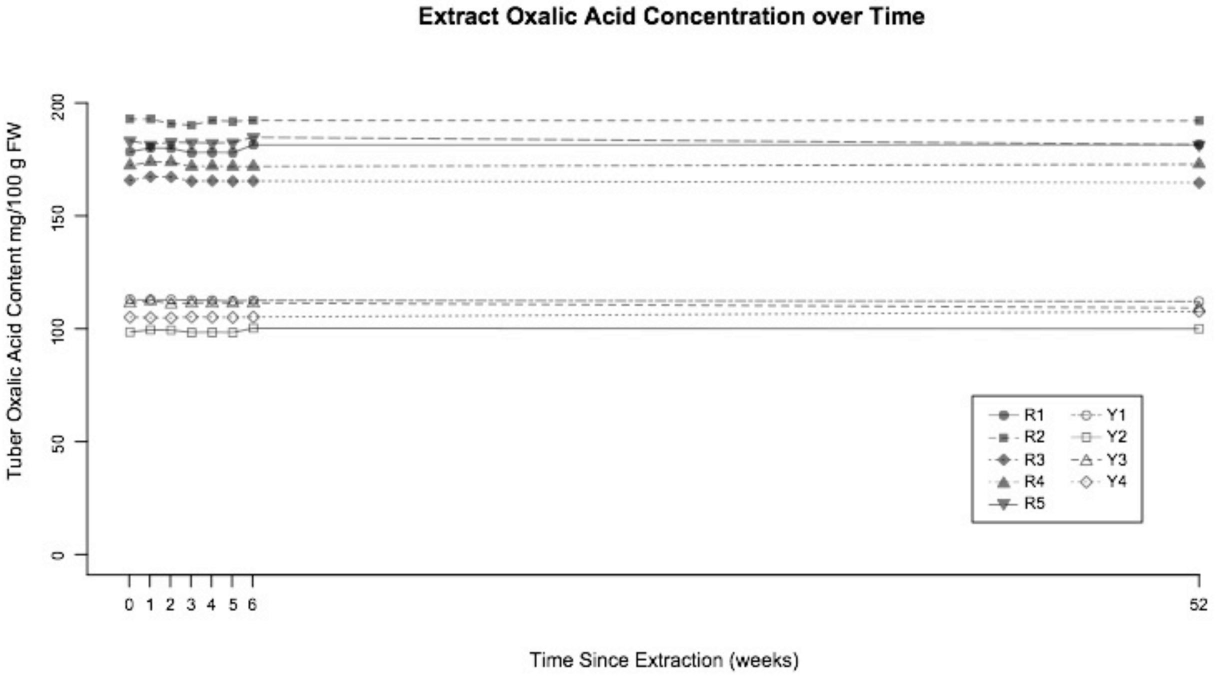


Figure 3. Histogram of oxalic acid (mg/100 g FW) in oca tubers. Each data point represents a single individual plant. Histogram bars are shaded to indicate which use-categories of tubers were found in each range. Acid ranges containing only tubers used for processing are shaded black; acid ranges containing only tubers not used for processing are white; acid ranges containing both use-categories are striped.

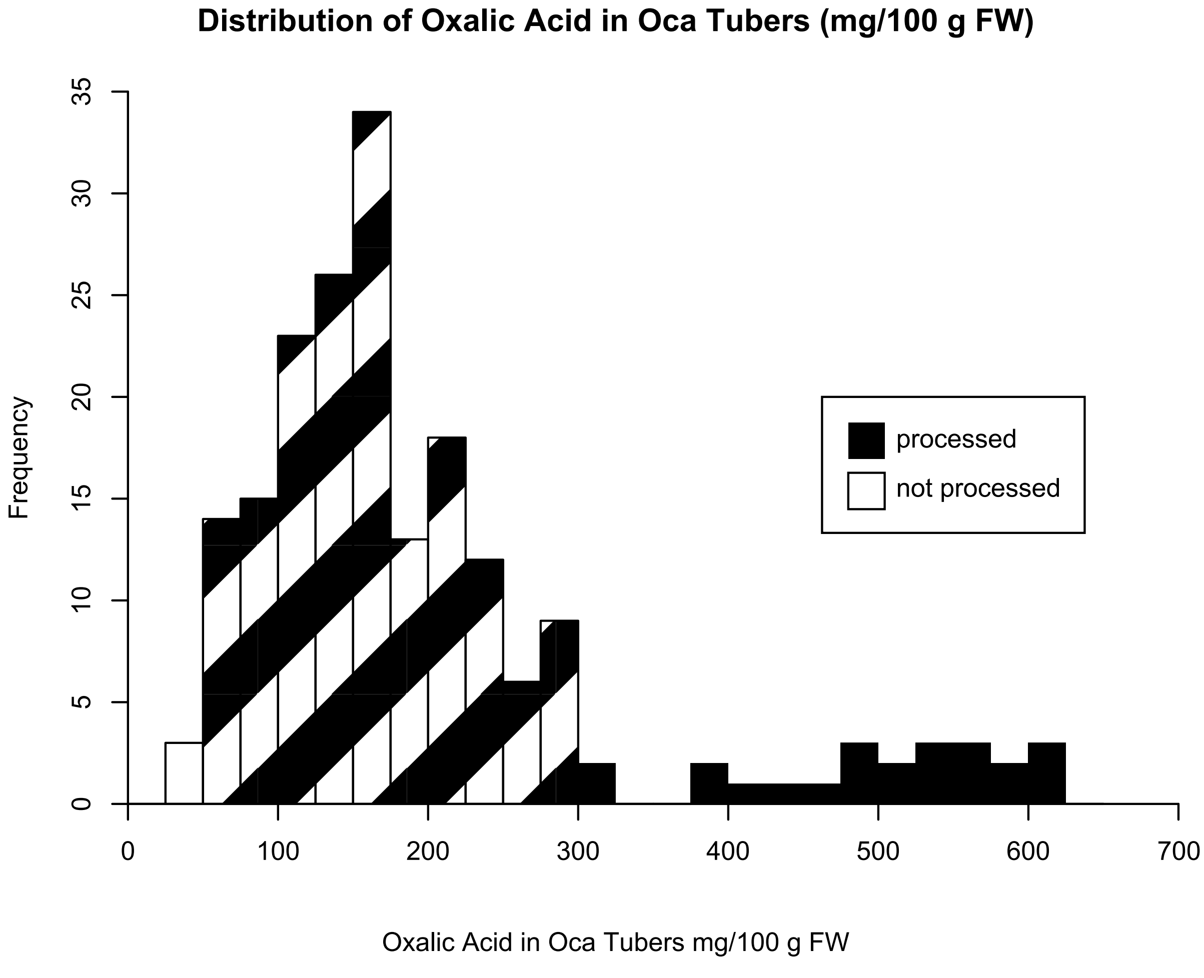


Figure 4. Stripchart of oxalic acid (mg/100g FW) in oca tubers according to use-category. Each data point represents a single individual plant.

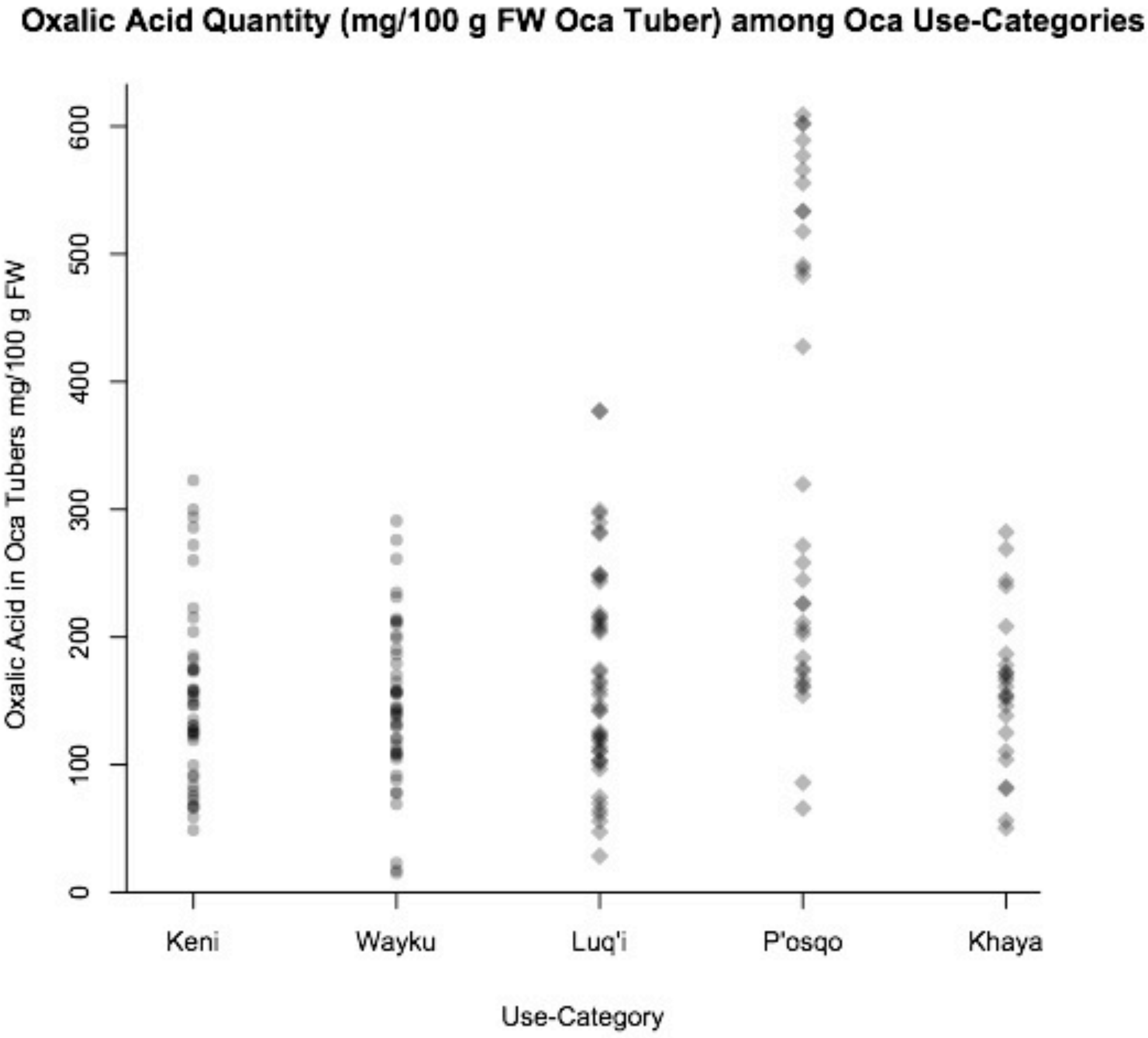
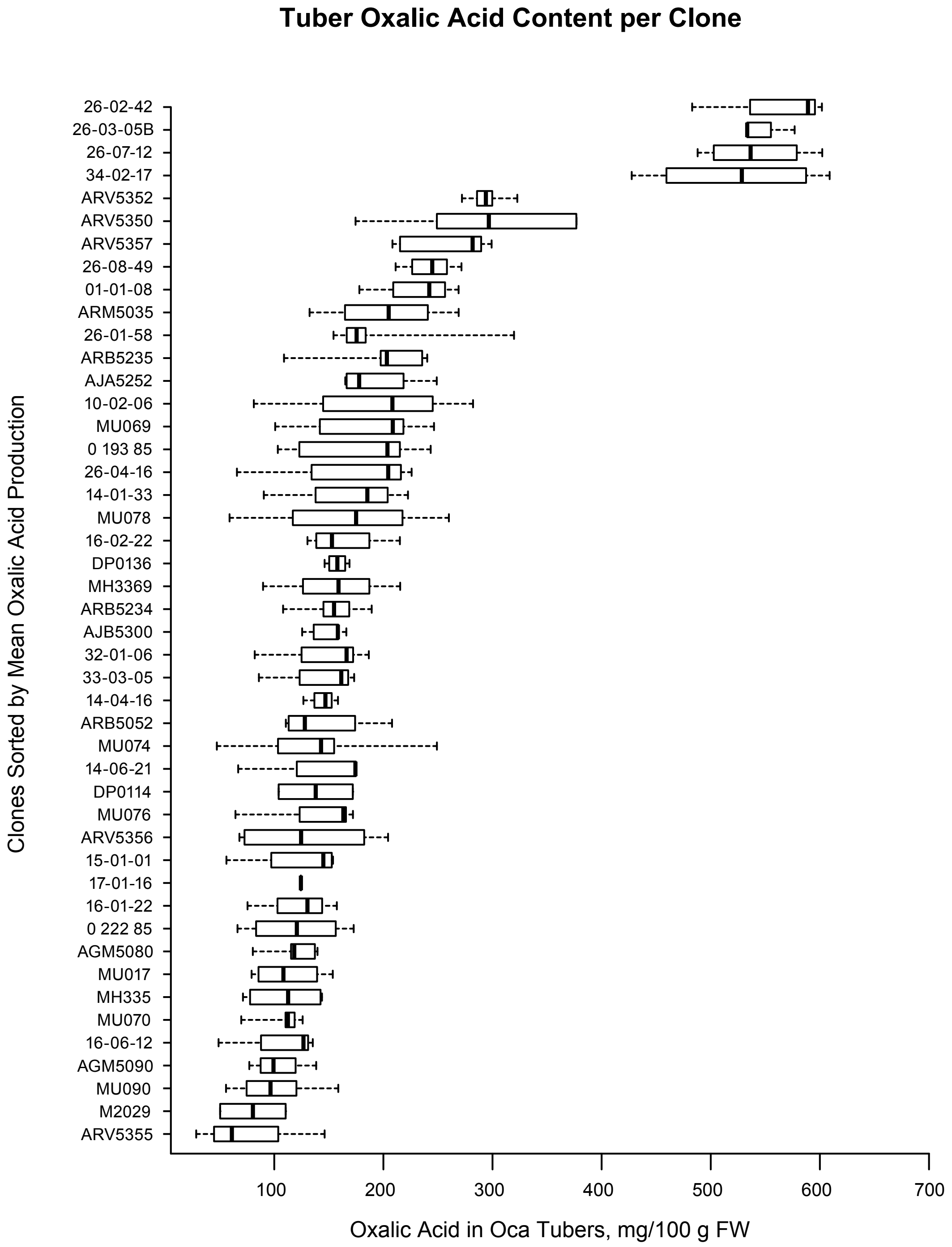


Figure 4. Boxplots of clonal oxalic acid (mg/100g FW). Each boxplot represents all of the oxalic acid measurements from a single genotype. Genotypes are arranged in order of increasing oxalic acid production.



CHAPTER 4.

Genetic differentiation of sweet and sour cultivars of oca (*Oxalis tuberosa*) from Quechua and Aymara communities suggest evidence for inconsistent ploidy and the importance of considering folk taxonomy during conservation efforts

E. Jane Bradbury, Kelly Vivanco, Ivan Manrique, Francisco Vivanco, Eve Emshwiller

Abstract

The recent discovery of bimodally distributed oxalic acid concentrations in the tubers of *Oxalis tuberosa* raised questions about the existence of unusual, under-sampled oca varieties and the implications for conservation and reconstructing the history of the crop and crop uses. As a first step to addressing these questions, we have genotyped the samples reported by Bradbury and colleagues (Ch. 3) using eight SSR loci. We analyzed patterns of genetic differentiation with respect to three primary variables: culture of origin (Aymara or Quechua), use-category and folk taxonomy (processed or not processed; varietal designations when available), and tuber oxalic acid content (high or low). To assess significance of group structuring we used a permutation test calculating the average distance between each randomly-assigned group compared with the mean of pairwise distances found when samples are grouped by the variables concerned. Differentiation was visualized using double principal coordinate analysis (DPCoA). Results showed that the primary structuring variable is tuber acid content with evidence that difference in tuber acid content may be correlated with inconsistent ploidy in the species. A secondary conclusion is that there are some significant patterns of structuring within the “sweet” ocas, with some significant differences between Quechua and Aymara ocas. These results support the need for collaborative conservation efforts between Peru and Bolivia and suggest that collecting

"processed" tubers and *p'osqo* tubers are important strategies for targeting under-collected diversity.

Introduction

Oxalis tuberosa Molina, also called "oca", is a tuber crop native to the Andean highlands of Peru and Bolivia (National Research Council [NRC], 1989). Though under-studied, oca is an integral part of the traditional system of crop rotation (NRC, 1989; Trivelli 1996), which serves as the primary form of agricultural subsistence for Quechua- and Aymara-speaking farmers in Peru and Bolivia, together totaling approximately 12 – 13 million people in the Andean highlands (Chile National Census, 2011; Peru National Census, 2007; Bolivia National Census, 2001). Recent studies have shown that oca, with evidence for multiple potential hybridization and/or polyploidization events in its history (Emshwiller & Doyle, 2002; Emshwiller, 2002; Emshwiller et al., 2009) could also hold value for the scientific community as an interesting study organism for questions related to the genetic mechanisms of plant domestication and the effects of domestication events on plant biochemistry (Bradbury & Emshwiller, 2011; Bradbury et al., Ch. 3). Oca is usually reported to be octoploid (with eight sets of each chromosome) with a base number of eight chromosomes ($2n = 8x = 64$) (reviewed in Emshwiller 2002), and genetic evidence from DNA sequences of the chloroplast-expressed, but nuclear-encoded, form of glutamine synthetase (ncpGS; Emshwiller & Doyle, 2002) supports oca as an allopolyploid and possibly an autoallopolyploid, though inferring oca's precise relationship to multiple potential wild progenitors remains unclear (Emshwiller et al., 2009).

Recently, Bradbury and colleagues (2011; Ch. 3) presented evidence suggesting that oca's domestication may have been further influenced by the production of oxalic acid in oca tubers, with the possibility of divergent domestication towards high-acid and low-acid cultivars

(Bradbury & Emshwiller, 2011). Both Quechua and Aymara farmers separate oca tubers into two distinct culinary use-categories, those that are baked, boiled, or roasted after a few days to "sweeten" in the sun (Hermann & Erazo, 2000; Emshwiller, 2006) and those tubers that are processed into an indefinitely storable food product called *khaya* (Johns, 1996; Emshwiller, 2006). Tubers belonging to the category of ocas that are not processed into *khaya* are usually called "wayk'u" ("boiling") or "miski" ("sweet") in Quechua or "k'eni" ("sweet") in Aymara. These names are use-category names denoting culinary function and consisting of several varietal sub-categories (Emshwiller, 2006). Ocas that are processed into *khaya* belong to the use-category "*khaya*". In Quechua, *khaya* can be made from many varieties of ocas. In the Pisac district of Peru, he two most commonly-used varieties for *khaya* are *p'osqo* (meaning "sour", "fermented") and *kusipata* (L. Moscoe, pers. comm.). Sometimes *khaya* ocas are also called *luk'i* in Aymara, in apparent parallel with *luk'i*, or bitter, potatoes.

Though these linguistic names would suggest that *khaya* ocas produce more acidic tubers, studies based on tuber pH (Bradbury & Emshwiller, 2011) and oxalic acid concentrations (Bradbury, Ch. 3) found that only a small subset of *p'osqo* cultivars produce elevated oxalic acid, whereas *luk'i* and other non-*p'osqo khaya* ocas do not. Similarly, ocas belonging to the Quechua folk variety *p'osqo* clustered separately from all other Quechua ocas in neighbor-joining analyses based on AFLP data (Emshwiller, 2006; Emshwiller et al., 2009). Though the same accessions of *p'osqo* oca were not used in the two studies, together the AFLP data and oxalic acid data suggest that certain accessions of *p'osqo* ocas may be genetically and phenotypically distinct from the rest of the species. Additionally, no studies haveyet investigated what genetic patterning there may be among sweet oca cultivars or how this genetic patterning relates to farmer ethnicity or traditional folk taxonomy. In this paper we seek to determine if oca accessions of different acid levels, use-categories, or collected from farmers of different ethnic groups are genetically differentiated.

Methods

Sample collection

Sampling information for the samples genotyped in this study can be found in Bradbury et al. (Ch. 3; Table 1). The primary sampling strategy was to obtain a sample representative of oca's diversity based on pH measurements of oca tubers and folk taxonomy as recorded during collection. Tubers were supplied by the germplasm collections of both the International Potato Center (CIP, Lima, Peru) and the National Institute for Agrarian Innovation (INIA, Lima, Peru). Based on the limitations of regional germplasm collections, we were able to include a much higher diversity of ocas from Peru than from Bolivia, with only two accessions originally collected in Bolivia. This greatly limits our ability to draw crop-wide conclusions from our data set. Table 1 displays the sampling information for the wild tuber-bearing taxa included in this study. All leaf tissue was collected under the permits 0027-2011-INIA-SUDIRGEB-DIA/J and shipped to Madison, WI with certified Phytosanitary Statement PS-2011-19.

DNA Extraction and microsatellite genotyping

DNA was extracted following the Alexander et al (2006) protocol and eluted twice with 50 µL of hot (40º C) H_2O. Each sample was genotyped using eight microsatellite loci (SSR024, SSR142, SSR595, SSR636, SSR842, SSR876, SSR959, SSR976 [Bonnave et al., *in press*]). Loci were grouped into three multiplex groups for joint amplification using the Qiagen (Venlo, Netherlands) Multiplex PCR Kit following the manufacturer's recommendations, in a final volume of 10 µL with 4 ng of DNA. Amplifications were conducted after an initial denaturation phase of 15 min at 95ºC; with 28 cycles of 30 s denaturation at 94ºC, 30 s annealing at 57ºC for

Mix 1 (SSR142, and SSR976) or 60ºC for Mix 2 (SSR595, SSR636, and SSR842) and Mix 3 (SSR024, SSR876, and SSR959), and 60 s elongation at 72ºC; followed by a final elongation phase of 30 min at 60ºC. Genotyping was performed on an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, California, USA). Alleles were scored using the program GENEMARKER v. 1.4 (SoftGenetics, State College, Pennsylvania, USA) and visually confirmed. Two negative control wells containing 1 µL of ddH_2O in lieu of DNA were included in each 96-well reaction plate. Only genotypes from plates with negative results from water samples were included. Typing error rate was assessed by genotyping one sample 95 times, resulting in no genotyping discrepancies (type error < 0.0105%).

Statistical analysis

Genetic differentiation of the samples was analyzed using the program R 2.13 (R Development Core Team, 2011). Significance of group structuring was assessed with a permutation test over 10,000 permutations. Pairwise permutations were carried out such that for each pairwise permutation test only the distance data for individuals amongst the two groups in question was permuted. Permutation distribution was compiled of calculated pairwise individual distances from two randomly-assigned groups and comparing this distribution to the observed mean of pairwise distances. Genetic differentiation was visualized using principal coordinate analysis (DPCoA). Multiple analyses were conducted with samples assigned to different *a priori* groupings. Groupings were as follows: grouping 1 based on origin of oca tubers in relation to farmer ethnicity (Aymara or Quechua) and cultural use-category (Aymara processed, Aymara not processed, Quechua processed, and Quechua not processed); grouping 2 based on oca origin and use-category but with the Quechua processed group split between *p'osqo* and non-*p'osqo* cultivars; grouping 3 based on origin and acid content (Aymara low acid, Quechua low acid, Quechua high acid); and grouping 4 based on origin and acid content with the Quechua low acid split into *p'osqo* low acid and non-*p'osqo* low acid.x

Results

Genetic differentiation of oca cultivars in relation to farmer ethnicity and cultural use-categorization—Figure 1 shows the differentiation of oca cultivars based on ethnicity of the farmer from which the oca was originally collected (Aymara or Quechua) and the use-category of the oca (processed or not processed). This figure represents a gradient of diversity, with significant differentiation between the two most different sub-groupings, Quechua processed ocas and Aymara processed ocas ($p = 0.0007$), though nonsignificant differentiation between Quechua use-categories ($p = 0.138$), between Quechua not processed and either Aymara not processed ($p = 0.1462$) or Aymara processed ($p = 0.0335$), or between Aymara use-categories ($p = 0.7842$).

Genetic differentiation of oca cultivars in relation to farmer ethnicity and tuber oxalic acid content—When we group ocas based on acid content, we see that the underlying patterns of differentiation are most strongly correlated with acid content (Figures 2a and 2b). Quechua high acid cultivars are strongly differentiated from all low-acid cultivars (Figure 2a, Table 2), though Aymara low acid cultivars also differentiate from Quechua low acid cultivars ($p < 0.0001$). Further subdivision of categories incorporating use-category information, geography and acid reveals that the high-acid *p'osqo* ocas consistently segregate from all other ocas (Figure 2b, Table 2), but all sweet ocas from Quechua or Aymara farmers cluster with no significant differentiation (Table 2), except between the Aymara ocas and the *p'osqo* low-acid (Table 2).

Genetic analyses of the wild tuber-bearing taxa—Unfortunately, we experienced very low PCR-amplification success with the wild tuber-bearing taxa (Table 3). "Amplification success" is defined as returning scored genotypes for the locus being amplified. The two taxa with highest rates of amplification success were *O. chicligastensis* (71.21% amplification success rate over all loci) and *O.* sp. w/t BOL (70.53% success rate over all loci). Only the Bolivian wild-tuber-bearing *Oxalis* produce enough complete genotypes to be included in analyses, where it clustered separately, but somewhat between, the high-acid *p'osqo* ocas and the rest of the low-acid ocas (Figure 3).

However, allelic analysis of the ocas showed that the high-acid *p'osqo* ocas have significant differences in allelic composition and number from the sweet ocas (Table 3). Specifically all low-acid accessions presented five or more alleles at 2 or more loci, whereas all high-acid accessions presented between one and four alleles. Additionally, 20 alleles across all eight loci are present in ocas from all of the sweet groups but are absent from the high-acid *p'osqo*.

Discussion

Overall, these results carry important implications for informing future conservation efforts of oca's genetic diversity. Specifically, these analyses show the importance of considering folk taxonomy and classification when collecting oca, particularly with respect to ocas collected from Quechua-speaking farmers. These data serve as strong evidence for the significance of the *p'osqo* cultivars, as this is the only varietal designation which correlates thus far with either elevated acid production or evidence for low-ploidy in the species. However, these analyses also underscore the importance of diversifying the representation of Aymara ocas in analyses of genetic structuring of oca groups. Though we found no differentiation between Aymara use-cateogories and no evidence of structuring within Aymara ocas, given the limited geographical range of ocas from Aymara farmers that were included in the study, it is highly likely that we have inadequately addressed these questions in relation to ocas from Aymara farmers. Certainly, the differentiation between low-acid ocas from Aymara-speaking farmers and low-acid ocas from Quechua-speaking farmers signifies that conservation efforts must be collaborative between Peru and Bolivia if we are going to be able to accurately assess, locate, and preserve oca's existing genetic diversity.

Of particular interest, both for implications for conserving crop genetic resources and for our understanding of oca's evolutionary history, is the evidence that high-acid ocas may possess low-ploidy relative to low-acid ocas. In addition to the data presented here, flow cytometry data for two of the high-acid *p'osqo* accessions confirms that these two accessions have approximately one-half of the 2C DNA content as 8*x* oca (K. Vivanco, unpublished data). Should the existence of low-ploidy oca be confirmed, and should the apparent correlation between low-ploidy and high oxalic acid concentration be upheld, oca could become an important plant model for understanding the effects of polyploidization events on plant biochemistry. Overall, these results lend further evidence to suggest that tuber oxalic acid content is tightly correlated with a potential polyploidization event in oca's evolutionary history.

Though genotyping of wild tuber-bearing *Oxalis* was inconclusive, perhaps due to age of sample DNA extractions and inability of the researchers to experiment with PCR protocols due to time and funding constraints, the results from amplification attempts also support the wild tuber-bearing taxon from Bolivia and *O. chicligastensis* as the two most closely-related wild species to cultivated ocas. Microsatellites are well-known for species specificity in amplification (Lowe et al., 2002; Guo et al., 2006), and varied amplification success in the wild tuber-bearing species correlates with previously inferred evolutionary relationships among the taxa based on nuclear loci (Emshwiller & Doyle, 2002) and AFLP analyses (Emshwiller et al., 2009). Our data should provide additional support for the need for expanded sampling and representation of wild tuber-bearing taxa, particularly from the southern regions of Peru and throughout Bolivia and northern Argentina.

These new data strongly support the need for future investigations to evaluate the strength of the hypotheses presented here. In particular, the ploidy level of high-acid ocas has been strongly called into question by these data. Should high-acid ocas prove to be tetraploid as opposed to octoploid, this would signify an important new understanding of oca's evolutionary history. Additionally, the high-acid ocas in this study represent only four genotypes. Further field work must be done to expand collection of high-acid ocas to see if the genetic patterns presented here are sustained with greater sampling. Similarly, more thorough sampling of Aymara ocas should be conducted, including Aymara ocas from Bolivia, to confirm that there are no high-acid ocas being cultivated by Aymara farmers. Finally, sampling of the wild tuber-bearing relatives greatly needs to be enhanced, in the hopes that the addition of fresh, high-quality DNA extractions may be able to increase microsatellite amplification success and subsequent resolution of evolutionary relationships among oca and its wild relatives.

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Figures and Tables

Table 1. Collection information for wild tuber-bearing *Oxalis*.

Collection Number	**Taxon**	**Country**	**1st Pol. Div.**	**2nd. Pol. Div.**
EE259	*Oxalis* sp. (Bol W/T)	Bolivia	Cochabamba	Ayopaya
EE259.2	*Oxalis* sp. (Bol W/T)	Bolivia	Cochabamba	Ayopaya
EE260	*Oxalis* sp. (Bol W/T)	Bolivia	Cochabamba	Ayopaya
EE262_I	*Oxalis* sp. (Bol W/T)	Bolivia	Cochabamba	Ayopaya
EE262	*Oxalis* sp. (Bol W/T)	Bolivia	Cochabamba	Ayopaya
EE260_3_3	*Oxalis* sp. (Bol W/T)	Bolivia	Cochabamba	Ayopaya
EE279	*Oxalis* sp. (Bol W/T)	Bolivia	Cochabamba	Ayopaya
EE284	*Oxalis sp. (Bol W/T)*	Bolivia	Cochabamba	Chapare
EE1003	*O. chicligastensis*	Argentina	Tucumán	Chicligasta
EE1001_C	*O. chicligastensis*	Argentina	Tucumán	Chicligasta
EE1002_D	*O. chicligastensis*	Argentina	Tucumán	Chicligasta
EE_xxx_LJM_3_1	*O. chicligastensis*	Argentina	Tucumán	Chicligasta
EE_xxx_LJM_1_2	*O. chicligastensis*	Argentina	Tucumán	Chicligasta
EE1110	*Oxalis* sp. (Lima W/T)	Peru	Lima	Huarochirí
EE1110_LJM	*Oxalis* sp. (Lima W/T)	Peru	Lima	Huarochirí
EE1169	*Oxalis* sp. (Lima W/T)	Peru	Lima	Canta
EE1185	*Oxalis* sp. (Lima W/T)	Peru	Lima	Canta
EE1185.2	*Oxalis* sp. (Lima W/T)	Peru	Lima	Canta
EE917	*Oxalis picchensis*	Peru	Cusco	Calca
EE546	*Oxalis picchensis*	Peru	Cusco	Anta
EE1152	*Oxalis picchensis*	Perú	Apurimac	Cotabambas
EE531	*Oxalis picchensis*	Peru	Cusco	Calca

Figure 1. Double principal coordinate analysis of oca accessions grouped by farmer ethnicity and cultural use-category. Accessions are grouped by the ethnicity of the farmer from which the tuber was originally collected (either Quechua or Aymara) and the cultural use-category assigned to the accession by the farmer (processed or not processed). “ANP” stands for “Aymara Not Processed;” “AP” stands for “Aymara Processed”; “QNP” stands for “Quechua Not Processed”; and “QP” stands for “Quechua Processed”.

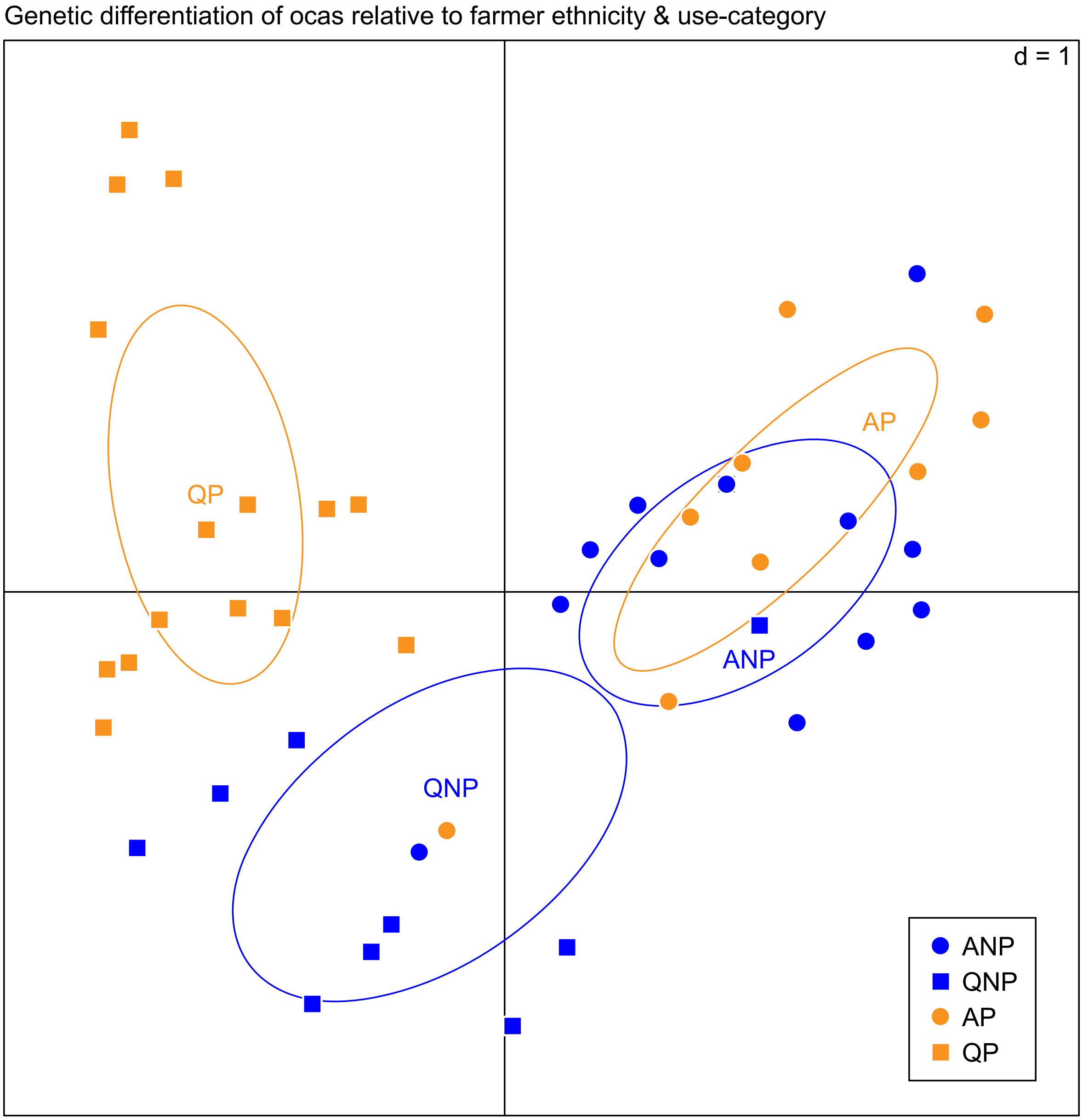

Figure 2a. Double principal coordinate analysis of oca accessions grouped by farmer ethnicity and tuber oxalic acid concentration. Accessions are grouped by the ethnicity of the farmer from which the tuber was originally collected (either Quechua or Aymara) and the measured tuber oxalic acid concentration (designated “high” for over 400 mg/100 g FW or “low” for under 400 mg/100 g FW). “ALA” stands for “Aymara Low Acid”; “QLA” stands for “Quechua Low Acid”; and “QHA” stands for “Quechua High Acid”. Quechua High Acid ocas are differentiated from Aymara Low Acid ocas ($p < 0.0001$) and Quechua Low Acid ocas ($p = 0.0004$). Aymara Low Acid and Quechua Low Acid ocas are also differentiated ($p < 0.0001$).

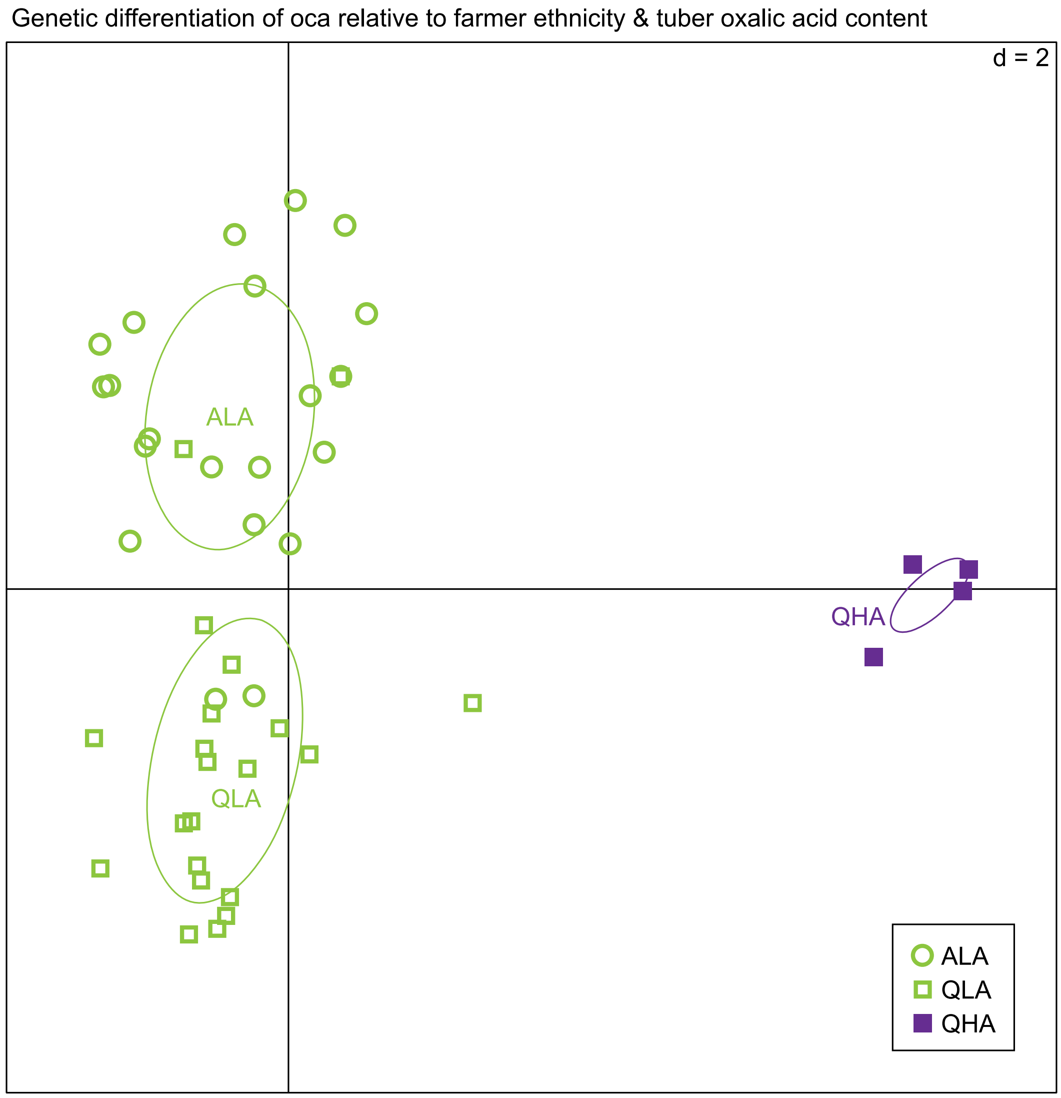

Figure 2b. Double principal coordinate analysis of oca accessions grouped by farmer ethnicity, tuber oxalic acid concentration, and use-category. Accessions are grouped by the ethnicity of the farmer from which the tuber was originally collected (either Quechua or Aymara), the measured tuber oxalic acid concentration (designated "high" for over 400 mg/100 g FW or "low" for under 400 mg/100 g FW), and the cultural use-category assigned to the accession by the farmer (processed or not processed, with the Quechua processed use-category subdivided into cultivars taxonomically grouped as *p'osqo* and processed cultivars designated only as *khaya*). "ANPLA" stands for "Aymara Not Processed Low Acid;" "APLA" stands for "Aymara Processed Low Acid"; "QPLA (Khaya)" stands for "Quechua Processed Low Acid—Khaya"; "QPLA (P'osqo)" stands for "Quechua Processed Low Acid—P'osqo"; "QPHA" stands for "Quechua Processed High Acid". All of the QPHA ocas also belong to the *p'osqo* group.

Genetic differentiation of oca relative to farmer ethnicity, folk taxonomy & tuber oxalic acid content

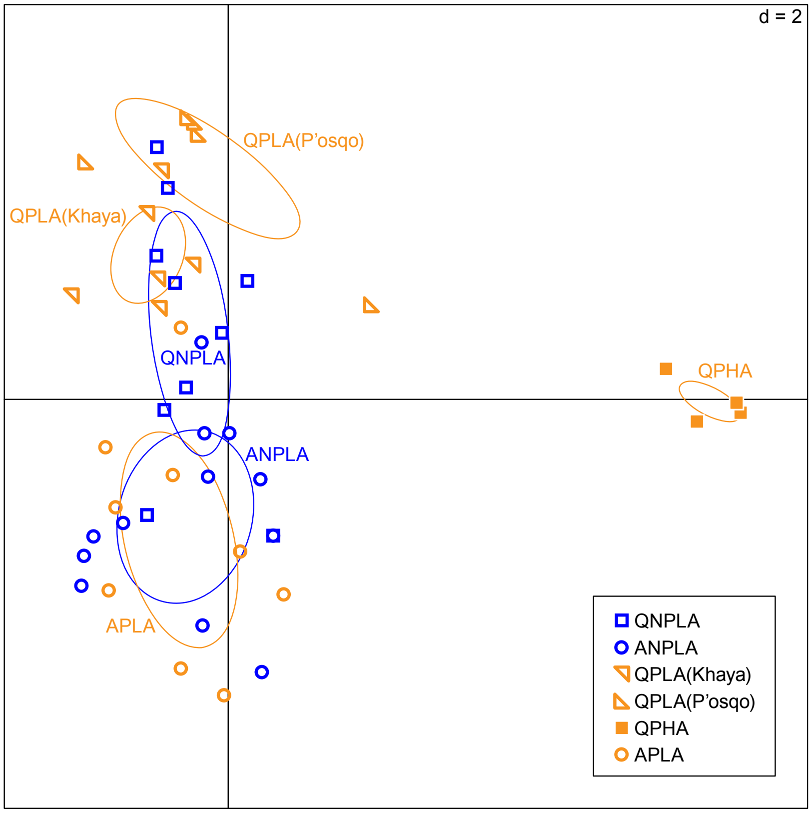

Table 2. P-values for all pairwise permutations of the *a priori* groupings visualized in Figures 1, 2a, and 2b. Groupings with p-values less than 0.01 are in bold. In table 2a, "ANP" stands for "Aymara Not Processed;" "AP" stands for "Aymara Processed"; "QNP" stands for "Quechua Not Processed"; and "QP" stands for "Quechua Processed". In Table 2b, "QLA" stands for "Quechua Low Acid"; and "QHA" stands for "Quechua High Acid". In Table 2c, "ANPLA" stands for "Aymara Not Processed Low Acid;" "APLA" stands for "Aymara Processed Low Acid"; "QPLA (Khaya)" stands for "Quechua Processed Low Acid—Khaya"; "QPLA (P'osqo)" stands for "Quechua Processed Low Acid—P'osqo"; "QPHA" stands for "Quechua Processed High Acid".

Table 2a.

	QNP	**QP**	**ANP**
AP	0.0335	**0.0007**	0.7842
ANP	0.1462	**< 0.0001**	-
QP	0.138	-	-

Table 2b.

	QHA	**QLA**
ALA	**< 0.0001**	**< 0.0001**
QLA	**0.0001**	-

Table 2c.

	QPHA	**QPLA--p'osqo**	**QPLA--khaya**	**QNPLA**	**ANPLA**
APLA	**0.0019**	**0.0028**	0.0107	**0.0321**	0.7896
ANPLA	**0.0009**	**0.003**	0.0435	**0.1405**	-
QNPLA	**0.0004**	0.0237	0.1982	-	-
QPLA--khaya	**0.0039**	0.062	-	-	-
QPLA--p'osqo	0.0175	-	-	-	-

Table 3. PCR amplication success for wild tuber-bearing *Oxalis*. PCR amplification success was defined as PCR that returned scoreable SSR genotypes. In the tables below, each column represents one SSR locus with the top table showing number of successful PCR and the bottom table displaying the same data as % successful. Total successes for all loci are in the final column. "Bol" denotes *O.* sp. BOL w/t, the wild tuber-bearing taxon from Bolivia. "Chicli" denotes *O. chicligastensis*. "Lim" denotes *O.* sp. LIM w/t, the wild tuber-bearing taxon from the Lima Department of Peru. "Picch." denotes *O. picchensis.*

TOTAL SUCCESS PER TAXON PER LOCUS; NUMBER (#)									
Taxon	**SSR976**	**SSR142**	**SSR001**	**SSR595**	**SSR636**	**SSR842**	**SSR959**	**SSR876**	**Total:**
Bol	11/13	08/13	11/13	11/14	11/14	11/14	7/7	7/7	**67/95**
Chicli	8/8	7/8	8/8	4/6	4/6	4/6	6/12	6/12	**47/66**
Lim	4/12	4/12	4/12	3/8	2/8	2/8	0/10	0/10	**19/80**
Picch	6/12	2/12	6/12	2/7	2/7	2/7	3/6	3/6	**26/69**

TOTAL SUCCESS PER TAXON PER LOCUS; PERCENT (%)									
Taxon	**SSR976**	**SSR142**	**SSR001**	**SSR595**	**SSR636**	**SSR842**	**SSR959**	**SSR876**	**Total:**
Bol	84.62%	61.54%	84.62%	78.57%	78.57%	78.57%	100.00%	100.00%	**70.53%**
Chicli	100.00%	87.50%	100.00%	66.67%	66.67%	66.67%	50.00%	50.00%	**71.21%**
Lim	33.33%	33.33%	33.33%	37.50%	25.00%	25.00%	0.00%	0.00%	**23.75%**
Picch	50.00%	16.67%	50.00%	28.57%	28.57%	28.57%	50.00%	50.00%	**37.68%**

Figure 3. Double principal coordinate analysis of *O.* sp. BOL w/t and *Oxalis tuberosa* accessions grouped by farmer ethnicity, tuber oxalic acid content, and cultural use-category. Oca accessions are grouped by the ethnicity of the farmer from which the tuber was originally collected (either Quechua or Aymara), the measured tuber oxalic acid concentration (designated "high" for over 400 mg/100 g FW or "low" for under 400 mg/100 g FW), and the cultural use-category assigned to the accession by the farmer (processed or not processed, with the Quechua processed use-category subdivided into cultivars taxonomically grouped as *p'osqo* and processed cultivars designated only as *khaya*). "ANPLA" stands for "Aymara Not Processed Low Acid;" "APLA" stands for "Aymara Processed Low Acid"; "QPLA (Khaya)" stands for "Quechua Processed Low Acid—Khaya"; "QPLA (P'osqo)" stands for "Quechua Processed Low Acid—P'osqo"; "QPHA" stands for "Quechua Processed High Acid". All of the QPHA ocas also belong to the *p'osqo* group. "Bol" stands for the wild tuber-bearing *Oxalis* from Bolivia.

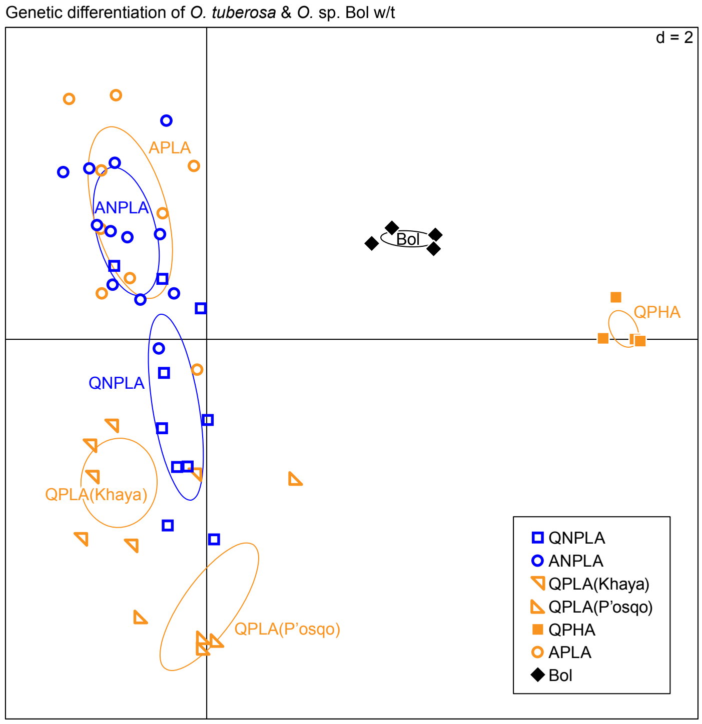

Table 3. Allele distribution among groups of oca cultivars. Analyses returned 74 alleles over eight loci. Twenty-one alleles were present in all groups. Twenty other alleles were found in all groups of oca except the high acid *p'osqo* ocas. Other alleles were private to one use-category or to one ethnic group. Twenty-two alleles presented heterogeneous patterns of distribution.

	Number of alleles
Present in all groups	21
In all groups except high acid *p'osqo*	20
Private to Aymara	2
Private to Quechua	2
Private to Quechua processed	1
Private to Quechua not processed	3
Private to Aymara not processed	1
Private to high acid *p'osqo*	2
With heterogeneous patterns	22
Total	74

CHAPTER 5.

Flow Cytometry Reveals Inconsistent Ploidy Levels in *O. tuberosa*

E. Jane Bradbury, Lauren Moscoe, Peter Gregg, Daniel Huaman, Raul Blas, Eve Emshwiller

Abstract

The evolutionary history of the traditional Andean tuber crop oca (*Oxalis tuberosa* Molina) remains unresolved. Recent analyses using SSR loci raised the possibility of inconsistent ploidy in the species. The primary aim of this study was to investigate the existence of inconsistent ploidy in *O. tuberosa* using flow cytometry to quantify nuclear DNA content in 79 oca samples from the Pisac District, Cusco Department of Peru. Analyses returned 19 samples containing approximately one-half of the nuclear DNA content (pg/2C) as the confirmed octoploid. These results strongly support inconsistent ploidy in oca, though karyological studies should be undertaken to confirm the cytological nature of low 2C DNA content in oca. The existence of tetraploid ocas has significant impact on our understanding of oca's domestication history, increasing the likelihood of a step-wise history with multiple polyploidization and/or hybridization events.

Introduction

The genus *Oxalis* comprises over 500 species with greatest numbers of species found in North and South America and southern Africa (Lourteig, 2000). *Oxalis* is notable for its variable morphology and habitat, ranging from tropical rainforest shrubs to high-altitude cushion plants adapted to highly seasonal variation in water availability. Though plant morphologies adapted to water limitations, such as seasonal deciduousness, fleshy stems, and bulbs, are common throughout the genus, the "*Oxalis tuberosa* alliance clade" (Emshwiller & Doyle, 1998;

Emshwiller & Doyle, 2002) is notable for the presence of a handful of taxa that produce tubers. One of these taxa, *O. tuberosa*, is distinguished as being the only domesticated *Oxalis*. *Oxalis tuberosa*, oca, is a high-altitude tuber crop critical to the traditional system of crop rotation and important as a subsistence crop for traditional farmers in Peru and Bolivia (National Research Council, 1989).

The "*O. tuberosa* alliance clade" is also noteworth in the evolutionary history of the genus due to its base chromosome number of $x = 8$, which is rare in *Oxalis* where base chromosome number generally ranges from $x = 5$ to 12, with $x = 7$ being most common (Cronquist, 1981). Additionally, the *O. tuberosa* alliance clade is notable for its multiple transitions in ploidy level, consisting of species that are $2x$, $4x$, $6x$, and $8x$ (Emshwiller, 2002). Previously, *O. tuberosa* had been determined to be octoploid ($2n = 8x = 64$) based on cytological analyses and flow cytometry (de Azkue & Martínez, 1990; reviewed in Emshwiller, 2002). However, conflicting chromosome counts have been reported, specifically with lower euploid chromosme numbers reported for some oca accessions in Bolivia (Guamán, 1997) and analyses based on AFLP data show differentiation of some *p'osqo* ocas, which could be due to difference in ploidy (Emshwiller, 2006). Recently, analyses of genetic differentiation of oca cultivars based on eight SSR loci supported the possibility for inconsistencies in the ploidy level of oca, specifically finding four genotypes that present significantly fewer copies of SSR alleles than would be expected for an octoploid sample (Bradbury et al., Ch. 4), two of which have accompanying flow cytometry data confirming tetraploidy (K. Vivanco, unpublished data).

The origins of *O. tuberosa* remain unresolved and molecular analyses based on nuclear sequences (Emshwiller & Doyle, 1998; Emshwiller & Doyle, 2002) and AFLP analyses (Emshwiller et al., 2009) have returned somewhat conflicting results. Though we are confident that *O. tuberosa* has an allopolyploidization event in its history (Emshwiller & Doyle, 2002), without knowing the precise wild progenitor species involved in oca's origins, we cannot rule out the possibility that oca was formed via an autopolyploidization of a wild allotetraploid nor can we eliminate the possibility that oca originated via hybridization of two or more wild taxa (Emshwiller et al., 2009). Additionally, without a clear understanding of oca's evolutionary history, we remain unable to postulate a center, or centers, of oca domestication.

Confirming the growing possibility of low-ploidy oca would further convolute the already-complicated history of oca's domestication. Were the existence of lower-ploidy ocas to be confirmed, this would greatly contribute to the as-yet-unresolved domestication history of this crop, disproving one potential hypothesis of a domestication event arising from a single hybridization of multiple crop progenitors and instead supporting alternative hypotheses of step-wise polyploidization events or multiple, independent origins. Therefore, the primary aim of this study is to investigate the existence of lower-ploidy *O. tuberosa* using flow cytometry to estimate genome size.

Materials and Methods

Sampling

Leaf tissue was collected from 79 ocas collected from Amaru and Viacha, two villages in Pisac district in the Cusco Department of Peru (Table 1). Ocas selected for this study all belonged to the cultural use-category *khaya*, designating ocas that are traditionally reserved for processing into a dried, storable food product. This sample strategy was based on the results of Bradbury and colleagues (Ch. 4), which showed that the only ocas with suspected low-ploidy all belong to the use-category *khaya*, and, more specifically, are ocas belonging to the *khaya* variety *p'osqo* and originated in the Vilacanota River Valley. Other collections of ocas from other areas of Peru, including ocas reserved for processing, all showed clear allelic evidence of octoploidy, so were not sampled here (Bradbury Ch. 4). We specifically wanted to confirm ploidy levels of the *p'osqo* ocas. Leaves were collected in small paper envelopes and immediately dried in silica gel. Collection of fully expanded leaves was prioritized so as to minimize presence of 4N G2-phase peaks. Samples were collected and exported under permit 0248-2013-INIA-SUBDIRGEB-DIA/J. Additionally, one sample of known ploidy, *O. tuberosa* MHG865 (Emshwiller, 2002), was processed as both fresh leaves and dried leaves to provide a reference value for octoploidy in oca and to demonstrate the comparability of results obtained from fresh and dried leaf tissue.

Seeds for internal standard *Oryza sativa* ssp. *japonica* cv. Nipponbare were obtained from the Simon Gilroy Lab at the University of Wisconsin-Madison Botany Department.

Flow cytometry

Sample preparation for flow cytometry followed the procedure using the modified two-step Otto method (Otto, 1990; Doležel and Göhde, 1995), using only 0.5 mL of Otto I buffer for chopping, so as to increase concentration of nuclei in sample preparations, and eliminating centrifugation, so as to avoid damaging nuclei. One large, fully expanded leaflet was chopped using a double-sided razor blade, along with 1 cm^2 of fresh rice standard in 0.5 mL of ice-cold "Otto I" solution [0.1 M citric acid monohydrate, 0.5% (v/v) Tween 20) 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. "Otto II" solution (0.4 M $Na_2HPO_4 \cdot 12H_2O$ containing 2 µL/mL β-mercaptoethanol) with propidium iodide and RNase (each at a final concentration of 50 µg/mL) was prepared at 0.75 mL/sample, of which 0.5 mL was added to sample preparation 5 minutes prior to being analysed in a cytometer.

Samples were run on an Accuri™ C6 Flow Cytometer (BD Bioscience, San Jose, California) in the Abbott Research Group Laboratory for Molecular Engineering at the University of Wisconsin-Madison, with a 488 nm 50 mW solid state laser. Pulse area was detected using FL2-A (585 mean/42 bandwidth). Software "CFlow Plus" (BD Bioscience) was used to visualize histogram peaks and calculate means, SD, and other statistics.

DNA content was calculated first as Mbp in accordance with the published values for *Oryza sativa* cv. *Nipponbare* (International Rice Genome Sequencing Project, 2005), and then converted to pg of DNA using the standard conversion 1 pg DNA = 980 Mbp (Bennett et al., 2000; Doležel et al., 2003).

Results

Analyses showed that samples form to two distinct groups of genome-size (Fig. 1, Table 2). The average of the 2C values in one of the groups, consisting of 60 samples, is 3.43 pg DNA, which includes the known-octoploid sample MHG864 (3.28 pg DNA [fresh] and 3.32 pg DNA [dry]). The remaining 19 samples all were found to have 2C values averaging 1.74 pg DNA, ranging from 1.68 pg DNA to 1.85 pg DNA in size. The comparative ratio of the two groups is 2.04:1, or approximately 2:1, indicating that the second group contains almost exactly half of the DNA quantity of the octoploid group and can be inferred to have a DNA ploidy of tetraploid. Histograms of relative fluorescence for known octoploid MHG864 (Figure 2a) and one tetraploid sample (Figure 2b) show distinctly different peak placement. When run together, tetraploid ocas and octoploid ocas do not overlap (Figure 2c).

Discussion

These data confirm the existence of multiple levels of DNA ploidy in *O. tuberosa*. It should be noted that without karyological data, we cannot confirm that the low-ploidy samples possess the same base chromosome number as 8*x* oca (Suda et al., 2006), though the fact that all of the members of the *O. tuberosa* alliance clade for which cytological studies have been performed possess base chromosome number $x = 8$ (de Azkue & Martínez, 1990) makes it unlikely that low-ploidy oca resulted from loss of base chromosomes as opposed to relating to the history of polyploidization in the species. For this reason, from here onward we will refer to the low-DNA ploidy ocas as "tetraploid". However, confirming the existence of ocas of low-DNA ploidy underscores the need for future karyological examinations. Given oca's complicated and unresolved evolutionary history, karyological studies will be critical to help determine which chromosomes or sets of chromosomes tetraploid and octoploid ocas share.

Additionally, this study does not disprove the finding that tetraploid ocas are consistently culturally categorized into the *p'osqo* variety of *khaya* ocas from Quechua-speaking farmers. All of the tetraploids discovered in this study belong to the *p'osqo* variety, and none of the other *khaya* varieties included in the sampling were found to be tetraploid. However, given that the original reports of lower chromosome numbers came from ocas in Bolivia (Guamán, 1997) and all of the ocas included in this study originated from the Villacanota River Valley in the Cusco Department of Peru, these results highlight the critical need for additional exploratory field work. Future studies investigating oca's ploidy with sampling throughout oca's range of cultivation are warranted.

These results have important significance for our understanding of the history of oca and for ongoing efforts to preserve crop genetic diversity. These data further support a multiple-origin hypothesis for oca's origin. However, we remain unable to fully distinguish between the many resulting possible hypotheses, including completely distinct domestication events for tetraploid and octoploid ocas, a single domestication event with some sort of multiple step-wise polyploidization process (either via hybridization or autopolyploidization), or domestication followed by loss of ploidy. Specifically, tetraploid and octoploid ocas could have arisen from distinct progenitor species or populations, or the tetraploid ocas may have been domesticated first, with a subsequent polyploidization (possibly involving hybridization) giving rise to the octoploid ocas. Alternately, octoploid ocas could have been domesticated first, with a subsequent loss of ploidy. Based on genetic data, including AFLP and SSR analyses, we support a step-wise domestication process as the most likely scenario, but further data are needed to test this hypothesis.

Domestication followed by subsequent polyploidization or hybridization—

Octoploid ocas are allopolyploids, and possibly autoallopolyploids, based on data from DNA sequences of the nuclear locus ncpGS (plastid-expressed glutamine synthetase) showing evidence of two contributing genomes (Emshwiller & Doyle, 2002). Further analyses using AFLP markers demonstrated that an as-yet unnamed tuber-bearing taxon in Bolivia is the most likely candidate for a progenitor of octoploid oca under current data and sampling (Emshwiller et al., 2009). Recent SSR analyses demonstrated significant differentiation between ocas possessing five or more alleles per locus (indicative of octoploidy) and ocas possessing four-or-fewer alleles per locus (now presumed to be tetraploid) (Bradbury et al, Ch 4). Additionally, the primary distinction between the putative-tetraploid ocas studied by Bradbury and colleagues (Ch. 4) and the octoploid ocas was the absence in putative-tetraploid ocas of many alleles characteristic of octoploid ocas. This could be due to the absence in tetraploids of at least one of the contributing genomes found in octoploids, which would also support a step-wise origin, with the tetraploid genome first being domesticated followed by a secondary hybridization to produce octoploid ocas. However, SSR data alone cannot allow us to determine which sets of chromosomes are shared by tetraploid and octoploid ocas and, therefore, which chromosomes (or sets of chromosomes) are absent in tetraploid oca as compared with octoploid oca. Indeed, absence in tetraploid ocas of alleles characteristic of octoploid ocas could also be viewed as support for the possibility that tetraploid oca arose from potential loss of genomic material in the octoploids (discussed in greater detail under "Alternative Hypotheses" below).

That oca originated from multiple step-wise hybridization or polyploidization events hypothesis is also supported by the inferred relationship of *O.* sp. BOL w/t to tetraploid and octoploid ocas. In SSR analyses, *O.* sp. BOL w/t clustered between octoploid and putative-tetraploid ocas, suggesting that it is a contributing progenitor to both 8*x* and 4*x* ocas (Bradbury et al., ch. 4). However, this pattern could also be observed if 8*x* and 4*x* ocas have independent origins but shared one progenitor in common. In order to further support a step-wise history of multiple polyploidization and/or hybridization events for oca's origins, further data using nuclear loci are required, which could allow us to observe evidence of all the contributing genomes or observe evidence of loss of homeologous loci, gene conversion, or concerted evolution (reviewed in Soltis & Soltis, 1999). Additionally, without further data from nuclear loci, we are unable to determine when in the course of oca's evolutionary history various polyploidization events occurred. Even though current evidence seems to suggest a step-wise history of the domesticate, we do not know if hybridization resulting in the allopolyploid ncpGS signal (Emshwiller & Doyle, 2002) occurred before oca's domestication (namely, in the history of oca's wild progenitor(s)) with a subsequent autopolyploidization to form 8*x* oca or if 4*x* oca was domesticated from a tetraploid progenitor with subsequent hybridization to produce allopolyploid 8*x* oca. Indeed, hybridization could have occurred both times—first to form oca's progenitor and then again during the transition from tetraploid to octoploid oca.

Alternative hypotheses—

Though the combination of data gained from nuclear-based phylogenetic, AFLP, SSR, and flow cytometry analyses supports a step-wise domestication history for oca, alternative hypotheses cannot be ruled out at this time. Two primary competing alternative hypotheses to the one presented above would be the possibility to two separate domestication events, one giving rise to 8*x* oca and one giving rise to 4*x* oca, or the possibility of one domestication event that produced 8*x* oca that then experienced an isolated loss of ploidy.

In particular, it is the isolated geographical distribution of tetraploid oca that supports either of these alternative hypotheses, coupled with the intermediary positioning of *O.* sp. BOL w/t between 8*x* and 4*x* ocas in SSR analyses. Therefore, in order to either support or refute these hypotheses, ploidy testing of a greatly expanded sample of oca from throughout Peru and Bolivia will be critical. Should 4*x* ocas be found to be truly confined to the Cusco Department of Peru, this would support a scenario in which 4*x* ocas were either domesticated independently in Peru or arose via an isolated chromosome-loss event. Indeed, genome evolution in polyploids is often characterized by loss of genetic material, gene conversion, concerted evolution, or other mechanisms to reduce ploidy and regain meiotic normality (reviewed in Soltis & Soltis, 1999). However, previous reports by Guamán (1997) of lower euploid chromosome numbers in oca suggest that 4*x* ocas are not confined to the Cusco Department of Peru, but are, nonetheless, so rare as to remain unsampled in subsequent molecular studies (Pissard et al., 2006; Malice et al., 2007; Maxime Bonnave, pers. comm.).

Indeed, the overall rarity of the tetraploid ocas carries important implications for conservation of oca genetic diversity. The confirmation of the existence of tetraploid ocas presents an entirely new subcategory of oca diversity to be discovered and maintained. Given that these ocas appear to be quite unusual and that tetraploidy appears to strongly correlate with elevated tuber oxalic acid content (rendering them unfit for consumption without extensive processing into *khaya*), tetraploid ocas are certainly at high risk for loss. Field studies to determine the geographical range of tetraploid ocas as well as their *in situ* conservation state should be undertaken posthaste.

Additionally, continuing studies are needed in order to further test the hypotheses of step-wise or separate origins of polyploidy in *O. tuberosa*. Specifically, SSR analyses of other wild tuber-bearing relatives, particularly *O. chicligastensis* are needed, as well as more thorough geographic sampling of each of the four wild, tuber-bearing taxa. Given the complicated evolutionary history that we are uncovering, in spite of the difficulty of determining allele copy-number in an octoploid, future SSR analyses should include steps to gain full genotypes for samples so as to gain additional analytic capacity that is not availabe to dominant markers. Additionally, sequencing of nuclear loci is critical for investigating separate origins of homeologous chromosomes. Should octoploid ocas be the product of hybridization between either wild *O.* sp. BOL w/t or 4*x* domesticated oca and *O. chicligastensis*, we would expect to see 8*x* oca cluster intermediately between either *O.* sp. BOL w/t or 4*x* ocas and *O. chicligastensis*, which was not observed based on AFLP analyses (Emshwiller et al., 2009). Additionally, SSR analyses of *O. chicligastensis* would allow us to evaluate whether the alleles typical of 8*x* ocas but missing from 4*x* ocas are similar to alleles found in *O. chicligastensis*. Given that preliminary analyses with very low sampling of *O. chicligastensis* echos Emshwiller and colleague’s (2009) suspicions about whether or not *O. chicligastensis* and *O.* sp. BOL w/t are actually separate taxa (E. J. Bradbury, unpublished data), the importance of including multiple distinct populations of *O. chicligastensis* underscores the need for additional field work to collect more accessions of this wild taxon.

Similarly, the range and diversity of 4*x* ocas needs to be established. The division of domesticated oca into 8*x* and 4*x* genotypes has critical implications for conservation of crop diversity. Without knowledge of this important biological distinction, collection of *p'osqo* ocas is often undervalued by researchers in the field, who are more likely to place emphasis on widely-used cultivars or cultivars belonging to the "sweet" oca use-category, which are significantly more widespread in geographic distribution and play a more significant role in the home and market economy of rural Andean peoples. Particularly given that 4*x* ocas have been observed only within a very limited geographical range, investigation of the true range of tetraploid ocas has important consequences for our the possibility to preserve these rare oca types from disappearing all together.

Finally, these data underscore the importance of future studies focusing on oxalic acid production in oca's tubers. Previously, a significant elevation in oxalic acid production was observed among some *p'osqo* ocas (Bradbury et al, Ch. 3). These high-acid ocas are also the same ocas that had fewer copies of alleles in SSR analyses (Bradbury et al, Ch. 4) and are now presumed to be 4*x* ocas. Confirmation that 4*x* ocas have four-to-six times the concentration of tuber oxalic acid in comparison to 8*x* ocas would indicate that *O. tuberosa* could become an extremely valuable model system for studying the phenotypic effects of polyploidization and hybridization, allowing us to investigate the genetic mechanisms of differential expression between 4*x* and 8*x* ocas. This research could contribute to understanding the complicated effects of genome doubling and/or combination.

Overall, the confirmation of inconsistent ploidy levels in *O. tuberosa* underscores the importance of additional field work and conservation efforts to preserve these rare tetraploid ocas. Together with previous studies on distribution of oxalic acid production in oca tubers, these data promote oca as a new, valuable model organism for studying polyploid mechanisms of domestication and the resulting phenotypic effects of genome combination.

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Figures and Tables

Table 1. Sampling information for 79 oca accessions analysed via flow cytometry. Accession number is found in the first column. The community from which the original tubers were collected is found in the second column. The varietal name of the accession, as given by the farmer at the time of collection, is listed in the third column. The use-category for the oca (K = processed into *khaya* and W = eaten without such processing, "*wayk'u*") is found in the fourth column. Latitude and longitude for the house from which the original tubers were collected is found in the last two columns, respectively.

Accession	Community	Varietal Name	Use-Category	Lat.	Long.
LJM 0201	Amaru	puka panti	K, W	-13.22425	-71.49284
LJM 0403	Amaru	?	K	-13.22379	-71.49058
LJM 0509	Amaru	p'osqo, puka kusipati	W, K	-13.22455	-71.48571
LJM 0511	Amaru	puka kusipata	W, K	-13.22455	-71.48571
LJM 0802	Amaru	?	W, K	-13.22455	-71.48571
LJM 0906	Amaru	kusipati	K	-13.2237	-71.49242
LJM 0912	Amaru	yuraq p'osqo	K	-13.2237	-71.49242
LJM 1106	Amaru	yuraq p'osqo	K	-13.2226	-71.4952
LJM 1108	Amaru	kusipata	K, W	-13.2226	-71.4952
LJM 1202	Amaru	kusipati	W, K	-13.2397	-71.49221
LJM 1305	Amaru	kusipata	K, W	-13.22306	-71.49256
LJM 1311	Amaru	p'osqo	K	-13.22306	-71.49256
LJM 1312	Amaru	puka p'osqo	K	-13.22306	-71.49256
LJM 1401	Amaru	oca de khaya	K	-13.22339	-71.49262
LJM 1503	Amaru	p'osqo oca	K	-13.22413	-71.49253
LJM 1701	Amaru	yuraq p'osqo	K	-13.22411	-71.49235
LJM 1702	Amaru	q'ello p'osqo	K	-13.22411	-71.49235
LJM 1904	Amaru	kusipata	K	-13.22472	-71.49339
LJM 2002	Amaru	puka p'osqo	K	-13.22134	-71.50086
LJM 2102	Amaru	kusipati	W, K	-13.22498	-71.49095
LJM 2305	Amaru	p'osqo	K	-13.22451	-71.48543
LJM 2501	Amaru	kusipata	K, W	-13.22393	-71.48555
LJM 2506	Amaru	sambo kishwar	W, K	-13.22393	-71.48555
LJM 2801	Amaru	yuraq p'osqo	K	-13.22439	-71.49158

LJM 2802A	Amaru	puka p'osqo, kusipati	K	-13.22439	-71.49158
LJM 2802B	Amaru	puka p'osqo, kusipati	K	-13.22439	-71.49158
LJM 2902	Amaru	kusipati	K	-13.22122	-71.50095
LJM 2903	Amaru	misitu	W, K	-13.22122	-71.50095
LJM 2904	Amaru	yuraq p'osqo	K	-13.22122	-71.50095
LJM 3104	Amaru	kusipata	K, W	-13.22491	-71.49268
LJM 3206	Amaru	yuraq p'osqo	K	-13.22448	-71.48563
LJM 3207	Amaru	kusipati	K	-13.22448	-71.48563
LJM 3208	Amaru	yuraq p'osqo	K	-13.22448	-71.48563
LJM 3401	Amaru	yuraq kishwar	W, K	-13.22481	-71.49356
LJM 3402	Amaru	q'ello panti	W, K	-13.22481	-71.49356
LJM 3403	Amaru	oca kishwar	W, K	-13.22481	-71.49356
LJM 3404	Amaru	wakankillay	W, K	-13.22481	-71.49356
LJM 3501	Amaru	puka panti	W, K	-13.22264	-71.49385
LJM 3506	Amaru	p'osqo oca	K	-13.22264	-71.49385
LJM 3602	Amaru	khayana ocaya	K, W	-13.22287	-71.49322
LJM 4005	Amaru	khaya apiña	K	-13.22324	-71.49062
LJM 4101	Amaru	kusipatiña	K	-13.22311	-71.49078
LJM 4301	Viacha	p'osqo	K	-13.23284	-71.50557
LJM 4602	Viacha	kusipata	K	-13.23386	-71.51019
LJM 4617	Viacha	p'osqo	K	-13.23386	-71.51019
LJM 4701	Viacha	p'osqo	K	-13.23364	-71.51041
LJM 4801	Viacha	p'osqo	K	-13.23358	-71.51069
LJM 4802	Viacha	kusipata	K	-13.23358	-71.51069
LJM 4901	Viacha	yuraq p'osqo	K	-13.23357	-71.51058
LJM 4902	Viacha	?	K, W	-13.23357	-71.51058
LJM 4903	Viacha	kusipata	K, W	-13.23357	-71.51058
LJM 4904	Viacha	tullu misitu	K, W	-13.23357	-71.51058
LJM 4905	Viacha	puka panti	K, W	-13.23357	-71.51058
LJM 4907	Viacha	misitu	K, W	-13.23357	-71.51058
LJM 4908	Viacha	yana misitu	K, W	-13.23357	-71.51058
LJM 4909	Viacha	yuraq ushpa	K, W	-13.23357	-71.51058
LJM 4910	Viacha	yuraq panti	K, W	-13.23357	-71.51058
LJM 4911	Viacha	yuraq kishwar	K, W	-13.23357	-71.51058
LJM 4912	Viacha	chachapiya	K, W	-13.23357	-71.51058
LJM 4913	Viacha	puka ushpa	K, W	-13.23357	-71.51058
LJM 5009	Viacha	kusipata	K	-13.23215	-71.51064
LJM 5010	Viacha	q'ello p'osqo	K	-13.23215	-71.51064
LJM 5204	Viacha	kusipata	K, W	-13.23234	-71.51013
LJM 5301	Viacha	p'osqo	K	-13.23495	-71.50588

LJM 5303	Viacha	kusipata	W, K	-13.23495	-71.50588
LJM 5401	Viacha	kusipata	K	-13.23275	-71.51051
LJM 5402	Viacha	q'ello k'aytu	W, K	-13.23275	-71.51051
LJM 5504	Viacha	p'osqo	K	-13.23362	-71.51074
LJM 5607	Viacha	kusipata	W, K	-13.23374	-71.51067
LJM 5710	Viacha	kusipata	K, W	-13.23396	-71.51103
LJM 5801	Viacha	kusipati	W, K	-13.23252	-71.51033
LJM 5817	Viacha	p'osqo	K	-13.23252	-71.51033
LJM 5902	Viacha	kusipata	W, K	-13.23206	-71.5058
LJM 5903	Viacha	p'osqo	K	-13.23206	-71.5058
LJM 6002	Viacha	p'osqo oca	K	-13.24186	-71.50459
RB025.3	Viacha	p'osqo	K	-13.24186	-71.50459
RB025.4	Viacha	p'osqo	K	-13.24186	-71.50459

Table 2. 2C nuclear DNA content for *Oxalis tuberosa* accessions.

Method, STD, & Sample Name	STD 2C Peak Mean	Sample 2C Peak Mean	STD Ref Value (Mbp)	Calc Sample Value (Mbp)	Est. Sample 2C Value (pg)	Avg. 2C DNA content per group
LJM 5504	32192.2	68328.1	777.6	1650.459756	1.684142608	1.73964555
LJM 1702	34125.5	73485.6	777.6	1674.478105	1.708651127	
LJM 4801	33468.5	72116.5	777.6	1675.5394	1.709734081	
LJM 5903	28032.7	60431.1	777.6	1676.300298	1.710510508	
LJM 2801	27330.4	58941	777.6	1676.979539	1.711203611	
LJM 1311	31145	67221.3	777.6	1678.320208	1.712571641	
LJM 1503	36833.2	79522.3	777.6	1678.826181	1.71308794	
LJM 4301	34998.4	75703	777.6	1681.981256	1.716307404	
LJM 2305	23893.9	51892.5	777.6	1688.782827	1.723247783	
LJM 5301	36241.8	79106.9	777.6	1697.308783	1.731947737	
LJM 4901	32848.3	72036.7	777.6	1705.285751	1.740087501	
LJM 1106	35906.2	78755.6	777.6	1705.564904	1.740372351	
LJM 5010	36109.4	79370.4	777.6	1709.206551	1.744088318	
LJM 3206	29734.2	65491.5	777.6	1712.714329	1.747667683	
LJM 0912	34378.7	75908.2	777.6	1716.941488	1.75198111	
LJM 5817	29024.1	65112.7	777.6	1744.468753	1.780070156	
LJM 4701	31619.4	71101.8	777.6	1748.570804	1.784255922	
LJM 4617	31064	70140.9	777.6	1755.780448	1.791612702	
LJM 2904	26564.3	61993.3	777.6	1814.690772	1.851725278	
MHG865 (fresh)	42077	174421.9	777.6	3223.387348	3.289170763	3.43015432
MHG865 (dry)	41251.9	173059.9	777.6	3262.186184	3.328761412	
LJM 5402	33280.4	137830.9	777.6	3220.433283	3.286156411	
LJM 4802	38519.7	159988.9	777.6	3229.707621	3.295620021	
LJM 5303	30913.1	128687.8	777.6	3237.062387	3.303124885	
LJM 1312	36199.8	151525.1	777.6	3254.877589	3.321303663	
LJM 1401	30933.1	129500.9	777.6	3255.409249	3.321846172	
LJM 1701	36,008.70	150825.7	777.6	3257.048	3.323518367	
LJM 5710	32078.4	134695.4	777.6	3265.098728	3.331733396	
LJM 0509	34832.3	146357.9	777.6	3267.309452	3.333989237	
LJM 0802	33779.3	142203.3	777.6	3273.52213	3.340328704	
LJM 2802A	30608.3	128954.2	777.6	3276.065182	3.342923655	
LJM 5204	37487.8	158042.3	777.6	3278.231651	3.345134338	
LJM 4902	36199.2	153109	777.6	3288.955513	3.356077054	
LJM 1904	34282.8	145034.9	777.6	3289.671154	3.3568073	
LJM 2506	32540.9	138196.7	777.6	3302.359613	3.369754707	

LJM 4903	34871.2	148209.2	777.6	3304.947175	3.372395076	
LJM 4904	35151.9	149422.9	777.6	3305.40446	3.372861694	
LJM 4912	32832.3	139821.9	777.6	3311.541057	3.379123527	
LJM 4911	28094.6	119734.3	777.6	3313.995988	3.381628559	
LJM 4910	31306.6	133473.1	777.6	3315.233291	3.382891114	
LJM 2902	35457	151237.4	777.6	3316.75557	3.384444459	
LJM 0511	31665	135125	777.6	3318.275699	3.385995611	
LJM 3208	29744.2	127005.1	777.6	3320.28314	3.38804402	
LJM 5607	36219.6	154741.7	777.6	3322.155571	3.389954664	
LJM 2802B	36645	156670.6	777.6	3324.520632	3.392367992	
LJM 5801	31705.6	135887.6	777.6	3332.729794	3.400744688	
LJM 5902	32970.4	141342.1	777.6	3333.523917	3.401555018	
LJM 1202	38826.3	166590.2	777.6	3336.412162	3.404502206	
LJM 3104	33724.7	144799.1	777.6	3338.674033	3.406810238	
LJM 0906	35569.9	152745.8	777.6	3339.203486	3.407350496	
LJM 2002	33,160.90	142,401.30	777.6	3339.211266	3.407358435	
LJM 5009	38611.7	165814	777.6	3339.323739	3.407473203	
LJM 3402	37013.1	159488	777.6	3350.647981	3.419028552	
LJM 6002	32652.6	140853.7	777.6	3354.337392	3.422793257	
LJM 4602	33356.4	144035.6	777.6	3357.738921	3.426264206	
LJM 0403	32743.1	141543.3	777.6	3361.443177	3.430044058	
RB025.4	37130.9	159977.6	782.6	3371.813497	3.440626017	
LJM 4913	31860.5	138176.7	777.6	3372.395346	3.441219741	
LJM 4907	33981.9	147503.3	777.6	3375.28408	3.444167429	
LJM 3602	32713.4	142163.3	777.6	3379.232427	3.448196354	
LJM 1305	40655.4	177855.9	777.6	3401.780522	3.471204614	
LJM 3501	38449.5	168220.6	777.6	3402.081654	3.471511892	
LJM 5401	33536.9	146755.1	777.6	3402.722546	3.472165863	
LJM 4005	33038.5	144803	777.6	3408.109109	3.477662356	
LJM 2102	34151.8	150509.9	777.6	3426.949626	3.496887374	
LJM 2903	40929.8	180514.9	777.6	3429.491135	3.49948075	
LJM 4101	33436.7	147494.6	777.6	3430.117235	3.500119628	
LJM 3207	35569.6	157050.9	777.6	3433.347011	3.503415317	
LJM 3404	34251.6	152070.8	777.6	3452.400883	3.522858044	
LJM 3403	36857.7	164010.2	777.6	3460.181496	3.530797445	
LJM 3506	35773.6	160511.8	777.6	3488.996793	3.560200809	
LJM 2501	39958.4	180093.1	777.6	3504.654705	3.57617827	
LJM 4908	31412.9	141653.3	777.6	3506.508666	3.578070068	
LJM 1108	32583.7	147196.7	777.6	3512.804068	3.584493947	
LJM 0201	33440.7	151443	777.6	3521.51949	3.593387234	
RB025.3	33520.2	151477.4	781.6	3532.041451	3.604123929	
LJM 4909	37351.4	170505.9	777.6	3549.676527	3.622118905	
LJM 3401	37466.4	172334.8	777.6	3576.739171	3.649733848	
LJM 4905	35186	164551.7	777.6	3636.542998	3.710758161	

Fig. 1. Distribution of all estimates of 2C values in samples of *Oxalis tuberosa*. The ranges of the octoploid and tetraploid ocas are discontinuous.

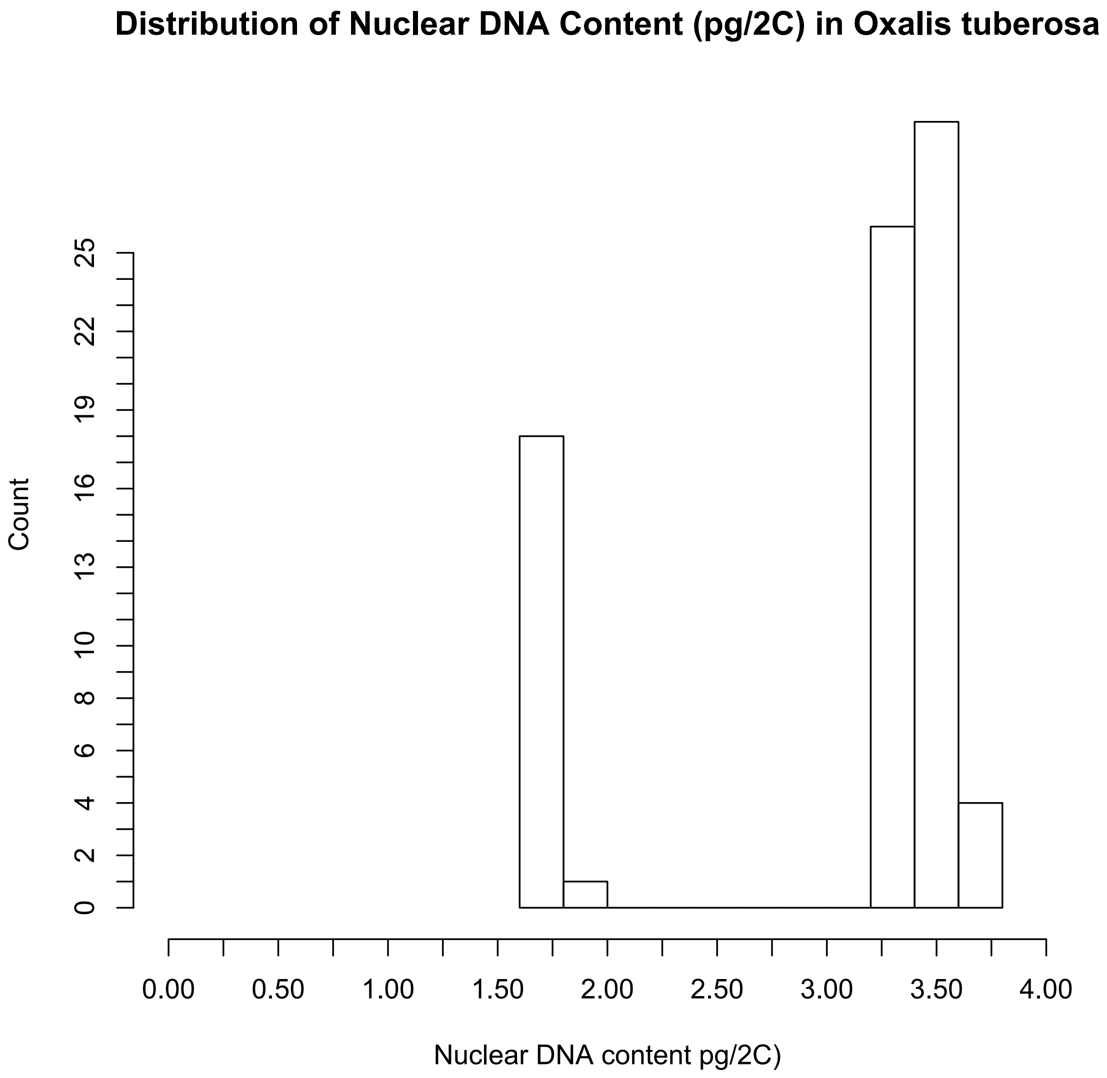


Figure 2a. Histogram of fluorescence at FL2-A of known octoploid *Oxalis tuberosa*. The peak labeled “M1” is the rice standard peak with the peak labeled “M3” being the octoploid oca peak.

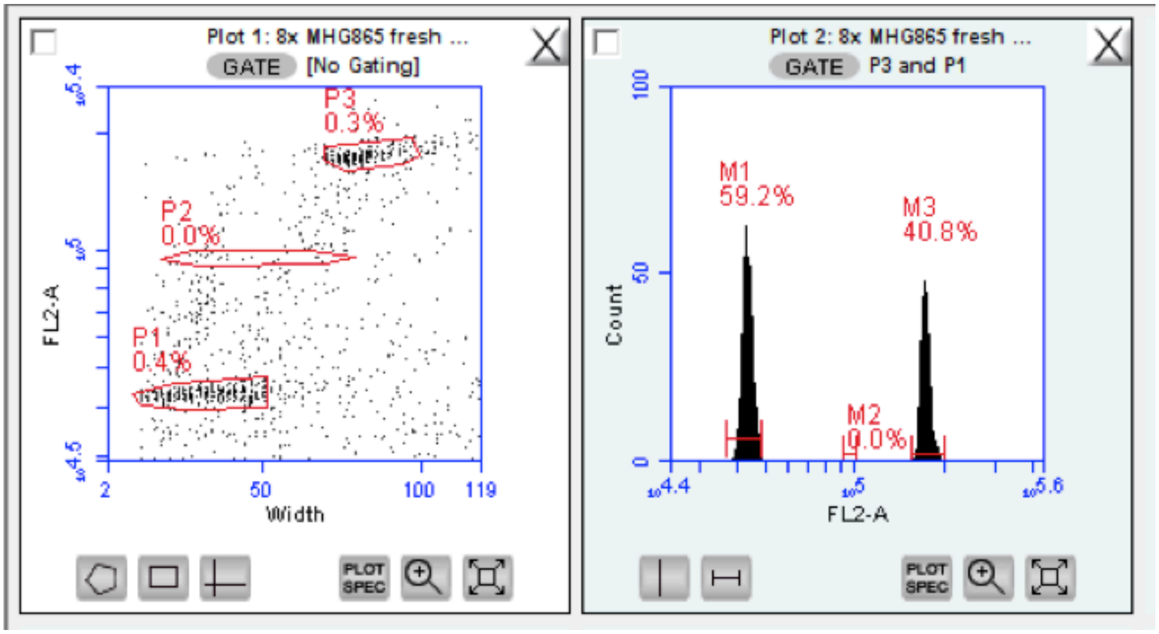


Figure 2b. Histogram of fluorescence at FL2-A of inferred tetraploid. Peak labeled “M1” is the rice standard peak. Peak labeled “M3” is the tetraploid peak.

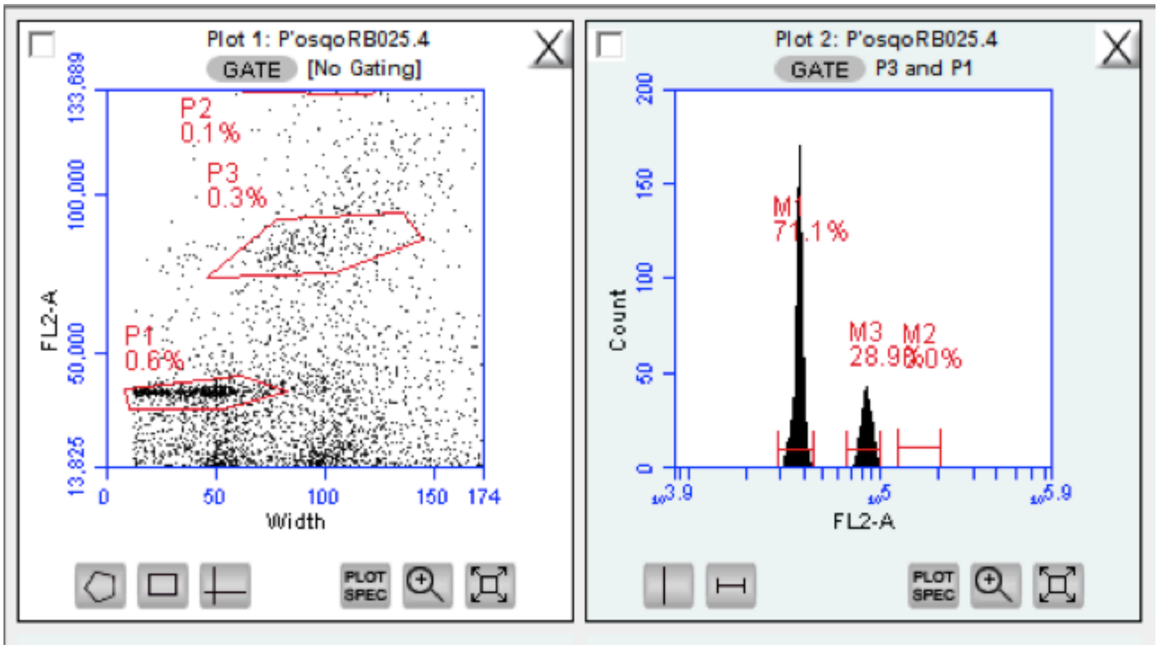
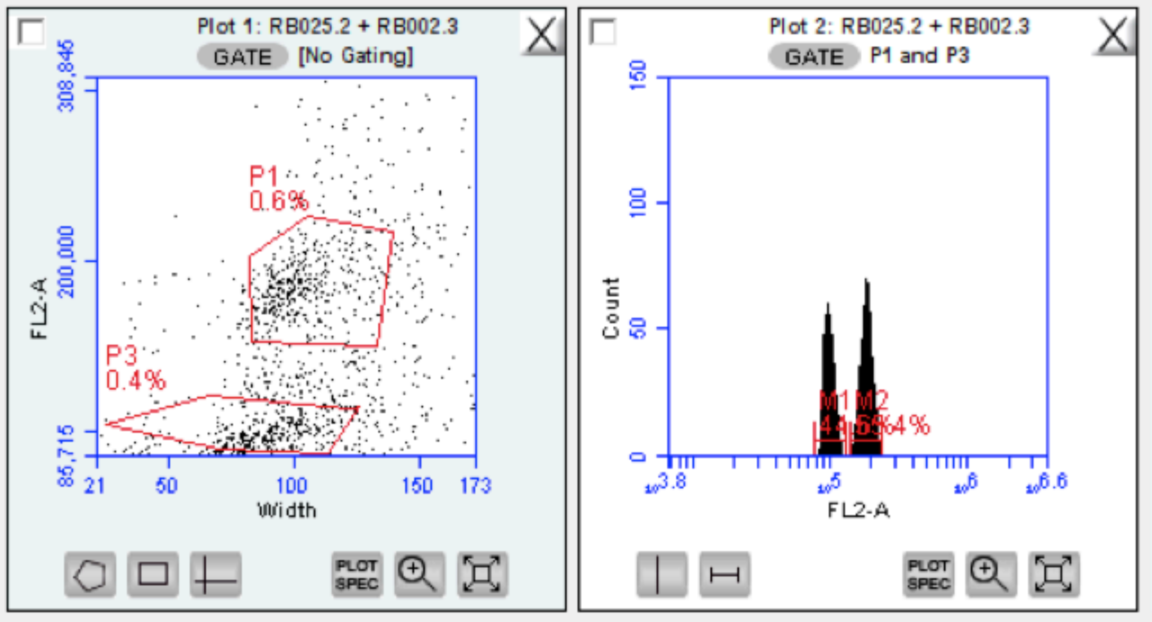


Figure 2c. Histogram of fluorescence at FL2-A of known octoploid and inferred tetraploid *Oxalis tuberosa*. The peak on the left is the inferred tetraploid peak and the peak on the right is the octoploid peak. Notice that the two peaks are non-overlapping.



Conclusion

As stated in my Introduction, my primary goals for my thesis were: 1) to investigate genetic mechanisms of domestication in *Manihot esculenta* and *Oxalis tuberosa*, in particular focusing on the origins of "processed" vs. "unprocessed" cultivars; 2) to determine what, if any, biochemical difference exists between "processed" and "unprocessed" cultivars of oca and if any observable biochemical difference relates to toxicity for humans; 3) to explore the underlying reasons, both cultural and biological, for continued cultivation of toxic domesticates.

Genetic mechanisms of domestication in manioc and oca

Interestingly, though superficially manioc and oca appear to be the result of similar domestication histories, in that they share a similar syndrome of domestication in regards to toxicity and share similar patterns of cultural food practices, my research has shown that this "functional similarity" arose via very different types of domestication histories. Genetic differentiation in manioc appears to be not based on an ancestral pattern of domestication and rather instead on divergent selection via the maintenance of traditional separation of bitter and sweet manioc varieties from one another during cultivation (Mühlen-Santos cite, Bradbury et al., 2013). This, along with other research in the field, suggests that bitter and sweet manioc were both the result of a single domestication event, with secondary breeding that amplified an already naturally broad and continuous distribution of cyanogenesis in the species.

In contrast, my research on oca has shown that sweet and sour ocas did not arise from divergent selection or breeding exclusion via agricultural separation. Though the exact history of oca's domestication remains to be uncovered, at this point a hypothesis involving multiple polyploidization events, or possibly separate origins of polyploidy with a shared progenitor, is the most likely scenario, with a shift in polyploidy accompanying significant drop in oxalic acid levels. In this way, oca is more reminiscent of crops like wheat and the domestication histories of oca and manioc are almost as different as they could be.

I believe this difference between the underlying mechanisms of domestication in oca and manioc to be a significant contribution to our understanding of crop domestication and the effects on crop biochemistry and toxicity. My research demonstrates that the syndrome of domestication in oca and manioc are similar not because of the genetic mechanisms of their domestication, which suggests that instead these crops must fit a similar selective profile, that is, play similar roles in cultural food practices and food security and/or address some fundamental selective pressure in the agricultural ecosystem. This suggests that continued research in this area could be extremely valuable in our quest to understand what it is that people want from their food and how they get it. The "how they get it" portion of this question is now shown to be extremely variable, at least at the genetic level, even when the "what they want" part may be highly similar. Understanding all of the underlying biological mechanisms emergent food and agricultural patterns is critical for our ability to then establish or alter those patterns.

What, if any, biochemical difference exists between "processed" and "unprocessed" cultivars of oca?

This is another way in which oca and manioc differ from one another. Whereas there were very few "ambiguous" cultivars in manioc in the sample we studied, i.e. very few cultivars that were classified as both "bitter" and "sweet", there is a much looser correlation in oca between cultural use-category and actual toxicity of the crop. Indeed, only one subgroup of the two groups of "processed" ocas displayed any difference in biochemistry, that is, greatly elevated levels of oxalic acid. However, it should be noted that in one respect the similarity of the two crops' domestication syndrome is confirmed by the presence of oca cultivars rich in oxalic acid: both crops display cultural food traditions that, at their very root, detoxify otherwise inedible food to make it fit for consumption. While the levels of oxalic acid that I found in oca tubers do not approach the levels required for acute toxicity (as happens in some particularly toxic varieties of manioc), the levels of oxalic acid in tetraploid ocas are well above the threshold that would cause chronic health consequences if consumed regularly without processing.

Additionally, it should be noted that a true evaluation of the strength of correlation of manioc classification (sweet vs. bitter) was not accomplished with my data. In fact, my research contributes additional evidence to suggest that the biochemical difference between bitter and sweet manioc is not as distinct as previously believed. The need for future research in this area is great, particularly in light of recent outbreaks of cyanide-related diseases in several African nations. Cultural food practices and the associated patterns in cognitive ethnobotany can have critical impact on people's health and nutrition and in some respects, the pattern observed in oca is much "safer"—namely, detoxifying ocas that do not need to be, as opposed to with manioc, where we now know that there are many manioc that need to be detoxified, but are not.

Moreover, we should refrain from drawing overly stark conclusions about the lack of biochemical differentiation between Aymara oca use-categories. Given that our sample of Aymara ocas was limited to ocas from Peruvian Aymara and included no ocas from Bolivian Aymara, and that our genetic analyses suggest that our sampling of Aymara ocas is not very diverse, a strong possibility remains that "sour" ocas, either tetraploids with elevated oxalic acid content, or other ocas with other elevated organic acids, could be found with a more diverse sample set. Additionally, though the different levels of polyploidy in the sour ocas (tetraploid) and the sweet ocas (octoploid) are compelling, this correlation has only been observed with one organic acid, namely, oxalic acid. In order to confirm that non-tetraploid ocas truly are biochemically identical in the “processed” and “not processed” categories, future analyses measuring the levels of other organic acids are necessary.

What are the underlying reasons, both cultural and biological, for continued cultivation of toxic domesticates?

Though the majority of my research did not directly address this question, the results of my biochemical and genetic analyses suggest that toxic manioc and ocas may be grown for very different reasons. For manioc, pest resistance favorability of high cyanide cultivars is almost always cited (reviewed in Chapter 1). However, the bitter manioc are also described as being the only manioc suitable to making manioc flour, which is a product that plays an important role in subsistence and food security and also is a product that is culinarily favored. Bitter manioc is often described as, after processing, simply tasting better (reviewed in Chapter 1). So, in some regards, the retention of toxicity in bitter manioc does not represent a conflict between anthropogenic conscious selective “wants” and ecosystemic pressures, because the varieties with elevated cyanide are more pest resistant, play an important role in subsistence, and are regarded as culinarily superior. In fact, the only “conflict” present is also a health one—namely, cyanide is toxic to humans and detoxifying manioc takes a lot of time and energy. I postulate that this is the selective criterion behind the development of sweet manioc, thus allowing societies to maintain a crop that has elevated pest resistance and produces a tasty, storable food product while simultaneously cutting down on intensive food processing time by growing a non-toxic variety suitable for cooking fresh. It would be relatively easy to test this concept, since there are regions of the world where only one kind of manioc is grown in exclusion of the other and variables relating to pest prevalence and the need for storable food products could be assessed in each of the regions.

In contrast, I can find no evidence for why high-acid ocas continue to be grown, and, in fact, it appears that they are, based on current sampling, very limited in geographic distribution. Whereas bitter manioc are the only ones capable of making a culinarily valued storable food product, I found a much higher percentage of ocas used for *khaya* are sweet rather than sour. Additionally, my research showed no evidence for greater pest resistance among sour ocas than sweet ocas, though ethnographic data suggests that farmers often perceived sour ocas as growing better and having fewer pests. Though my data show no difference in oca weevil prevalence, I do not believe we should wholly discount the farmers' perceptions. My pest resistance studies were secondary to my primary aim and were only focused on post-harvest prevalence of oca weevils. Elevated pest resistance in sour ocas easily could be more strongly related to foliar pests or microbial pests, neither of which were evaluated in my studies. Additionally, there could be a link between oxalic acid, particularly soluble oxalic acid, and calcium accumulation in a traditionally calcium-depleted ecosystem. As calcium plays an important role in frost resistance in plants, high-altitude crops of the Andean *altiplano* could benefit greatly from increased efficiency of calcium accumulation. Additional studies into the physiological role of oxalic acid in tetraploid oca tubers strongly merits additional investigation. Similarly, since all of this together calls into question the traditional utility of tetraploid ocas for food processing and/or pest resistance, ethnographic surveys of farmers who maintain tetraploid ocas are highly needed to continue to develop hypotheses relating to the selective pressures that may have contributed to the retention of domesticated sour ocas.

The broader picture

Often, it is difficult to see how a doctoral dissertation truly contributes to the broader dialogue on fundamental scientific concepts. Similarly, many may wonder how the investigation of toxicity in manioc and oca will successfully help our species overcome the agricultural challenges we are facing. However, my research has demonstrated several broadly important concepts.

First, it reinforces traditional agricultural systems as good systems to study for understanding the fundamental nature of domestication and cultural food practices. *Subsistence* is no longer a primary aim of modern, industrialized agricultural systems. The prevalence of monocropping alone reveals this—no subsistence farmer would dream of putting all of their “eggs in one basket”, or rather, all of their “calories in one crop”. When investigating the motivations behind maintenance of toxic domesticates in manioc and oca, the primary concerns of the agriculturalists are almost always subsistence related—the ability to process food to store it during the non-growing season and for future years to guard against possible crop failures and/or the ability of a crop to yield well in relation to predation. In fact, there are many other crops that are fit this same cultural purpose and also display a similar syndrome of domestication (e.g. bitter melon and bitter almond).

Some of these crops even persist in cultures dominated by industrialized agriculture, such as sweet and sour cherries. Sour cherries are often vaunted for their superior culinary quality in processed foods, such as canned, juiced, or cooked, whereas sweet cherries are traditionally eaten fresh. Similar to the sour ocas, however, distribution of sour cherries is dramatically more limited than sweet cherries and continues to shrink. The fact that these crops that display this “split” syndrome of domestication have greater cultural value when subsistence is a primary concern (such as in traditional systems for manioc and oca or historical contexts like cherries) suggest that there is an important set of selective pressures in the agricultural system that changes dramatically when subsistence is no longer the primary focus of the enterprise.

Additionally, this research helps us focus future research endeavors on the most pertinent aspects of investigations. In the end, if we are going to be forced to rapidly adapt our agricultural system to a new set of selective pressures that are more similar to those found in existing subsistence scenarios, then we are going to need to investigate more specifically how these selective pressures affect crop biology. Previously, we had been operating on the assumption that crop toxicity is advantageous to the plant because of decreasing predation. However, the evidence presented and discussed in this thesis suggests that this may be not as important as previously thought and emphasizes the need for future studies that directly address these questions. That being said, based on the concepts presented here, perhaps future research should focus first on a crop's utility for storage and food security, since these seem to be the primary anthropogenic selective pressures involved in these systems and only sometimes correlated with elevated suitability of the crop for the agricultural ecosystem. Certainly, this research underscores the necessity of ethnographic studies of crop importance. Whereas we had previously been assuming that a crop would be important for one reason, such as pest resistance, my work on oca has shown that we know very little about why the high-acid ocas are grown.

Overall, this research suggests that understanding what people want from their food and how they get it is a highly complicated and diverse enterprise. Though there may be over-arching patterns that emerge from additional study of these crop systems and others, the primary contribution of this work is to demonstrate that "what people want from their food" is surprisingly poorly understood and "how they get it" consists of, at times, extraordinarily different evolutionary mechanisms. In order to have the most complete and diverse set of tools with which to address future threats to the agricultural ecosystem, we must continue to explore the selective forces and desires of agriculturalists around the globe and the evolutionary mechanisms by which these desired ends are achieved.