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Landscape Genetics of *Phaedranassa* Herb. (Amaryllidaceae) in Ecuador

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

LANDSCAPE GENETICS OF *PHAEDRANASSA* HERB. (AMARYLLIDACEAE) IN
ECUADOR

A dissertation submitted in partial fulfillment of the

requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOLOGICAL SCIENCES

by

Nora Helena Oleas Gallo

2011

To: Dean Kenneth Furton
College of Art and Sciences

This dissertation, written by Nora Helena Oleas Gallo, and entitled Landscape Genetics of *Phaedranassa* Herb. (Amaryllidaceae) in Ecuador, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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Date of Defense: June 30, 2011

The dissertation of Nora Helena Oleas Gallo is approved.

Dean Kenneth Furton
College of Arts and Sciences

Interim Dean Kevin O' Shea
University Graduate School

Florida International University, 2011

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DEDICATION

To my family, Luciana and Cristian

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ABSTRACT OF THE DISSERTATION
LANDSCAPE GENETICS OF *PHAEDRANASSA* HERB. (AMARYLLIDACEAE) IN
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Nora Helena Oleas Gallo

Florida International University, 2011

Miami, Florida

Professor Javier Francisco-Ortega, Major Professor

Speciation can be understood as a continuum occurring at different levels, from population to species. The recent molecular revolution in population genetics has opened a pathway towards understanding species evolution. At the same time, speciation patterns can be better explained by incorporating a geographic context, through the use of geographic information systems (GIS). *Phaedranassa* (Amaryllidaceae) is a genus restricted to one of the world's most biodiverse hotspots, the Northern Andes. I studied seven *Phaedranassa* species from Ecuador. Six of these species are endemic to the country. The topographic complexity of the Andes, which creates local microhabitats ranging from moist slopes to dry valleys, might explain the patterns of *Phaedranassa* species differentiation. With a Bayesian individual assignment approach, I assessed the genetic structure of the genus throughout Ecuador using twelve microsatellite loci. I also used bioclimatic variables and species geographic coordinates under a Maximum Entropy algorithm to generate distribution models of the species. My results show that *Phaedranassa* species are genetically well-differentiated. Furthermore, with the exception of two species, all *Phaedranassa* showed non-overlapping distributions.

Phaedranassa viridiflora and *P. glauciflora* were the only species in which the model predicted a broad species distribution, but genetic evidence indicates that these findings are likely an artifact of species delimitation issues. Both genetic differentiation and non-overlapping geographic distribution suggest that allopatric divergence could be the general model of genetic differentiation. Evidence of sympatric speciation was found in two geographically and genetically distinct groups of *P. viridiflora*. Additionally, I report the first register of natural hybridization for the genus. The findings of this research show that the genetic differentiation of species in an intricate landscape as the Andes does not necessarily show a unique trend. Although allopatric speciation is the most common form of speciation, I found evidence of sympatric speciation and hybridization. These results show that the processes of speciation in the Andes have followed several pathways. The mixture of these processes contributes to the high biodiversity of the region.

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CHAPTER I.

INTRODUCTION

Taxonomic history of *Phaedranassa*

Phaedranassa is a small genus of Amaryllidaceae established by Herbert (1845) from a specimen originally named *P. chloracra*. The name *Phaedranassa* is derived from the greek “phaidos” (gay) and “anassa” (queen) (Quattrocchi 2000). In its native Ecuadorian highlands it is known as “ashpa cebolla” in Quichua (fake onion), “cebolleta” or “papa de lobo” in Spanish (fox’s potato). Twenty two species have been described in this genus (www.tropicos.com). *Phaedranassa chloraca* was later considered synonymy and changed to *P. dubia* by Macbride (1931), because of the description of a previous basionym *Haemanthus dubius* H.B.K. (1815). Lindley (1845) presented a figure and a description of *Phaedranassa chloracra*, which share the same description as Herbert’s (1845) and was published at the same volume. However, Lindley’s description is supposed to be from a specimen collected at Saraguro in Loja whereas Herbert’s corresponded to a specimen collected in Guayllabamba. Other synonyms of *P. dubia* are *Collania dubia* Schult. f. (1830), *Crinum quitense* Spreng. (1825), *Phycella obtusa* (Herbert) Lindley (1844) and *Phaedranassa obtusa* Herb (1845). During the late 1800’s, Baker described four new species of *Phaedranassa*: *P. carmioli* in 1869, from a cultivated specimen from Costa Rica; *P. viridiflora* (1877); *P. ventricosa* (1887) and *P. schizantha* (1880). Through the same period, Regel (1883) described *P. lehmanni* from material collected in Colombia. Kraenzl (1917) described *P. megistophylla*, which was later changed to *Rauhia multiflora* (Ravenna 1969). Ravenna (1969) published a synoptic treatment of *Phaedranassa* in which he recognized eight species in the genus: *P.*

carmioli, *P. dubia*, *P. lehmanni*, *P. schyzantha* (with “y” in the original paper), *P. viridiflora*, *P. eucrosoides*, *P. tunguraguae*, *P. loxana* (the latter two as new species). He also presented the first key for the genus, and he assigned *Phaedranassa* to the Tribe Stenomessaeae, recognizing the genus as related to *Rauhia*, *Stenomesson*, *Eucrosia* and *Paramongaia*. Ravenna (1984) described *P. cinerea* and *P. viridilutea*. Meerow (1987b) described *P. brevifolia* and proposed three changes: *P. eucrosoides* as a synonym of *Eucrosia stricklandii* var. *stricklandii* (Meerow 1987a), *P. loxana* as a synonym of *Eucrosia stricklandii* var. *montana* Meerow (Meerow 1986) and *P. viridilutea* as *P. viridiflora*. Meerow (1990) treated Ecuadorian Amaryllidaceae, describing a new species: *P. glauciflora* and a new variety, *P. schizantha* var. *igneae*. He also provided a new key for the Ecuadorian *Phaedranassa* species with descriptions of each species and notes about geographic distribution and habitat (Meerow 1990). Currently a total of nine species and one variety are recognized in *Phaedranassa* (Govaerts 2011) (Table 1). In this research I follow Meerow’s (1990) species classification.

Phylogenetic relationships

A phylogenetic study of the internal transcribed spacers (ITS) of nuclear ribosomal DNA revealed that *Phaedranassa* is part of a subclade in the tetraploid-derived Andean clade (n=23) of the American Amaryllidaceae (Meerow et al. 2000). The Amaryllidaceae has a total of 59 genera and 850 species worldwide (Meerow and Snijman 1998). In Ecuador there are 11 genera and 32 species (Jørgensen and León 1999). *Phaedranassa* was resolved as a well-supported monophyletic group within the tribe Eucharideae, a clade representing all of the petiolate-leafed Andean genera (Meerow et al. 2000). *Rauhia* resolved as a sister taxa of *Phaedranassa* (Meerow et al. 2000). In a

previous cladistic analysis of plastid *rbcL* and *trnL-F* genes, *Eucrosia* resolved as a sister taxon of *Phaedranassa* (Meerow et al. 1999). The phylogenetic relationships among *Phaedranassa* species are unknown.

Overview of the study organism

Phaedranassa species are hysteroanthous geophytes. They produce bulbs with a brown tunic and contractile roots. They have one or two petiolate leaves that are usually absent at flowering. The lamina is elliptic to lanceolate with an acute apex and an attenuate base that tapers towards the petiole (Meerow 1990). The leaves have a prominent central vein throughout their length and are sometimes glaucous. Glaucous leaves, as well as the size and the color of the leaves, are features that vary among species and have been used in taxonomic keys (Meerow 1990).

Phaedranassa develops a scape, with a pseudo-umbellate inflorescence consisting of six to 20 tubular flowers. The flowers in *Phaedranassa* are actinomorphic and perfect. They have six tepals, usually green at the base and apices, with an intervening area of red, pink, yellow or orange. The flowers have six free stamens adnate at the base to the tepals. The staminal filaments are 3-seriate in length and the anthers are yellow or green. The stigma is capitate. The ovary is inferior and 3-locular with multiple ovules. The fruit is a loculicidal capsule with many flat black or dark brown winged seeds. Many of the floral characters (such as color, size and number of the flowers, color of the filaments) are used to identify species (Meerow 1990).

A new alkaloid called fedranamina has been found in *P. dubia* (Osorio 2008); and some anti-parasitic effect against *Plasmodium falsiparum* has been reported from other alkaloids of this species (Osorio 2008).

Biogeography and Ecology

Phaedranassa is found in Ecuador, Colombia and Costa Rica (Meerow 1990).

Three species have been recorded in Colombia: *P. lehmani*, *P. ventricosa* and *P. dubia* (Meerow pers com); the latter is also distributed in the Northern part of Ecuador.

Phaedranassa carmioli was described from a cultivated specimen that was reportedly collected in Costa Rica and apparently recollected later (according to records in Herbertia 1940, according to Ravenna 1969). Information in the TROPICOS database (www.tropicos.org), cite a collection by Leon, which is reportedly deposited in the National Herbarium in San Jose, Costa Rica (CR). However, I did not find the specimen in the museum during my visit to CR in 2005. Six species are endemic to Ecuador (*P. brevifolia*, *P. cinerea*, *P. glauciflora*, *P. schizantha*, *P. tunguraguae* and *P. viridiflora*).

In Ecuador, *Phaedranassa*'s populations are isolated and occur in one of the most deforested regions of the country, where little native vegetation is remaining (Valencia et al. 1999). Originally, these species lived in areas that corresponds to Cloud Forest and Dry and Wet Inter-Andean Shrubs (Valencia et al. 1999); these areas are now dedicated to agriculture, mining or, increasingly residential development. The species have never been found in a forest, and their natural habitat might be any open spot, such as between rocky mountain slopes or near rivers deep canyons flanks. In general, each species is restricted to a particular valley (Fig. 1). It seems that certain species have adapted to particular climate conditions (e. g., *P. cinerea* and *P. tunguraguae* are found in moist habitats, while *P. brevifolia* and *P. dubia* inhabit mesic areas). However, the specific climatic tolerance of each species is unknown and studies are needed to more accurately define their ecological niche.

Understanding plant evolution in the tropical Andes: a landscape genetics approach

The tropical Andes have been recognized as a biodiversity hotspot on the basis of their confluence of species endemism and degree of threat (Brummit and Lughadha 2003; Mittermeyer et al. 1999; Mittermeyer et al. 1998). Within the hotspot, Ecuador is acknowledged as a megadiverse country with probably the highest concentration of species per area in the world (Mittermayer et al. 1999). More than 4000 endemic plant species have been reported from the country, of which more than half are restricted to the Andes (Valencia et al. 2000). The current variety of habitats in Ecuador and consequent species richness has been influenced by Andean geological history (Hughes and Eastwood 2006; Jørgensen et al. 1995; Luteyn 2002). However, the biological richness in this area is not matched by a commensurate level of understanding of speciation processes in the region.

In general, it has been understood that mountains constitute either bridges for plant colonization or isolating barriers, both of which play a role in speciation (Hewitt 2004; Ohsawa and Ide 2007). The inter-Andean valleys are naturally isolated from each other because of deep and narrow topographic contours (Young et al. 2002). This topographic complexity creates local microhabitats ranging from moist slopes to dry valleys.

The recent molecular revolution in population genetics has created a pathway towards answering evolutionary questions that were previously difficult to address, such as how species evolve (Barraclough and Vogler 2000). It has been proposed that there is a continuum of evolutionary processes that influence genetic variation at different levels, from populations to species to higher level taxa (Carstens et al. 2004). One of the most

problematic questions of speciation is the geographical model of speciation (Losos and Glor 2003). There are two different views of speciation: sympatric and allopatric. In general terms, sympatric speciation occurs when competition among populations leads to ecological niche differentiation, while allopatric speciation requires a spatial barrier leading to genetic isolation (Mayr 1963). Parapatric speciation is somehow in between both models because it allows speciation to occur even with migration among adjoining populations (Gavrilets et al. 2000). With the increased availability of molecular tools as well as geographic information systems (GIS), it is now possible to study the genetic variation in a geographic context, an approach called landscape genetics (Manel et al. 2003).

Population genetic studies in plants

Molecular markers changed the scenery of population genetic studies. In conjunction with the neutral theory, they allowed a new understanding of isolation and migration in populations (Frankham et al. 2002). Microsatellites (SSRs), one of these molecular markers, are two to six nucleotides that are repeated many times in tandem and that display high dissimilarity in repeat number among individuals. For nearly two decades, DNA microsatellites have been used as a preferred method to address population genetic inquiries in plants (Morgante and Olivieri 1993, Chase et al. 1996, Aldrich et al. 1998, Ouborg et al. 2010). They are popular in part because SSRs are highly reproducible among laboratories. Because they are codominant, it is possible for SSRs to distinguish homozygotes and heterozygotes. They are also highly variable and relatively easy to analyze, and they occur regularly throughout the genome (Frankel et al.

1995; Cruzan 1998; Rossetto et al. 1999; Susol et al. 2000; Zane et al. 2002; Squirrell et al. 2003).

Molecular markers are powerful tools for species conservation because they can be used to estimate levels of genetic variation among and within populations (Frankham et al. 2002). The amount of neutral genetic variation in a population is caused by the equilibrium between drift (loss of variability) and mutation (gain of variability) (Frankham et al. 2002). In general, it is expected that without migration from external sources, populations with a reduced number of individuals will experience an increase of genetic drift and increase of inbreeding (Frankham et al. 2002). Such data are important to better design conservation strategies to preserve genetic diversity, which provides species with the means to evolve and adapt in a changing environment (Geffen et al. 2006).

Despite the advantages of molecular markers, studies using them to address the conservation of undomesticated plants from the tropical Andes are largely lacking. Only two studies with microsatellites of threatened plant species of the tropical Andes have been published. One concerned the microevolution of the wax palm (*Ceroxylon echinulatum* Galeano, Arecaceae) (Trenel et al. 2008), and another addressed mating patterns and their role in genetic variation of the Andean Oak (*Quercus humboldtii* Bonpl., Fagaceae) (Fernandez and Sork 2005).

Modeling species distribution of tropical plants

Understanding landscape genetics requires knowledge of species distributions across the landscape. Species Distribution Models (SDMs) are a general suite of models that estimate the ranges of species on the basis of relationships between the species'

probability of occurring and environmental factors such as temperature, precipitation, seasonality, soil type, etc. Because of the multiple applications of SDMs, these methods have rapidly gained in popularity and are in widespread use by ecologists, biogeographers, conservation scientists and practitioners.

In recent years there has been an increase in the availability of species occurrence data on the World Wide Web (Canhos et al. 2004, Soberón and Peterson 2004, Jiménez-Valverde et al. 2008, Feeley and Silman 2011). International initiatives' such as the Global Biodiversity Information Facility (GBIF <http://www.gbif.org>), SpeciesLink (<http://splink.cria.org.br/>), Mantis (<http://insects.oeb.harvard.edu/mantis/>) and others, have compiled data from thousands of disparate sources (e.g., herbarium and museum collections and sighting records), allowing users to rapidly acquire vast amounts of occurrence data. As a result of the low cost and ease of acquiring data and generating distributions along with their many potential important applications (e.g., conservation of endangered species, prediction of invasive species, propagation, and predicting species distributions under different past and future climate change scenarios [Peterson 2003, Martinez-Meyer 2005; Powell et al. 2005; Guisan et al. 2006; Peterson 2006; Thuiller et al. 2006; Peterson and Nakazawa 2008; Feeley and Silman 2010]). Species Distribution Models have rapidly gained in popularity and are in widespread use by ecologists, biogeographers, conservation scientists and practitioners.

However, accessibility to data may come at the expense of data quality or reliability. In general, SDMs rely on three primary components: (1) species occurrence data, (2) environmental variables and (3) the underlying statistical model. The accuracy of the SDM's range predictions will be directly influenced by the accuracy of these three

components. In the case of species occurrence data, there are several issues associated with data obtained from large online databases and natural history collections that may limit their utility in SDMs (Newbold 2010). For example, one problem linked with historical records from herbarium and museums specimens is the lack of geographical coordinates - information that is essential for the implementation of SDMs (Beaman and Conn 2003). Geographic coordinates can often be obtained from location data, that accompany the records, in conjunctions with maps, gazettes or software (Guralnick et al. 2006; Murphey et al. 2004); however, in many instances the geographical information found in the specimen labels/annotations is vague, and/or of uncertain accuracy.

General overview and outline of the dissertation

The goal of my dissertation is to contribute to a better understanding of speciation in the tropical Andes, one of the most diverse places on Earth. The specific objectives of my dissertation are to (1) unravel intra-specific patterns at the population level in Ecuadorian *Phaedranassa* and (2) investigate the connection between the genetic differentiation among species and spatial distribution patterns.

The results of my dissertation are presented in three chapters (Chapters II to IV). In Chapter II, I report the development of microsatellite markers from *P. schizantha*. This chapter is published in Conservation Genetics (Oleas et al. 2009). Chapter III corresponds to an empirical assessment of the use of species' occurrences for the development of Species Distribution Models using three different sources: (a) databases only, (b) specialist evaluation and (c) field corroboration. Chapter III. In Chapter IV, I discuss the species genetic patterns in a geographic context. Finally in Chapter V, I summarize the conclusions of my investigation.

Table 1. Recognized species of *Phaedranassa* Herb. (Amaryllidaceae)

Species name	Author	Year of publication
<i>P. brevifolia</i>	Meerow	1987
<i>P. carmioli</i>	Baker	1869
<i>P. cinerea</i>	Ravenna	1984
<i>P. dubia</i>	(HBK) J.F. Macbride	1931
<i>P. glauciflora</i>	Meerow	1990
<i>P. lehmanni</i>	Regel	1883
<i>P. schizantha</i> var. <i>schizantha</i>	Baker	1880
<i>P. schizantha</i> var. <i>ignea</i>	Meerow	1990
<i>P. tunguraguae</i>	Ravenna	1969
<i>P. ventricosa</i>	Baker	1887
<i>P. viridiflora</i>	Baker	1877

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CHAPTER II.

EIGHT MICROSATELLITE LOCI IN *PHAEDRANASSA SCHIZANTHA* BAKER (AMARYLLIDACEAE) AND CROSS-AMPLIFICATION IN OTHER *PHAEDRANASSA* SPECIES

Abstract

Phaedranassa schizantha is a species endemic to Ecuador from which eight polymorphic microsatellite loci were isolated from an enriched genomic library. A total of 31 alleles with an average of four alleles per locus were detected across 29 individuals from a single natural population of *P. schizantha*. Observed heterozygosity ranged from 0.05 to 0.68. Most of the eight loci were successfully amplified in six *Phaedranassa* species. Five of those species are either “Vulnerable” or “Endangered” under IUCN criteria. These loci will be used to investigate patterns of inter- and intraspecific variation of *Phaedranassa* species, which will contribute data relevant to their conservation status.

Keywords: Microsatellite primers, *Phaedranassa schizantha*, Ecuador, Amaryllidaceae, Andes, Endangered species, Conservation, Population genetics

Phaedranassa Herb. is a small monophyletic genus restricted to the dry valleys and wet slopes of the northeast Andes (Meerow 1990; Oleas 2000a). Ten species are known of which six are endemic to Ecuador. Because of their small population numbers as well as habitat alteration, these six species are considered either endangered or vulnerable to extinction according to the IUCN Red List (Oleas 2000b). In order to design adequate strategies for their conservation, it is necessary to understand their population genetic structure. In this paper, I report new primers generated from genomic DNA of

Phaedranassa schizantha, developed in an order to increase the number of microsatellite loci available to study the population genetic structure across the genus in Ecuador.

A microsatellite-enriched library was built with methods described previously (Oleas et al. 2005) using DNA of *P. schizantha*. All cloning of isolated fragments used the pGEM-T and pGEM-T Easy Vector Systems (Promega, Madison, WI, USA) following manufacturer's protocols. The PCR primer sequences were designed with Primer 3 software (Rozen and Skaletsky 1998) using the default options with the exception of the primer GC content range of 40-80, self-complementarity and 3' self-complementary of 5 and CG clamp of 1. Amplification and polymorphism were screened by PCR cold primers using dUTPs. Polymerase chain reactions were carried out with polymorphic loci in a 10 µl reaction mix containing 1 µl template DNA (~20ng/µl), 6.25 µl dH₂O, 1 µl of standard Buffer (New England BioLabs, Inc. Ipswich, MA, USA), 0.25 µl 10mM dNTP, 0.05 µl Taq (5U/µl) (New England BioLabs, Inc. Ipswich, MA, USA), 0.25 µl fluorescent 5' end-labelled forward primer (10µM), 0.25 µl reverse primer (10 µM). The PCR temperature profile consisted of 5 min at 94 °C; 45 s at 94 °C; 45 s at 60 °C; 60 s at 72 °C; steps 2 repeated 14 times (<1 degree/cycle); 45 s at 46 °C; 60 s at 72 °C; steps 6 though 8 repeated 25 times and 4 °C storage. The products were genotyped in an ABI 3730 Genetic Analyzer (Applied Biosystems Inc. Carlsbad, CA, USA) and allele size identified by capillary gel electrophoresis. Allele calls were made in GeneMapper 4.0 (Applied Biosystems Inc. Carlsbad, CA, USA). Descriptive statistics, exact tests for Hardy-Weinberg equilibrium and linkage disequilibrium were calculated with Arlequin 3.1 (Excoffier et al. 2005).

Overall, 74 unique microsatellite-containing sequences were identified through sequencing the inserts of 288 positive clones. Almost half of the sequences were not suitable for primer design mainly because of short flanking region on either side of the repeats, inadequate GC content, lack of CG clamp or too high or too low melting temperature (T_m). A total of 34 sequences were suitable for primer design, from which sets of primers were screened by PCR using dUTPs. Fluorescently labeled forward primers were obtained from 18 sets of primers out of the 34 that amplified and showed polymorphism in eight individuals.

Ultimately, eight primer pairs were selected that amplified highly polymorphic repeats (Table 1). An average of 3.9 alleles per locus was detected, and observed heterozygosity ranged from 0.048 to 0.679. With the exception of ps9, ps16, and ps33 all the loci departed significantly from Hardy-Weinberg Equilibrium in my test population. A total of 23 out of the 28 comparisons between loci showed linkage disequilibrium ($P < 0.001$). An explanation for this high level of linkage disequilibrium could be that *Phaedranassa* species are inbred, self-compatible and reproduce clonally by bulbs (Oleas unpublished data). Furthermore, *Phaedranassa*'s populations are small, living in a mosaic of agricultural and rural landscapes. Recent bottlenecks in the history of the populations could also contribute to non-random association of alleles among loci (Frankham et al. 2002). With the exception of ps4, ps9 and ps33 with *P. glauciflora*, loci were amplified in other *Phaedranassa* species (Table 2). Furthermore, most of them showed high levels of polymorphism, which make them useful for the investigation of genetic variation and conservation of *P. schizantha* and other species of *Phaedranassa*.

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Table 1. Primer sequences and basic descriptive statistics of eight microsatellite loci from *Phaedranassa schizantha*.

Locus	GenBank accession no.	Forward primer (5'-3')	Reverse primer(5'-3')
Ps2	FJ427510	TCTTGATGTACGCCCTCGG	TTTCGGCTCACACCCTCC
Ps4	FJ427511	ATGAAGGGGAGGCCAAAGG	ACCACACACTACACCCACC
Ps9	FJ427512	CCGCCCCGATTACCCAACTC	CACGCGTTGCCATATTAAGC
Ps13	FJ429258	AGCTTGGAAGAGGTCAGGC	AGCTTTCTCACATCTTGGCTTC
Ps16	FJ429259	TGGACATTGAAGTCGAACTCTTATC	TCGTGTCAACAAATATCAAGTGTG
Ps27	FJ571145	TCCTGGTGTTTCTTCCCCTC	TGAATTGTCTGAAGTCTACTACTGC
Ps28	FJ429260	TGGCAATCAACCAAAGGCTC	GCTTGGCATCTCCTCTTTGC
Ps33	FJ429261	TGACGACGACCCTATCTTCC	TTGATTCCGCGATTACAGGC

Table 1. (continued)

Locus	Repeat	<i>n</i>	A	Allele size range (bp)	<i>H_O</i>	<i>H_E</i>
Ps2	(GA) ¹⁶	29	5	193-207	0.655	0.731***
Ps4	(AG) ⁹	29	3	148-162	0.483	0.580***
Ps9	(CT) ¹⁰	29	5	189-213	0.571	0.518 ^{ns}
Ps13	(AG) ⁷	19	3	217-225	0.158	0.243**
Ps16	(AAG) ⁷	29	3	176-185	0.517	0.490 ^{ns}
Ps27	(CT) ¹²	21	5	151-203	0.048	0.505***
Ps28	(AAG) ⁷	28	2	203-206	0.679	0.456*
Ps33	(GA) ⁸	29	5	166-182	0.643	0.529 ^{ns}
Mean		26.4	3.9		0.469	0.498

n, number of samples, *H_E*, expected heterozygosity; *H_O*, observed heterozygosity; A, Number of Alleles. Departure from HWE: ns=not significant, * P<0.05, ** P<0.01, *** P<0.001

Table 2. Cross-amplification of eight sets of microsatellite primers of *Phaedranassa schizantha*.

Locus		<i>P. brevifolia</i>	<i>P. cinerea</i>	<i>P. dubia</i>	<i>P. glauciflora</i>	<i>P. tunguraguae</i>	<i>P. viridiflora</i>
Ps2	n	8	7	7	6	7	8
	A	6	3	6	4	4	1
	Range	167-187	189-215	191-211	191-197	189-199	191-215
Ps4	n	6	4	8	0	6	28
	A	8	2	7	-	5	5
	Range	130-168	148-150	122-162	-	142-154	130-152
Ps9	n	5	3	6	0	8	8
	A	5	2	3	-	8	1
	Range	205-237	183-201	201-206	-	177-213	209
Ps13	n	8	7	7	7	8	12
	A	5	2	6	5	5	7
	Range	211-227	219-221	221-241	213-225	213-235	183-227
Ps16	n	8	8	6	8	8	19
	A	3	2	3	3	1	3
	Range	179-185	176-182	179-185	179-185	179	176-185
Ps27	n	7	6	8	6	8	8
	A	3	6	7	2	1	3
	Range	187-193	121-189	157-203	187-201	189	149-187
Ps28	n	8	5	8	7	8	8
	A	4	3	4	3	1	1
	Range	164-206	122-206	155-206	194-206	203	200
Ps33	n	3	7	7	0	8	8
	A	5	3	3	-	4	1
	Range	170-198	172-188	178-186	-	120-182	186

n, number of amplified samples (maximum 8); A, Number of Alleles.

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CHAPTER III.
MUDDY BOOTS BEGET WISDOM: A CAUTIONARY TALE FOR SPECIES
DISTRIBUTION MODELS ON ENDANGERED SPECIES

Abstract

Aim: I aim to investigate the impact of species occurrence data quality on estimates of species ranges. I highlight the data quality impact in poorly collected plant species using tropical endangered plants.

Location: Northern Andes, Ecuador

Methods: I used species distribution models (SDMs) to predict potential species distributions for seven species of *Phaedranassa* (Amaryllidaceae) with three progressively edited species occurrence sources: (1) database, which are occurrence records available online; (2) taxonomy-based, specimens which species identification was revised by experts and (3) field-based records taken with GPS in situ.

Results: The SDMs generated out of different occurrence record sources did not generally agree. The SDMs with species occurrence data from records obtained from online databases performed worse than the taxonomically-revised and the field-based models.

Main conclusions: In order to generate accurate models, species occurrence records to be used for SDMs need to be carefully evaluated with: (1) appropriate filters (e.g., altitude range, ecosystem); (2) taxonomy monographs and/or specialist corroboration; and (3) confirmation in the field. This study is a cautionary tale that speaks against extrapolating the results of SDMs produced with unverified online records to species' extinction risks associated with climate change.

Key words: Andes, Species Distribution Models, endangered plants, geo-referencing error, species occurrence data, taxonomy, *Phaedranassa*

Introduction

In recent years there has been an increase in the availability of species occurrence data on the World Wide Web (Canhos et al. 2004; Soberón and Peterson 2004; Jiménez-Valverde et al. 2008; Feeley and Silman 2011). International initiatives' such as the Global Biodiversity Information Facility (GBIF <http://www.gbif.org>), SpeciesLink (<http://slink.cria.org.br/>), Mantis (<http://insects.oeb.harvard.edu/mantis/>) and others, have compiled data from thousands of disparate sources (e.g., herbarium and museum collections and sighting records), allowing users to rapidly acquire vast amounts of data. These data can then be used in Species Distribution Models (SDMs). The SDMs are a general suite of models which estimate the ranges of species on the basis of relationships between the species' probability of occurring and environmental factors such as temperature, precipitation, seasonality, soil type, etc. (Franklin 2009). As a result of the low cost and ease of acquiring data and generating distributions along with their many potential important applications (e.g., conservation of endangered species, prediction of invasive species propagation, and predicting species distributions under different past and future climate change scenarios [Peterson 2003; Martinez-Meyer 2005; Powell et al. 2005; Guisan et al. 2006; Peterson 2006; Thuiller et al. 2006; Peterson and Nakazawa 2008; Feeley and Silman 2010]). SDMs have rapidly gained in popularity and are in widespread use by ecologists, biogeographers and conservation scientists.

Accessibility to data, however, may come at the expense of data quality or reliability. In general, SDMs rely on three primary components: (1) species occurrence

data, (2) environmental variables and (3) the underlying statistical model. The accuracy of the SDMs' range predictions will be directly influenced by the accuracy of these three components. In the case of species occurrence data, there are issues associated with data obtained from large online databases and natural history collections that may limit their utility in SDMs (Newbold 2010). For example, historical records from herbarium and museums specimens typically lack geographical coordinates - information that is essential for the implementation of SDMs (Beaman and Conn 2003). Geographic coordinates can often be obtained from location data that accompany the records and using maps, gazettes or software (Murphey et al. 2004; Guralnick et al. 2006); however, in many instances the geographical information found in the specimen labels/annotations is vague, and/or it is uncertain to what extent errors were made when the information was originally recorded. For example, in one instance I found a herbarium label indicating that a specimen of *P. cinerea* had been collected from the Ecuadorian Paramo (vegetation > 4000 m) at higher altitudes than had been previously reported for this species. After further research (which involved contacting a crewmember of the botanic group that originally collected the plant), I found that there was an error in the label: the species was actually collected in a different location on the way to the Paramo site but the information in the specimen label was that of the Paramo plot where the group was focusing their collecting efforts.

It has been suggested that GIS analysis (Hijmans et al. 1999) and the use of environmental filters can help to screen for errors in species occurrence data (Feeley and Silman 2010). In the anecdotal note above, using an automatic "altitude" or "ecosystem" filter would have eliminated the erroneous record.

Another, potentially important, but underappreciated source of error in collection records is the need for taxonomic validation by experts, since misidentifications will clearly have implications for the ability of SDMs to accurately portray species ranges (Guralnick et al. 2007; Lozier et al. 2009).

In order to understand the impact of data accessibility vs. data quality, we need to quantify the degree that different types of data have on SDMs. The objective of this study is to investigate the impact of differing data quality as associated with different data sources on estimates of species ranges produced using SDMs. I am specifically interested in addressing the problems in generating SDMs using species records from large online databases, since this is a common practice. Towards this goal, I compared species range estimates in Ecuador for seven species in the genus *Phaedranassa* generated from three data sources: (1) standard species occurrence data available online through GBIF and other sources; (2) a combination of records available online and specimens from physical herbaria in Ecuador for which taxonomic identity was corroborated by a specialist; and (3) locations of plants in the field as recorded during field work for this research from 1999 to 2009. By comparing the range estimates resulting from these three datasets I hope to highlight the importance of data quality and taxonomic verification. I anticipate that my study will serve as a cautionary tale exemplifying how conclusions on the basis of SDMs may be misleading if the source and quality of data is not carefully considered.

Methods

Study species

Phaedranassa (Herb.) is a small genus of bulbous plants in the family Amaryllidaceae. This genus is represented by ten species that, except for one species

described from Costa Rica, are restricted to open moist slopes and xeric valleys in the Andes mountains of Colombia and Ecuador (Meerow 1990). Out of the ten *Phaedranassa* species, seven are native to Ecuador with six of them endemic to the country. Within Ecuador, each of the species is known by no more than 10 populations, each with a distribution smaller than 100 km² (Oleas 2000). Because of their restricted geographic distribution, as well as the limited number of populations, all the Ecuadorean endemic *Phaedranassa* species are listed as either “endangered” or “vulnerable to extinction” under the International Union for the Conservation of Nature (IUCN) criteria (Oleas 2000).

Collection of occurrence data

I used three sources of species occurrence data to generate SDMs. First, I compiled records from sources available through the Global Biodiversity Information Facility (GBIF <http://www.gbif.org/>) online data clearing house, as is common practice in many SDM studies. These data (hereafter referred to as “database” records) came primarily from individual herbarium collections at the (1) the Missouri Botanical Garden (MO, <http://www.TROPICOS.org>), (2) the University of Aarhus, Denmark (AAU, http://herb42.bio.au.dk/aau_herb/default.php), (3) the New York Botanical Garden (NYBG, <http://www.nybg.org/>), (4) the Royal Botanical Garden Kew (K, <http://www.kew.org/>), and (5) the University of Florida Herbarium (FLAS, <http://www.flmnh.ufl.edu/herbarium/>). When geographical coordinates were available with the database records, they were included with no modification. Besides downloading records available online, I took some additional steps to ensure that my database set had the best possible quality for this source. I excluded any record that was obviously

incorrect (e.g., located in a large body of water) or located in a country other than Ecuador. All the records collected before the year 1900 were also excluded because of uncertainties in associated locations. In an effort to increase the number of records, localities without coordinates were geo-referenced from the online data using regional maps. With the exception of *P. dubia*, all *Phaedranassa* species in Ecuador are listed at some level of threat under IUCN criteria. Geo-referenced records of endangered species are not provided in some online databases to avoid over-collection. Thus, coordinates of endangered *Phaedranassa* records in TROPICOS were not available through GBIF and were obtained directly from MO. I did not calculate the distribution for *P. brevifolia* and *P. glauciflora* with the database set because there was only one specimen for each species available online.

My second dataset (hereafter referred to “taxonomy-based” records) includes all the records accessible online as well as records that are available only as physical specimens in Ecuador in the two main herbaria in Ecuador: Herbario de la Pontificia Universidad Catolica del Quito (QCA), and the Herbario Nacional del Ecuador (QCNE). Many of these specimens were used in the latest the taxonomic treatment of the family (Meerow, 1990), which greatly reduces taxonomic uncertainty. Herbarium specimens not included in the taxonomic treatment were inspected by myself at the QCA, QCNE and MOBOT herbaria and identified to the species level using the keys in Meerow's (1990) treatment.

My third dataset (hereafter referred to as “field-based” records) includes only the geographic coordinates (GPS-based) of plants encountered during my fieldwork in Ecuador from 1999 to 2009. It was during the fieldwork that I noticed errors in the

specimen's labels. These data were originally collected for use in an extensive study of the population genetics of the genus. In collecting field data, all of the locations listed in the previous two datasets (database and taxonomy-based) were visited, at least once, to collect samples for the population genetic portion of this dissertation (Chapter IV) and to verify occurrence.

Model building

Distribution models for *Phaedranassa* spp. were generated through a maximum entropy approach as implemented in the program MAXENT ver. 3.3.3a (Phillips et al. 2006). The maximum entropy method is appropriate for presence only data (Newbold et al. 2010) and is one of the most widely programs used to generate species distribution models. Maximum entropy has shown superior prediction accuracy compared to other methods (Elith et al. 2006) and is less sensitive to sample size than other SDMs (Hernandez et al. 2006; Pearson et al. 2007; Hernandez et al. 2008; Wisz et al. 2008), an important consideration because small sample sizes are expected for collections of endangered species from the tropics. When implementing MAXENT, I used the default options but removed duplicate records.

I obtained the environmental variables to use in MAXENT from WorldClim (www.worldclim.org, spatial resolution of 30 arc second or $\sim 1 \text{ km}^2$). I conducted the SDMs using two different sets of climatic variables. First, I used all 19 bioclimatic variables (Hijmans et al. 2005) available through WorldClim - a common approach for SDMs. I also used a subset of five bioclimatic variables known be to be important for species distribution and uncorrelated for the region: temperature annual range, annual mean precipitation, annual mean temperature, precipitation seasonality (Loiselle et al.

2007; Hernandez et al. 2008; Seo et al. 2009) and minimum temperature of the coldest month.

The output of MAXENT is a continuous probability field. I transformed this into a predicted presence vs. absence map by thresholding at the point of maximum training sensitivity plus specificity (Jiménez-Valverde et al. 2008). In all, I generated six sets of range predictions for each of the seven species occurring in Ecuador, using the “database”, “taxonomy-based” and “field-based” data sets and the two sets of climatic variables (Fig. 1).

Evaluation of the models

I assessed model performance with the receiver operating characteristic analysis (ROC) as implemented in MAXENT (Phillips et al. 2006). The ROC value shows the relationship between the false positive error versus the true positive rate (Franklin 2009), and is usually reported as the area under the curve (AUC). The area under the curve is a threshold-independent measure that shows the probability that a random selection of species presence will show higher probability than an absence site chosen at random (Franklin 2009). Values of AUC higher than 0.9 are considered to indicate high model performance, values of 0.7- 0.9 indicate moderate model performance, and values lower than 0.7 are considered to show poor model performance (Manel et al. 2001; Franklin 2009).

I evaluated the degree of agreement between the range predictions produced from the different occurrence datasets using the Kappa test statistic. Kappa is a threshold-dependent categorical measure of the difference between observed versus random agreement (Franklin, 2009). Kappa values closer to one represents a good agreement

between range predictions. For this study I used Kappa to compare the probability threshold range predictions of each species generated with the three data sources (database, taxonomy-based and field-based).

I also compared the total area and elevation range of the probability threshold distribution for each species obtained with each one of the three datasets. For the total area I counted the number of pixels of the distribution model multiplied by 0.86 to obtain the approximate area in km². Elevation value was obtained from the altitude layer available in WorldClim (www.worldclim.org), for each distribution prediction as well as the altitudinal range of the data points themselves using the extracting option of ArcToolBox in ESRI ArcMap 9.2. For field-work records, altitude was also measured using an Etrex Garmin GPS unit on site.

Results

I found 65 unique records available through GBIF for *Phaedranassa* in Ecuador. The number of records per species in my “database” set ranged from one to 40 (mean = 9). The total number of records in my “taxonomy-based” set was 73 with a range of zero to 25 records per species (mean = 10). My “field-based” dataset had 184 records with a range of 16 to 41 records per species (mean = 26).

The model performance as judged by AUC was generally high (Table 2). In terms of Kappa, overall there was poor agreement between the range predictions generated with each of the three datasets (Table 1). The exception was *P. dubia*, with high Kappa values between all datasets, and *P. schizantha* in the “database” vs. “taxonomy-based” assessment with five bioclimatic variables but not when all bioclimatic variables were included (Table 1).

The area of the range predictions generated using field-based data were smaller than the areas of range predictions created with either “database” or “taxonomy-based” data (Table 2, Fig 1). The areas of the range predictions obtained using all the bioclimatic variables were smaller than the area obtained with the models using just five bioclimatic variables (Table 2, Fig 1).

The average difference between the altitudes of each collection point estimated by GPS and the altitude extracted from the altitude layer was 86 meters and ranged from -247 m to +617 m. In general, both “database” and “taxonomy-based” records resulted in wider altitudinal range predictions for each species compared to the altitudinal range predictions with “field-based” data (Table 2). The difference was highest in “database” vs. “fieldwork-base” comparisons. I found the same pattern for both models obtained with either five or all bioclimatic variables (Table 2).

Discussion

The increased accessibility of free data from disparate sources through online clearing houses such as the GBIF has the potential to greatly advance our understanding of large-scale geographic and ecological patterns in species distributions. However, the indiscriminate use of these data also has the potential to produce inaccurate or misleading results, clouding our understanding of the species distributions and the extrapolations made with them. To highlight this risk, I compared range predictions generated by SDMs with data garnered directly from GBIF (with standard levels of data filtering) to those generated with data that has undergone increased levels of scrutiny (i.e., taxonomic verification) and data on the basis of expert observations in the field.

In this study my principal interest was to highlight the implications of different species occurrence datasets on SDMs. In most cases, the degree of agreement among models was low. The agreement was weaker when I compared “database” vs. “field-based” predictions (Table 1) as was expected because of the large differences in data quality in terms of both geo-referencing and taxonomic certainty.

I found two opposite patterns for total amount of area classified as suitable under the SDMs made with different data sources. For species known from only a very small number of collections (e.g., *P. brevifolia*, *P. viridiflora*, Table 2), the estimated area for the field-based distribution was larger than the areas of either database or taxonomy-based range predictions. This result is likely because of the greater number of records in the field-based dataset compared to the limited number of records available online. In contrast, for species with larger sample sizes, the area of field-based range predictions was smaller than those produced with the other two datasets. The reduction in area is believed to occur as the result of errors found in the database and taxonomy-based dataset which tend to inflate species ranges (Feeley and Silman 2010).

Altitudinal range is an important feature that, because of its association with temperature ($\sim 5.5^{\circ}\text{C}$ colder per km elevation gain), it is often used for ecological inference, especially within the context of climate change (Körner 2007). If I translate the differences I found in altitude ranges predicted by “field-based” vs. either “database” or “taxonomy-based” data, into differences in thermal niche breadths, I find an average difference of -5.6°C ($+30^{\circ}\text{C}$ to -21°C range) in my five climatic variable models, or an average difference of -4.6°C ($+18^{\circ}\text{C}$ to -24°C range) when all 19 bioclimatic variables are included. In other words, the differences in thermal niche breadths predicted for

species on the basis of online records vs. data collected and verified in the field is higher than some of the worst warming scenarios predicted for the Andes over the next century (Urrutia and Vuille 2009). This unrealistic result should raise a cautionary "red flag" against extrapolating the results of SDMs produced with unverified online records to species' extinction risks related to climate change.

In this study I dealt with two types of errors common in species occurrence data records: geo-referencing errors and taxonomic misidentification. There are ways to minimize error records in species occurrence data. Automatic filters using known features of the species range, such as altitude or ecosystem, might help to eliminate erroneous species records before generating species distribution models (Feeley and Silman 2011). Another simple way to avoid errors is reading the actual specimen herbarium label, instead of relying solely on the geographic coordinates that can be downloaded in a spreadsheet. One mistake that can be identified by reading the label is ex-situ collections. For example, in one case I found one living specimen for which the geo-referenced collection information available online corresponded to the address of a botanical garden where it is cultivated rather than indicating the location of natural origin of the specimen. By chance this false record fell between the natural altitudinal range of the species and thus the error may not have been caught by an automatic altitude based filter.

The effects of taxonomic misidentifications are potentially as important as geo-referencing errors but have received considerably less attention. Checking for taxonomic errors requires access to the physical herbarium specimen and a specialist willing to review the data. As such, checking for taxonomic errors cannot be easily automated and thus may be infeasible for large scale, multi-taxa studies. I suggest that taxonomic errors

can be minimized by using the records reported in recent taxonomic monographs because in these cases the taxonomy of the specimens has been previously verified by experts. I anticipate that for species without recent taxonomic revisions, the level of error of SDMs with data originating from databases alone will be even higher than in my study because, even at my dataset level, many of the specimens were relatively recently curated by the specialist before I conducted this study.

One of the main problems for SDMs of tropical plant species in general, but endangered species in particular, is the lack of species occurrence data. In order to obtain accurate models of species distribution range, a minimum of 20 records per species is needed (Hernandez et al. 2006; Wisz et al. 2008). In this study, only the three sets of data for *P. dubia* met this minimum. A need for more collections in the tropics has been recognized previously (Feeley and Silman 2011). I also found that there were specimen records in local herbaria in the country of origin that were not included in the World Wide Web databases like GIBF. In this case, most of the collection was made by a local botanist who had not collected extra vouchers to share with international herbaria. A program that circumvents this problem through quick data and specimen sharing is the partnership between the Missouri Botanical Garden (MOBOT, USA) and the Herbario Nacional (QCNE, Ecuador). More exchange programs between international institutions and local herbarium and museums are greatly needed and provide a relatively inexpensive way to increase species representation in international databases.

Finally, SDMs, even those generated with fewer than the optimal number of species occurrence records, can be helpful for other purposes. The information obtained from SDMs can be applied to find new populations of endangered species. For example, I

used a preliminary SDM to predict habit for *P. brevifolia*, which I then searched for specimens; this approach enabled me to find three new records for this species. Another successful example of this use was reported for *Byrsonima subterranean* (Malpighiaceae), an endemic plant from Brazil (de Siqueira et al. 2009). Ideally, new populations can be incorporated to the SDM; these additions in turn upgrade the model; and then the use of the improved SDM increases the chances of finding new populations (Guisan et al. 2006).

Species Distribution Models are clearly useful for conservation purposes. The results presented here, however, show that it is indispensable to address the limitations of the species occurrence data first, in order to avoid erroneous outcomes of posterior extrapolations. Data quality certainly matters, and databases do not have the certainty associated with taxonomic revisions and field work.

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Phaedranassa brevifolia

Figure 1. Distribution maps for seven *Phaedranassa* spp. Dark areas indicate predicted presence. Lower case letter indicates dataset source type: (a) database; (b) taxonomy-based; (c) field-based; numbers are (1) SDMs with 5 bioclimatic variables (www.WorldClim.org) and (2) SDMs with all 19 bioclimatic variables

Phaedranassa cinerea

Figure 1 (continued)

Phaedranassa dubia

Figure 1 (continued)

Phaedranassa glauciflora

Figure 1 (continued)

Phaedranassa schizantha

Figure 1 (continued)

Phaedranassa tunguraguae

Figure 1 (continued)

Phaedranassa viridiflora

Figure 1 (continued)

Table 1. Kappa statistic evaluating the degree of agreement between species distribution models for species of *Phaedranassa*. Numbers without parenthesis corresponds to kappa values for models with five bioclimatic variables, while the number inside parenthesis is kappa from model built with all 19 bioclimatic variables

Species	(Database vs. Field-based)	(Taxonomy-based vs. Field-base)	(Database vs. Taxonomy-based)
<i>P. brevifolia</i>	0	0.169 (0.147)	0
<i>P. cinerea</i>	0.531 (0.150)	0.322 (0.260)	0.396 (0.486)
<i>P. dubia</i>	0.775 (0.333)	0.826 (0.514)	0.935 (0.725)
<i>P. glauciflora</i>	0	0.567 (0.655)	0
<i>P. schizantha</i>	0.275 (0.116)	0.276 (0.157)	0.797 (0.673)
<i>P. tunguraguae</i>	0.054 (0.022)	0.322 (0.306)	0.239 (0.108)
<i>P. viridiflora</i>	0.456 (0.043)	0.474 (0.350)	0.521 (0.181)

Table 2. Comparison among *Phaedranassa* species distribution models (SDMs). Datasets are represented with superscript numbers in each species: (1) database, (2) taxonomy-based, (3) field-based. Numbers without parenthesis are values for models with five bioclimatic variables, numbers inside parentheses are values of model built with all 19 bioclimatic variables. Altitudinal range (a) geographic coordinates, (b) SDMs

Species	Number of records	SDMs Area (Km ²)	Altitudinal range (m) ^a	Altitudinal range (m) ^b	Area under curve (AUC)
<i>P. brevifolia</i> ¹	1				0.500
		N/A	1533	N/A	(0.500)
<i>P. brevifolia</i> ²	3			0 – 3549	0.938
				(1211 – 1674)	(1.000)
		9811 (66)	1533 - 1674		
<i>P. brevifolia</i> ³	22	13138		0 – 5068	0.923
		(412)	924 - 1533	(798 – 1818)	(0.999)
<i>P. cinerea</i> ¹	11	43421		79 – 4810	0.955
		(34285)	585 - 3394	(30 – 4267)	(0.969)
<i>P. cinerea</i> ²	11	72980		0 – 6169	0.791
		(18890)	585 - 3439	(48 – 3175)	(0.932)
<i>P. cinerea</i> ³	26	23704		430 – 3920	0.980
		(3235)	583 - 2276	(531 – 3175)	(0.996)
<i>P. dubia</i> ¹	26			1500 – 3996	0.966
		20289		(1164 – 4114)	(0.977)
		(22929)	1803 - 3541		
<i>P. dubia</i> ²	25	18367		1522 – 3846	0.969
		(13752)	1803 - 3541	(1164 – 4130)	(0.978)
<i>P. dubia</i> ³	41			1640 – 3892	0.989
		13777		(1851 – 3897)	(0.995)
		(4947)	2255 - 3394		
<i>P. glauciflora</i> ¹	1				0.500
		N/A	2152	N/A	(0.500)
<i>P. glauciflora</i> ²	6	59316		1404 – 6169	0.937
		(25518)	2609 - 3296	(126 – 5217)	(0.955)
<i>P. glauciflora</i> ³	39	28972		1265 – 3809	0.944
		(20715)	1690 - 2916	(266 – 3430)	(0.967)
<i>P. schizantha</i> ¹	15	13339		1536 – 6169	0.938
		(19703)	1177 - 4860	(1092 – 6169)	(0.967)
<i>P. schizantha</i> ²	13	13103		1813 – 5415	0.934
		(13948)	1177- 4860	(1573 – 4997)	(0.977)
<i>P. schizantha</i> ³	23	2227		1851 – 3374	0.996
		(1305)	2431 - 3376	(2189 – 3345)	(0.997)

Table 2 (continued)

Species	Number of records	SDMs Area (Km ²)	Altitudinal range (m) ^a	Altitudinal range (m) ^b	Area under curve (AUC)
<i>P. tunguraguae</i> ¹	9	58191 (22473)	939 - 2477	639 – 6169 (4 – 4338)	0.751 (0.990)
<i>P. tunguraguae</i> ²	12	16342 (1532)	939 - 2477	789 – 3167 (1219 – 2633)	0.989 (0.998)
<i>P. tunguraguae</i> ³	17	3322 (280)	1337 - 2018	886 – 2814 (1260 – 1590)	0.997 (0.999)
<i>P. viridiflora</i> ¹	2	5198 (623)	2502 - 2692	126 – 6169 (1590 – 3027)	0.980 (0.998)
<i>P. viridiflora</i> ²	3	12915 (5937)	2502 - 2911	126 – 6169 (1458 – 3809)	0.979 (0.985)
<i>P. viridiflora</i> ³	16	13753 (18002)	2194 - 2966	839 – 3546 (794 – 3512)	0.986 (0.991)

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CHAPTER IV.
POPULATION GENETIC STRUCTURE OF *PHAEDRANASSA* HERB.
(AMARYLLIDACEAE)

Introduction

Ecuador is recognized as a megadiverse country, with one of the highest concentrations of species per area in the world (Mittermeier et al. 1999; Myers et al. 2000). This massive number of species is not distributed evenly in the country. In fact, more than half of Ecuador's endemic plant diversity (more than 2000 plant species), is restricted to the Andean mountains (Valencia et al. 2000). The Andes in Ecuador are shaped by 86 major volcanoes, whose modern altitudinal range evolved during the lower Holocene-Pleistocene in a process that might have taken place for over 4 Ma (Coltorti and Ollier 2000). The influence of Andean geological history on the current variety of habitats in Ecuador and consequent species richness has been recognized previously (Jørgensen et al. 1995; Luteyn 2002; Hughes and Eastwood 2006). The inter-Andean valleys are naturally isolated from each other as a consequence of deep and narrow topographic contours (Young et al. 2002). The topographic complexity creates local microhabitats ranging from moist slopes to dry valleys (Bush 2002). In this intricate topography, species have extended north and south and also up and down in altitudinal range during climate change events (Struwe et al. 2009). In general, it has been recognized that mountains constitute either bridges to plant colonization or isolating barriers, which indeed play a role in speciation (Hewitt 2004; Ohsawa and Ide 2007). Understanding the speciation process in such a diverse area as the tropical Andes is a fundamental question in Neotropical biology.

A recent metapopulation analysis of speciation time in the Andes showed that the process in this part of the world occurred gradually since the Tertiary, with less than half of the plant species evolving during the Quaternary (Rull 2008). Recent explosive plant speciation during the late Pliocene or Pleistocene has been reported in these mountains ranges (Hughes and Eastwood 2006). The majority of data has been gathered for taxa located in the highlands, e.g., Valerianaceae in the Paramos (Bell and Donoghue 2005), *Halenia* (Gentianaceae) (von Hagen and Kadereit 2003) and *Lupinus* (Fabaceae) (Hughes and Eastwood 2006). An example of rapid speciation in low to mid altitudes is *Costus* (Kay et al. 2005). Furthermore, a recent investigation on *Cyathostegia mathewsii* (Benth.) Schery (Fabaceae) from the seasonally dry forest in the inter-Andean valleys of Ecuador and Peru, demonstrated an older diversification as a result of isolation in the valleys for at least 5 million years (Pennington et al. 2010). All of these examples of diversification have been explained by the environmental conditions created during the recent uplift of the Andes and climate change through the Quaternary (Richardson et al. 2001; Rieseberg and Willis 2007). At the same time, these cases show that high diversification rates in the Andes have occurred across various types of habitats. However, these phylogenetic studies account only for a small percentage of tropical Andean plant biodiversity, and the speciation phenomenon remains largely understudied.

One of the most controversial aspects of the speciation process is how this process occurs geographically (Losos and Glor 2003). There are two different views of speciation: sympatric and allopatric. In general terms, sympatric speciation occurs when competition among populations leads to ecological niche differentiation, while allopatric speciation requires a spatial barrier leading to genetic isolation (Mayr, 1963). A middle

point between both allopatric and sympatric speciation is parapatric speciation because it allows speciation to occur even with migration among adjoining populations (Gavrilets et al. 2000). It has been proposed that there is a continuum of evolutionary processes that influence genetic variation at different levels: from populations to species to higher level taxa (Carstens et al. 2004). The early stages of speciation will be population genetic divergence. In this respect, divergence can be higher among peripheral populations (peripatric speciation), because these populations might be smaller, more isolated and affected by different selection pressures (Mayr 1963). The opposite view proposed that divergence will be more likely in the central and potentially more diverse populations (centrifugal speciation) (Brown 1957). A comprehensive review of speciation models was provided by Gavrilets (2003). Key factors promoting speciation are small populations, mutation, genetic drift and selection to local environment adaptation (Gavrilets 2003).

With the increased availability of molecular tools as well as geographic information systems (GIS), it is now possible to study genetic variation in a geographic context, an approach called landscape genetics (Manel et al. 2003). Interest in landscape genetics among researchers has increased steadily, with a broad array of taxa being studied (Holderegger et al. 2010; Storfer et al. 2010). The geographic aspects of speciation in the tropical Andes have been poorly investigated with empirical approaches, and there are only scattered examples of landscape genetic research available in the literature (Storfer et al. 2010). One recent study describes the complex evolutionary patterns linked to demographic history, dispersal patterns, interspecific divergence and hybridization of two wild tomato species (*Solanum lycopersicum* and *S. pimpinellifolium*

Solanaceae) (Nakazato and Housworth 2011). There are only two other studies of threatened plant species of the Tropical Andes using microsatellites. One concerned the microevolution of the wax palm *Ceroxylon echinulatum* Galeano, (Arecaceae) (Trenel et al. 2008), and the other addressed mating patterns and their role in genetic variation of the Andean Oak (*Quercus humboldtii* Bonpl.) (Fernandez and Sork 2005).

The prospect of unraveling genetic relationships in a spatial context is made possible through the use of molecular markers. Among the different molecular markers available (Schlotterer 2004), microsatellites are one of the most popular. Microsatellites, known also as simple sequence repeats (SSRs), are two to six nucleotide motifs repeated many times in tandem. Microsatellites are a preferred molecular tool to study genetic relationships because they capture a high level of polymorphism; they are highly reproducible and codominant; they occur regularly all over the genome; they are easy to automate; they usually are inherited in a Mendelian pattern; and they are generally neutral markers (Frankel et al. 1995; Jarne and Lagoda 1996; Cruzan 1998; Rossetto et al. 1999; Susol et al. 2000; Li et al. 2002; Zane et al. 2002; Squirrell et al. 2003). Furthermore, the high mutation rate of microsatellites offers excellent resolution of recent or current microevolutionary events (Wang 2010).

One of the disadvantages of this technique is that microsatellite primers need to be developed specifically for the species to be studied. In some cases, but not always, microsatellite primers can be used for other closely related species. Despite the fact that the design of microsatellite primers can be outsourced from specialized laboratories, the method is still relatively expensive, costing approximately \$10,000 to design 10 to 15 loci (Selkoe and Toonen 2006). Another disadvantage of this molecular technique is the

possibility of undetected null alleles and allele size homoplasy (Selkoe and Toonen 2006). Currently, next generation sequencing technology has even further reduced the cost and time for microsatellite development in non-model species (e.g., development of 17 polymorphic microsatellite markers at \$5,000 in six weeks, Csencsics et al. 2010).

The main mechanism of microsatellite mutation is the gain or loss of repeat units as a consequence of DNA-replication slippage (Weber and Wong 1993; Chambers and MacAvoy 2000; Lai et al. 2003; Schlotterer 2004). Several theoretical mutation models for microsatellites have been proposed (reviewed by Chambers and MacAvoy 2000). The most common are: infinite allele model (IAM, Kimura and Crow 1964) and stepwise mutation model (SMM, Kimura and Ohta 1978). In the IAM, mutations could result in a gain or loss of any number of repeat units, but always produce a new allele (Symonds and Lloyd 2003). As a result, this model does not allow homoplasy, i.e., identical alleles that are identical by descent (Balloux and Lugon-Moulin 2002). In the SMM only one repeat unit is gained or lost per mutation and therefore alleles could mutate to allele states already present in the population (Estoup and Cornuet 1999; Symonds and Lloyd 2003). Therefore, this mutation model has a memory of allele size and alleles of very different sizes will be less closely related (Balloux and Lugon-Moulin 2002).

Population genetic structure can be better explained through models. The simplest is the Wright-Fisher model (Fisher 1930; Wright 1931), which corresponds to an ideal population without change because of a constant number of diploid individuals, random mating, non-overlapping generations, and a random number of offspring per individual. In reality, natural populations are more dynamic and complex: diverse in biogeographical history, changing in size, density and dimension over time, and exchanging gene material

with other populations (Hey and Machado 2003). As a result, genetic structure can be distinguished among populations (Hedrick 2000). In subdivided populations, the amount of genetic connection between subpopulations depends on the effective gene flow, genetic drift and selection (Hedrick 2000). Different models of population genetic structure have been proposed, including mainly additions like direction and rates of genetic flow. The simplest, describing unidirectional gene flow, is the island model of migration (Wright 1940). More complex variants of this model have been proposed, such as the stepping-stone model (Kimura and Crow 1964). The stepping-stone model describes genetic exchange between close populations. Another variant is the isolation-by-distance model, where individuals are genetically more similar to their neighbors than to distant individuals (Wright 1943). The matrix model incorporates migration among populations as a matrix with or without symmetric gene flow (Bodmer and Cavalli-Sforza 1968). Models can also incorporate more complexities like extinction and colonization among populations, e.g., in the metapopulation models (Hanski and Gilpin 1991). Both mutational models and populations models represent a baseline for statistical inference to address questions about the genetics of populations. In recent years, population genetics have benefited by the development of coalescence theory (Hudson 1990). The coalescence approach describes the genealogical process of neutral genes backward in time (Kingman 1982). Statistical tools such as Bayesian analysis and the Markov Chain Monte Carlo method contributed to inferring demographic genealogies (Beaumont and Rannala 2004). These two approaches make it possible to estimate population genetic aspects such as recent population bottlenecks (Cornuet and Luikart 1996) and migration rates (Beerli 2008).

Endemic plants are an excellent subject to study the dynamic processes of speciation and evolution (Matolweni et al. 2000). At the same time, many of these species are in serious risk of extinction, which makes their study a priority (Matolweni et al. 2000; Pitman and Jørgensen 2002). In this paper I used *Phaedranassa* spp. (Amaryllidaceae) to investigate the speciation process in the tropical Andes. *Phaedranassa* can be used as a model group to study speciation in the Northern Andes. This genus is a monophyletic group (Meerow et al. 2000). It is represented by ten species that, with the exception of one species described from Costa Rica, are restricted to the Northern Andes. Six species are endemic to Ecuador, having one of the highest proportions of endemic species per genus in Ecuador (Borchsenius 1997). *Phaedranassa dubia* has been reported from both Ecuador and Colombia, and there are two endemic species in Colombia (Meerow 1990). The geographic distribution of each species in Ecuador has been investigated in depth since the last decade (Oleas 2000). The species of the genus appears limited to a particular inter-Andean valley or open moist slope along river canyons (Fig 2). In terms of conservation, all the Ecuadorian endemic species of *Phaedranassa* occur in one of the most deforested regions of the country, where little of the native vegetation remains (Valencia et al. 1999). In fact, all six *Phaedranassa* species endemic to Ecuador are classified as either “Endangered” or “Vulnerable” to extinction under the International Union for Conservation of Nature criteria (IUCN) for red-listing of endangered plants (Oleas 2000).

The objectives of this study were to (1) analyze the population genetic structure of the seven *Phaedranassa* species in Ecuador; and (2) explore the connection between genetic differentiation among these species and their spatial distribution patterns.

Methods

Sample collection and species delimitation

Fresh leaves from 1367 individuals were collected across the geographical distribution of the seven *Phaedranassa* species in 52 collecting sites (Fig. 2). These sites were visited in May and June of 2003, December 2005, July to August 2006, December 2007 and from July to December 2009. On average, I collected 25 specimens per collection site (Table 1). Overall, I located 71% of all the known populations for this genus in Ecuador. Six new localities for the genus were also discovered: three for *P. brevifolia*, two for *P. schizantha*, and one for *P. glauciflora*. Samples were collected from plants whose petiole bases were at least one meter distant in an effort to minimize collecting multiple ramets of a single genet. The leaves were fast-dried in silica gel.

Samples were classified into species following the last taxonomic review of *Phaedranassa* (Meerow 1990). If the individuals were sterile at the moment of collection, living vouchers were collected and kept ex-situ until flowers were obtained, or the locations were revisited until flowers were found in the wild. The name of the species assigned to each population is given in Table 1. Changes to species identifications based on subsequent fieldwork or identification made from living voucher specimens are marked in bold (Table 1).

DNA extraction, genotyping and DNA quality control

Total DNA extraction protocols followed the methods described in Oleas et al. (2005). For DNA quantification, I followed Livingstone et al. (2009). Individuals were genotyped with 13 microsatellite primers: eight developed from *P. tunguraguae* -locus pt14, pt21, pt32, pt39 (Oleas et al. 2005) and pt43, pt48, pt49 and pt61 (Oleas et al. in

prep)-; and five developed from *P. schizantha* - ps2, ps13, ps16, ps27 and ps28 (Oleas et al. 2009)-. Polymerase Chain Reaction conditions and genotyping procedures followed the protocols described by Oleas et al. (2005, 2009).

Polymerase Chain Reaction procedures were repeated up to three times if the sample did not amplified initially. Samples with alleles showing one base pair (bp) difference rather than a repeat unit variation as expected for microsatellite mutation were also repeated. If the sample still showed a difference of a single base pair, the sample was dropped from the analysis. I also used MICRO-CHECKER version 2.2.3 (Van Oosterhout et al. 2004) to identify genotyping errors, as well as null alleles, stuttering and large allele dropouts.

Statistical analysis

Descriptive population genetic statistics were estimated with GENALEX 6.4 (Peakall and Smouse 2006): number of alleles per locus, effective number of alleles per locus, number of private alleles, total heterozygosity, observed heterozygosity and fixation index. Significance of F_{is} and R_{is} was evaluated after 20,000 repetitions in SPAGEDI 1.3a (Hardy and Vekemans 2002). I used the program FREENA to evaluate difference in F_{ST} values when putative null alleles were detected (Chaupis and Estoup, 2007).

Linkage disequilibrium (LD) was estimated with GENEPOP 4.0.10 (Raymond and Rousset 1995; Rousset 2008) with the following parameters for the Markov chain: 10,000 batches of 10,000 iterations per batch and 10,000 dememorisation. Sequential Bonferroni correction (Rice 1989) was applied to avoid Type I statistical error. Multilocus estimate of the effective number of migrants (N_m) was done in GENEPOP

4.0.10 (Raymond and Rousset 1995; Rousset 2008). Analysis of molecular variance (AMOVA) was calculated in ARLEQUIN 3.5 (Excoffier et al. 2005); significance was calculated with 10,000 permutations.

I used the allele size permutation test (Hardy and Charbonnel 2003), as implemented in SPAGEDI 1.3a (Hardy and Vekemans 2002), to test the contribution of stepwise-like mutations to population differentiation, when a significantly greater value of observed R_{ST} against permuted R_{ST} corroborated that SMM was a factor in population differentiation (Hardy and Charbonnel 2003).

Tests for recent bottleneck events were conducted with BOTTLENECK 1.2.02 (Cornuet and Luikart 1996; Piry et al. 1998). All populations with less than 20 individuals were not included in this analysis because a low number of individuals can be misleading. The estimation was calculated with 100,000 replications, using the sign test and Wilcoxon sign-rank test to establish if populations were in mutation-drift equilibrium (Cornuet and Luikart 1996). Both tests were performed under two models of microsatellite mutation (IAM and SMM). I reported results of both types of mutation models as suggested by Cornuet and Luikart (1996). It is unlikely that microsatellite loci follow a strict IAM or SMM, and these two models represent the extremes. An additional test for recent bottlenecks known as the mode-shift, which is an indicator of shift of the allelic frequency distribution (Luikart et al. 1998), was also reported.

Genetic relationships among populations were estimated in POPULATIONS 1.2.30 (Langella 1999). A phenogram was generated with chord distance D_c (Cavalli-Sforza and Edwards 1967), and Neighbor Joining (NJ) as the clustering method. Chord distance was used because it is less influenced by the presence of null alleles (Chaupis

and Estoup, 2007), and it has been reported that Chord distance successfully recovers the correct topology in a tree from microsatellite data (Takesaki and Nei 1996). Bootstrap values across loci were calculated with 10,000 permutations by locus. The tree was visualized with TREE EXPLORER in MEGA 4 (Kumar et al. 2004).

Individual assignment was performed with the program STRUCTURE 2.2. (Pritchard et al. 2000), in BIOPORTAL (Kumar et al. 2009). First, individuals were assigned to a pre-defined genetic group (k) of seven, which represents the number of species. The analysis was made under the admixture model, with 1,000,000 repetitions after a burn-in of 500,000 and replicated 20 times. The results were loaded into STRUCTURE HARVESTER (Earl 2011), which calculates k value following Evanno et al. (2005) method. Another set of analyses following the same methods were conducted for each species separately and in groups formed by the populations that came together in the NJ cluster. In this case, to identify the most likely number of genetic groups (k) in each species, a series of analyses was performed using different values of k . Additionally, I analyzed three different cases of putative sympatric groups using the same method. A consensus of the results of the independent runs for the optimal k was done in CLUMPP 1.1.2 (Jakobsson and Rosenberg 2007), with the Greedy option, random input order and 100,000 repeats. The results were visualized in DISTRUCT 1.1 (Rosenberg 2004).

Principal Coordinates Analysis (PCA) of the *Phaedranassa* individuals from Pululahua Crater was performed in GENALEX 6.4 (Peakall and Smouse 2006). Pairwise genetic distance was calculated in POPULATIONS using Cavalli-Sforza chord distance.

In order to investigate the relationship between genetic structure and landscape, I analyzed the data in two ways. First I tested for isolation by distance with a correlation

between log genetic similarity (chord distance D_c) and log geographic distance. The significance of the correlation was tested with a Mantel test calculated with 9999 randomizations in GENEALLEX 6.4. Furthermore, I contrasted the population genetic structure with each Species Distribution Model (SDM). The methods for estimating the species distribution models are explained in Chapter III. In addition, I calculated the percentage of overlap among SDMs between species with the Kappa coefficient. Kappa is a categorical measure of the difference between observed versus random agreement (Franklin 2009). The closest the Kappa value to one of two distribution comparisons will mean that both are similar (Franklin 2009). My assumption was that overlapping models among species, showing genetic differentiation, will be indicative of random pattern in the landscape.

Results

Descriptive statistics among species

The mean effective number of alleles ranged from 1.28 to 5.39 per population (Table 1). Among species, *P. viridiflora* showed the lowest number of alleles (average of 1.39 effective allele number) and *P. brevifolia* had the maximum effective allele number (4.21) (Table 1). A total of 77 private alleles were found across 36 populations (Table 1). *Phaedranassa viridiflora* had the lowest number of private alleles, followed by *P. tunguraguae*. *Phaedranassa cinerea* had the highest number of private alleles per population (Table 1). With the exception of most populations of *P. viridiflora*, all of the species showed evidence of inbreeding (positive F values, Table 1). Overall, the inbreeding coefficient was highly significant (0.29 F_{is} , 0.33 R_{is} , $p = 0.001$) across all populations. Evidence of null alleles was found in the majority of populations, in an

average of seven out of 13 loci per population (range 0-12 loci per population). One way to overcome the presence of null alleles is to calculate allele frequencies acknowledging the possibility of null alleles. I did not find significant changes in F_{ST} when genotypes were corrected for null alleles using FreeNA (Chaupis and Estoup, 2007). Both corrected and non-corrected overall F_{ST} were 0.29 ($p < 0.0001$).

Linkage disequilibrium (LD) was found in roughly 5% of the combinations (190 out of 4057) after Bonferroni correction. Indication of LD was found in a total of 10 populations (Table 1). None of the populations of *P. tunguraguae* and *P. brevifolia* show evidence of linked loci. The majority of species had only one population presenting evidence of LD, whereas *P. schizantha* and *P. viridiflora* showed three or more populations with LD (Table 1).

None of the populations showed evidence of recent bottlenecks with all three tests: Wilcoxon, sign statistic test and the mode-shift test (Cornuet and Luikart 1996) (Table 2). The majority of populations (32 out of 43 of populations, excluding populations with less than 20 individuals) showed evidence of bottlenecks under the sign test with the SMM, whereas only three populations did under IAM using the same test. With the Wilcoxon test, seven populations tested positively for a bottleneck with the IAM, and only one under the SMM (Table 2). Only one population showed evidence of bottleneck with the mode-shift test (Table 2).

Analysis of Molecular Variance showed that 69% of genetic variation resided within populations, followed by 21% of the variance at the within-populations-between-groups level. Only 9% of the variation was found among groups, which in my study represented species ($p < 0.00001$) (Table 3). A permutation test of allele size between

observed R_{ST} against permuted R_{ST} was significantly greater, which corroborated SMM was a factor in population differentiation (Hardy and Charbonnel 2003). The multilocus estimate of the effective number of migrants (N_m) were two individuals in average with a range of four to one individual (Table 4).

Cluster analysis

The neighbor-joining tree showed populations generally clustered with other populations of the same species (Fig. 3), although none of the species clusters were supported by a high bootstrap proportion (Fig. 3). One of the exceptions to species clusters was *P. viridiflora*, populations which comprised two distinct groups, each formed by geographically adjacent populations (Fig. 3). Furthermore, one population of *P. viridiflora* (Population 44) was clustered with *P. dubia* populations. It is noteworthy that this population was found in sympatry with *P. dubia* (Pop 41) in the Pululahua crater (Fig. 2). The population Pop52 of *P. schizantha* was also included in the *P. dubia* cluster (Fig. 3). Along with those cases, populations of *P. glauciflora* were included in the *P. cinerea* clade (Fig. 3).

Individual assignment to species

Individuals' assignment to a given species was first investigated with $k = 7$, which is the number of recognized *Phaedranassa* species in Ecuador (Meerow, 1990). In most cases individuals were assigned to the corresponding species group (Fig 4). There were three exceptions: *P. viridiflora*, Population 52 (*P. schizantha*) and Population 35 (*P. cinerea* 2) (Fig. 4). The majority of the populations of *P. viridiflora* were assigned to the same two groups detected in the neighbor-joining tree (Fig 3, 4). These two groups were formed by geographically close populations, one located in the central part of the country

and the other in southern Ecuador (Fig. 2). As in the NJ tree, Population 44, which is located inside Pululahua crater, resolved as part of the cluster formed by the *P. dubia* group (Fig. 3 and 8). Also, the majority of the populations of *P. schizantha* formed a distinctive cluster (Fig. 4). The exception in *P. schizantha* was Population 52 that showed some individuals genetically similar to *P. dubia* (Fig. 4). Population 35 assigned originally to *P. cinerea* is more similar to *P. glauciflora* (Fig 6).

Among all *Phaedranassa* species, *P. brevifolia* and *P. dubia*, at the level of $k = 7$ analysis, were not distinguished as two different groups (Fig. 4). Also the neighbor-joining tree showed *P. brevifolia* forming a subcluster in the *P. dubia* group (Fig. 3). However, when analysis was done with *P. dubia* and *P. brevifolia* alone, the resulting k was 2 (Table 5, Fig. 5). Finally *P. cinerea* showed two groups: (1) the populations at the western portion of the geographical distribution of *P. cinerea*, (2) populations located at the eastern portion of the species range. Some populations of this later group appear to be similar to *P. glauciflora* as the $k = 7$ analysis indicates (Fig 4). A separate analysis of *P. cinerea* and *P. glauciflora* found an optimal k of 10. The same pattern described above was still present (Table 5, Fig. 6).

There were three possible cases of sympatry in *Phaedranassa*, all of them involving *P. viridiflora* and several other *Phaedranassa* species. First, I analyzed the individual assignment for three collection groups of *P. viridiflora* (1) and three populations of *P. cinerea* (2) that are located in southern Ecuador (Fig. 2). These two species are separated to a distance of three to 12 km. The result shows that these groups belong to three genetically defined populations (k) (Table 5). There was no evidence of

admixture among the individuals assigned to the *P. viridiflora* (1) and individuals of *P. cinerea* (2) (Fig 8).

The second case is geographically located in central Ecuador (Fig. 2) and it includes *P. viridiflora* and *P. schizantha* (Fig. 1). In this case, most of the individuals were not in flower, thus the identity of the samples were unknown. The analysis showed an optimal population number of $k = 2$: Population 10 and 24 corresponded to one group (*P. viridiflora*), whereas Population 27 was assigned to a different group (*P. schizantha*) (Fig. 8). There was no evidence of extensive admixture among collecting sites of *P. viridiflora* and *P. schizantha* (Fig 8).

In the third case, *P. viridiflora* (yellow flower type), *P. dubia* (red flower type) and a putative hybrid between the two (orange flower type) are located in the Pululahua crater (Fig. 2 and 8). The individual assignment retrieved three genetically differentiated groups ($k = 3$). The individual assignment analysis also showed admixture between the putative hybrid and *P. dubia* (Fig. 8). Also, three groups could be distinguished in the PCA: one formed by orange flowers only, other formed by yellow flowers only and one formed by all red flowers and some yellow and orange flowers (Fig. 9).

Individual assignment within species

Within species, in general the optimal k was either two or three populations, with the exception of *P. cinerea*, for which the optimal k was 4 (Table 5). The number of optimal k represents in some cases a quarter of the number of collecting sites (Table 5, Fig. 7). Most of the patterns observed at the analysis of $k = 7$ were still found in the individual species analysis. The main difference is the substructure found within species (Fig.7). Substructure was found in *P. schizantha*, separating some populations around the

cities of Ambato and Riobamba from the other populations. On the other hand, I did not find evidence of distinct clusters for the varieties of *P. schizantha* proposed by Meerow (1990): *P. schizantha* var. *schizantha* and *P. schizantha* var. *ignea* either in the entire genus analysis nor the individual species analysis (Fig. 3 and 7). *Phaedranassa tunguraguae* was divided in two groups, one at the west and other to the east of the species distribution (Fig 7).

Landscape genetics

I did not find evidence of overall isolation by distance among populations, when all samples were included in the analysis ($R^2 = 0.11$, $p = 0.0001$) (figure not shown). Moreover there was no significant isolation by distance when data was separated by species. On the other hand, with the exception of some comparisons including *P. glauciflora* and *P. viridiflora*, there was minimal overlap among species distributions, on the basis of Kappa, especially in the cases of adjacent species like *P. brevifolia*-*P. dubia*, *P. cinerea*-*P. glauciflora* and *P. tunguraguae* and *P. schizantha* (Table 6).

Discussion

Species identification

My initial assumption was that *Phaedranassa* species will show genetic structure among populations and that this structure will be influenced by geography. It turns out to that to address this apparently straight forward query, another issue must first be confronted. The species identity was not known for all the populations, and, in some cases the presumed identity (herbarium records), was likely not accurate. Species identification is relatively easy in the field when flowers are present. Not all populations were in flower at the time of collection. To solve this problem, I assigned an *a priori*

identity to species for each population. In cases where I was able to revisit the location during the flowering season, I made changes if needed (marked in bold font in Table 1). In general, the genetic signature at the species level was consistent with the a priori species definition. However, for Population 59, identified as *P. schizantha* in the field, some individuals were assigned to *P. dubia* in the Bayesian analysis of individual assignment of $k = 7$ (Fig. 4). Furthermore, this species was part of the *P. dubia* group of the NJ cluster (Fig. 3). One possible explanation of this incongruence is the possibility of some of yellow form individuals (*P. viridiflora*) present in this collection. This is possible because of the geographic proximity of this site to other populations of both *P. schizantha* and *P. viridiflora*. This supposition needs to be corroborated at the field.

Genetic mosaic in the landscape

Under isolation, relatedness of individuals will decrease with distance (Hardy and Vekemans 1999). Because of the landscape heterogeneity of the Northern Andes, I was expecting a strong genetic association with geography. I did not find evidence corroborating a correlation between genetic differentiation and geography as a linear distance. What I found was evidence of a more complex pattern of genetic structure associated with restricted species distributions in spatial heterogeneity of the Andes. The following is a case by case description of the genetic mosaic of *Phaedranassa* in the Ecuadorian Andes is described below.

The species that showed the least amount of genetic diversity was *P. viridiflora*, found in central and south Ecuador (Table 1, Fig. 2). *Phaedranassa viridiflora* had the lowest number of private alleles, the fewest alleles per population and the lowest heterozygosity values (Table 1). The majority of the populations of this species showed

an impressive number of putative clones (individuals with the same multi-locus genotype, MLG) (Table 1). This is characteristic of peripheral populations in marginal environments (Stoeckel et al. 2006; Millar et al. 2010). The high level of clonarity in *P. viridiflora* is not found in any other *Phaedranassa* species, including the populations of *P. viridiflora* found in the Pululahua crater. Additionally, both cluster and individual assignment method separate the populations of *P. viridiflora* of the central and south area into two different groups, which also includes geographically adjacent species. No evidence of admixture was found between the species in central and southern Ecuador, which is compatible to sympatric speciation. The origin of the yellow form of *Phaedranassa* is unknown, but my results suggest that it has evolved three times in different parts of Ecuador and from different species (Fig 8).

On the other hand, the data show a possible case of natural hybridization and introgression among *P. dubia* and *P. viridiflora* in the Pululahua crater. The Pululahua crater constitutes the remains of the collapse of the volcano flanks after intensive volcanism during the Quaternary (Hall and Mothes 2008). This is the only locality where the presence of two different *Phaedranassa* species was known prior to this study. In this particular collecting site flowers were gathered in an effort to analyze the three color types separately (red for *P. dubia*, yellow for *P. viridiflora* and orange for a presumed hybrid). As opposed to the central and south *P. viridiflora* groups described above, Pululahua's populations of *P. viridiflora* have almost double the effective number of alleles. In a Bayesian analysis to assign individuals to either three groups, only red individuals (*P. dubia*) showed in all cases a consistent assignment to a particular group (Fig. 8). This pattern was not always evident for the yellow and orange forms, because

some individuals showed admixture (Fig. 8). The group formed by yellow flowers showed seven times more identical MLGs than the group formed by the orange flowers.

This fact and the individual assignment results suggest a scenario of introgression between the hybrids and *P. dubia* individuals. Hybridization and probably introgression is occurring in the Pululahua crater. Hybridization has been proposed as a major role in plant speciation in general (Soltis and Soltis 2009). Introgressive hybridization has been found in plants, for example a well studied system is the *Iris fulva* asymmetric introgression into both *I. brevicaulis* and *I. hexagona*, which apparently have adaptative implications (Arnold et al. 2010). Even though the amount of hybridization and introgression in Andean plant species is unknown, evidence have been found across different taxa: wild tomato species *Solanum lycopersicum* and *S. pimpinellifolium* (Solanaceae) (Nakazato and Housworth 2011), *Vasconcellea* (Caricaceae) (van Droogenbroeck et al. 2006) and *Puya* (Bromeliaceae) (Schulte et al. 2010).

In the latest taxonomic revision of revision of the Amaryllidaceae in Ecuador, the yellow flower form of *Phaedranassa* was recognized as *P. viridiflora* (Meerow 1990). *Phaedranassa viridiflora* was described by Baker in 1877 from cultivated material supposedly from Peru (Baker 1877). Another yellow variety, *P. viridilutea*, was described from a specimen located at the south of the generic range in Ecuador (Ravenna 1984). In this study, I found two groups that appear to be genetically different from each other (Figs 3, 4, 6 and 8). My results seem to identify at least two different geographic areas where yellow flowers occur. The species distribution model for *P. viridiflora* is not showing the specificity as in other *Phaedranassa* species, and this might be a result of considering all the individuals as only one species. Unfortunately, I cannot test the

specificity of the distribution for these two groups because of the limited number of records for each one of the groups, which is not appropriate for building a reliable SDM. A revision of the species including at least morphological evidence to support the separation of *P. viridiflora* into two species is needed.

On the opposite side of the spectrum, *P. brevifolia* showed the highest number of alleles and one of the highest averages of private alleles (Table 1). *Phaedranassa brevifolia* occurs at the north-western portion of the generic distribution in Ecuador (Fig 2). Vegetatively, the most evident difference between this species and *P. dubia* is the length of the leaves. *Phaedranassa brevifolia* is the species with the smallest leaves (ca. 12 cm long) while *P. dubia* leaves length are ca. 20 cm long. *Phaedranassa dubia* has larger flowers with a slightly inflated perianth base and darker red color (Fig 1). Moreover, the Species Distribution Models (SDM) for these two species do not overlap, indicative of niche differentiation (Table 5, Fig. 5). In the NJ cluster analysis, both species formed a group in which *P. brevifolia* was a subcluster (Fig. 3). The assignment of individuals of these two species together shows a higher degree of admixture between the geographically closer populations of the two (Fig 5). The results suggest that these two species seems to be an example of peripatric speciation and may be a result of a recent divergence.

A more extreme example of geographic divergence is *P. tunguraguae* and *P. schizantha*. Despite the relatively proximity of both species, they showed no overlapping geographic distribution. The former prefers more humid habitats than the later. Both are identified as separate groups by NJ clustering and individual assignment (Fig 3, 4 and 9). Differences between these two species may be influenced by the Tungurahua volcano.

Tungurahua is one of the most active volcanoes in Ecuador. During the last 1300 years, there is evidence of eruptions at least once every hundred years (Hall, 1999; Le Pennec et al. 2008). Throughout these events, pyroclastic flows and large lahars occurred along the volcano flanks. A large avalanche with a projected deposit volume of 8 km³ took place 3000 years BP (Le Pennec et al. 2008). The avalanche flowed through the Rio Patate and Rio Chambo valleys, which limit the western geographical distribution of *P. tunguraguae*. Moreover, I witnessed the last eruptive period since 1999, with episodes of activity in 2004 (Ruiz et al. 2006), 2006 (Sheridan et al. 2007) and late 2007- early 2008 (Le Pennec et al. 2008), and most recently in December 2010. Tungurahua was especially active in 2006 with more than 20 pyroclastic flows on ravine cuts in the north and western part of the volcano (Sheridan et al. 2007). The direction of the flow of both pyroclastic and lahars might have separated these two species either by a physical barrier or indirectly by creating different habitats during the development of the Tungurahua volcano.

Finally, the southernmost species, *P. cinerea* and *P. glauciflora* also present a pattern that appears to be linked to geography (Fig. 7). Both species distributions do not overlap (Table 6). Some level of admixture is evident among the western populations of *P. cinerea* and *P. glauciflora* populations Andes (Fig. 7). What I considered a priori *P. cinerea*, seems to be actually two different species (Fig. 7). One putative species is located at the western side of the Andes, which corresponds to the elevation of the original description of *P. cinerea*. The other group ("*P. cinerea* 2" in all Tables and Figures) is located at the central area of the Andes in a higher altitude than the original *P. cinerea*. The populations of "*P. cinerea* 2" might correspond to a new *Phaedranassa*

species. The hypothesis of two different species for *P. cinerea* requires further morphological and molecular analysis. Another issue that needs additional investigation is the monophyly of *P. glauciflora*.

Molecular markers are an indirect measure of demographic patterns. Indication of inbreeding was found for all *Phaedranassa* species except *P. viridiflora*. These results are expected because individuals of these species can reproduce clonally by bulbs and are self-compatible, producing viable seeds. However the values of inbreeding coefficients need to be taken with caution because of the high number of putative null alleles reported in this study. Null alleles can inflate the inbreeding coefficients. Also, evidence of recent bottleneck was found in three, eleven, or 36 populations, depending on the statistical test and mutation model. None of the species seems to be more prone to bottlenecks (Table 2). Conservatively, I can considered three populations that might have experienced a recent bottleneck (Table 2, populations in bold). Some cautionary considerations about the bottleneck test is that the method can fail to detect a bottleneck if the event was not recent enough, thus allowing re-establishment of mutation-drift equilibrium, or when sample size and number of loci are small (Mock et al. 2004). Additional sources of error are Hardy-Weinberg disequilibrium and null alleles (Cornuet and Luikart 1996). Features of *Phaedranassa* such as self-compatibility and reproduction by bulbs suggest inbreeding and genetic drift as a more likely explanation for the high frequency of homozygotes over the presence of null alleles. Because of the contrasting results among statistic methods and the possibility of violations of the requirements for the bottleneck test, these results needs to be taken as a first evidence that requires further research.

Taking into consideration all the described scenarios (hybridization, allopatric, peripatric speciation and niche differentiation), this study supports the idea that in a complex, dynamic and hyper-diverse area as the Northern Andes, the speciation process is not uni-dimensional but the result of multiple intricacies. The uplift of the Andes plays an important role in the speciation process of *Phaedranassa*. In Ecuador the mountains would have achieved 1,500-2,500 m of altitude during the last part of the Tertiary, and it was only in the Pleistocene that elevations reached altitudes > 4,000 m (Sauer 1957). Furthermore, climatic fluctuations during the Plio-Pleistocene influenced the species distribution as well as speciation process in the Andes (Luteyn 2002, Young et al. 2002). The entire genus evolved in the Northern Andes, which is a younger area of this mountain range, located north to the Amotape-Huancabamba zone. The Amotape-Huancabamba zone is considered either a phytogeographical barrier between the Northern and Central Andean species or a blend zone for both neo- and paleoendemics (Weigend 2002). The related genera *Rauhia* Traub (a Peruvian endemic) and *Eucrosia* Ker Gawler (found in both southern Ecuador and northern Peru); both related to *Phaedranassa*, are more diverse to the south of the Amotape Huancabamba zone (Meerow et al. 2000).

The data presented in the study suggest mostly allopatric speciation in *Phaedranassa*. It appears that the mosaic of environments in the Northern Andes contributed to divergence in the genus. This biogeographical scenario is also reported for the genus *Macrocarpaea* (Gentianaceae- Helieae) from the Andes, in which local allopatric speciation was more common than sympatric speciation (Struwe et al. 2009). Also allopatric speciation was proposed as an explanation for high number of endemic

species of plants in seasonally dry patches in the Andes (Pennington et al. 2010) and in the highlands (i.e. *Lupinus*, Hughes and Eastwood 2006). Speciation among birds in the tropical Andes has also been linked to local conditions and topography (Fjeldsa and Irestedt 2009). Adaptation to different climatic conditions in allopatric or parapatric populations may influence the speciation process, causing phenotypic variance that leads to the evolution of reproductive isolation (Gavrilets 2003).

Phaedranassa species appear to be segregated into different distribution ranges. In general there was low or no agreement among species distributions. It appears that 3,500 meters is the natural limit for this genus in Ecuador (Fig 2 and Fig 9). According to the Species Distribution Model (Chapter III in this dissertation), there are differences in the altitudinal range where each *Phaedranassa* species is located (Fig. 9). *Phaedranassa schizantha* and *P. tunguraguae* are different both in morphology and genetically. However, these two species are located in adjacent areas; but at different altitudinal ranges. The former occurs between 2,200 and 3,300 m, whereas the latter reaches 2,300 m as its upper limit and continues down to 1,300 m. Despite their geographic proximity in terms of distance, they are separated altitudinally. A similar difference in altitudinal range is found between *P. brevifolia* and *P. dubia* (Fig. 9). Even in the case of species that share almost the same altitudinal range, as the case of *P. glauciflora* and *P. cinerea*, it is evident that the mean altitudinal distribution is different between both (Fig. 9). The apparently non-specificity of altitudinal range between these two species might be caused by unresolved taxonomic issues. Both genetic differentiation and elevation range are evidence that the populations of *P. cinerea* located to the central-east portion are in fact a

different species. An exhaustive search for populations in that area, along with a morphological revision, is needed.

Theoretically, allopatric and sympatric speciation are the two opposite ends of a continuum of gene flow among diverging populations (Fitzpatrick et al. 2008, Nosil 2008). The majority of *Phaedranassa* species appear to have evolved by allopatric speciation; both lack of admixture among species and non-overlapping distribution models corroborate this statement. The divergence among *Phaedranassa* species does not completely fit allopatric speciation. I found a case of admixture between species (*P. dubia*- *P. viridiflora* in Pululahua) and species sharing the same localities (*P. viridiflora*-*P. cinerea* 2 and *P. viridiflora*-*P. schizantha*). There is no general agreement about the probability of divergence either in the middle or in the periphery of the species distribution. My data suggests that the speciation process is occurring at the periphery of the species distribution, probably by migration and colonization along the Andes topography.

Conservation implications

All endemic *Phaedranassa* species (except *P. dubia*, which is also found in Colombia), were classified either as Endangered or Vulnerable to extinction under the IUCN criteria (Oleas 2000). I propose changing the status of *P. schizantha* and *P. cinerea* from Vulnerable to Endangered, because of the reduction of species occurrences (Table 7). The occurrence of these species was overestimated in past assessments as a result of incorrect identification of specimens collected from the southern portion of the genus geographic range in Ecuador. On the other hand, this study shows that molecular markers can contribute to conservation efforts providing information about the genetic structure of

the species' populations. Using low genetic variation as an indicator, my study can be used to discriminate populations at risk which are the ones with a reduced genetic variation.

Limitations of this study and further research

Molecular studies of adult individuals in long-lived perennial plants such as *Phaedranassa* might still show an historical condition in which the genetic erosion as a result of habitat fragmentation is less severe (Tomimatsu and Ohara 2003, Van Geert et al. 2008). Further studies should include analysis of field-collected seedlings, which will be affected by present processes (Van Geert et al. 2008).

The results of this study need to be taken as a first step towards understanding the genetic structure of *Phaedranassa* species. Pursuing these kinds of studies on non-model species in the tropics is still challenging. Even for species with a relatively recent taxonomic review, species identification is not always clear-cut. In the process of this investigation I changed the species name of seven out of the 52 populations (13%). My results point out the need for a taxonomic review of the genus, especially for *P. cinerea*, *P. glauciflora* and *P. viridiflora*. Additional collections are needed especially of the southern taxa, because the species delimitation in this group requires a taxonomic revision. New occurrence records can then be incorporated in the SDMs for a better model of species ranges. Furthermore, this study is based on one type of molecular marker and microsatellites studies can potentially have issues as interspecific differences in mutation rates, constraints on microsatellite evolution and homoplasy (Estoup and Cornuet 1999; Amos 1999). I hope that in the future the cost of study other molecular

markers (i.e. single nucleotide polymorphism) will be accessible for further investigation of *Phaedranassa* speciation.

Figure 1. Ecuador's species of *Phaedranassa*. a) *Phaedranassa brevifolia*; b) *P. cinerea*; c) *P. dubia*; d) *P. glauciflora*; e) *P. schizantha* ; f) *P. tunguraguae* ; g) *P. viridiflora*; h) Up: *P. viridiflora*; Down: possible *P. dubia* x *P. viridiflora* hybrid

Figure 2. Distribution map of *Phaedranassa* spp. in Ecuador. Squares are locations with more than one *Phaedranassa* species

● <i>P. brevifolia</i>	● <i>P. glauciflora</i>	◆ <i>P. dubia</i> (Pululahua)
● <i>P. cinerea</i>	● <i>P. schizantha</i>	◆ <i>P. viridiflora</i> (Pululahua)
● <i>P. dubia</i>	● <i>P. tunguraguae</i>	□ <i>P. vidiflora</i> ?
◆ <i>P. dubia</i> x <i>viridiflora</i> ?	● <i>P. viridiflora</i>	

Figure 3. Unrooted neighbor joining tree showing relationship among populations of *Phaedranassa* spp. in Ecuador. Number in branches are bootstrap values > 50% with 1000 replications

Figure 4. Plots of the individual assignment of *Phaedranassa* under the assumption of $k = 7$ (number of species). Individuals are represented as a thin vertical line with colors indicating cluster membership. Population ID is listed under the plot

Figure 5. Bayesian individual assignment for *Phaedranassa brevifolia* and *P. dubia*. A) Assignment with number of populations $k=2$ with both species analyzed together, B) Assignment for each species separately ($k=2$ each). Area in grey is altitude >3500 m

Figure 6. Analysis of population structure of *P. glauciflora*, and *P. cinerea*. Plot bar represent the estimated population structure per each one of the species' populations ($k=10$). Area in pink represent the species distribution model (SDM) for *P. glauciflora*, in green is SDM for *P. cinerea*.

Figure 7. Plots of the individual assignment of each species of *Phaedranassa* analyzed separately. Individuals are represented as a thin vertical line with colors indicating cluster membership

Figure 8. Population genetic structure of *P. viridiflora*. A) *P. viridiflora* and *P. dubia* in Pululahua, B) *P. viridiflora* (Pv) and *P. schizantha* (Ps), C) *P. viridiflora* (Pv) and *P. cinerea* (Pc). Plot bar represent the estimated population structure per each of the species' populations. Area in yellow is *P. viridiflora*'s distribution model, area in grey is area with altitude >3500m

Figure 9. Principal Coordinates Analysis (PCA) of *Phaedianassa* individuals in Pululahua crater. The first, second and third axes explained 44, 23 and 10% respectively, of the total variability. Symbols are: orange flowers (a), red flowers (b) and yellow flowers (c)

Figure 10. Altitudinal range (m) from the Species Distribution Model of seven *Phaedranassa* species in Ecuador

Table 1. Basic descriptive statistics of the genetic structure of *Phaedranassa* spp. Population name (Pop ID); Collection ID, (Col ID) on bold are species assignment changed after further study; Sample number (N); Clone individuals (C); Number of alleles (Na); Effective Allele number (Ne), Expected and Observed heterogosity (He, Ho), Fixation index (F), Number of pair of locus with Linkage disequilibrium (LD), Number of Private alleles (PA), Loci with evidence of null alleles (NL)

Species	Pop ID	Col ID	N	C	Na	Ne	Ho	He	F	LD	PA	NL
<i>P. brevifolia</i>	Pop28	4pb	29	3	6.92	3.47	0.38	0.68	0.46	0	1	12
<i>P. brevifolia</i>	Pop11	2pb	30	0	7.31	4.05	0.44	0.66	0.30	0	2	8
<i>P. brevifolia</i>	Pop20	3pb	30	0	7.38	4.12	0.40	0.65	0.35	0	2	10
<i>P. brevifolia</i>	Pop3	1pb	30	0	8.92	5.18	0.43	0.72	0.39	0	3	10
Mean			28.75	0.75	7.63	4.21	0.41	0.68	0.37	0	2	10
<i>P. cinereal</i>	Pop4	1pc	30	0	4.77	2.21	0.31	0.48	0.33	0	0	7
<i>P. cinereal</i>	Pop45	7pc	31	0	6.54	2.60	0.36	0.51	0.28	0	1	5
<i>P. cinereal</i>	Pop21	3pc	29	0	6.38	3.11	0.40	0.58	0.30	0	3	7
<i>P. cinereal</i>	Pop29	4pc	30	0	8.92	3.25	0.43	0.60	0.30	0	8	5
<i>P. cinereal</i>	Pop49	8pc	31	0	8.23	4.22	0.35	0.70	0.48	0	2	10
Mean			28.92	0	6.97	3.08	0.37	0.58	0.34	0	2.8	6.8

Table 1. Continued

Species	Pop ID	Col ID	N	C	Na	Ne	Ho	He	F	LD	PA	NL
<i>P. cinerea2</i>	Pop15	2ps	30	13	4.08	2.04	0.32	0.32	0.27	0	0	9
<i>P. cinerea2</i>	Pop12	2pc	27	4	5.69	2.50	0.41	0.49	0.20	18	1	7
<i>P. cinerea2</i>	Pop24	3ps	13	0	3.92	3.10	0.41	0.62	0.42	0	0	6
<i>P. cinerea2</i>	Pop35	5pc	29	0	8.46	4.47	0.41	0.68	0.39	0	2	8
<i>P. cinerea2</i>	Pop40	6pc	30	0	8.62	4.82	0.42	0.73	0.41	0	3	10
Mean			24.32	3.4	6.15	3.39	0.39	0.57	0.34	2.0	1.0	8
<i>P. dubia</i>	Pop22	3pd	12	0	3.54	2.43	0.63	0.52	-0.09	0	1	2
<i>P. dubia</i>	Pop46	7pd	9	0	5.00	3.29	0.47	0.57	0.19	0	1	2
<i>P. dubia</i>	Pop50	8pd	6	0	4.15	3.50	0.52	0.64	0.21	0	0	-
<i>P. dubia</i>	Pop30	4pd	23	1	6.85	4.07	0.41	0.63	0.31	0	0	9
<i>P. dubia</i>	Pop13	2pd	30	2	8.00	4.10	0.42	0.70	0.38	7	4	10
<i>P. dubia</i>	Pop41	6pd	27	0	8.08	4.13	0.45	0.69	0.34	0	3	8
<i>P. dubia</i>	Pop36	5pd	23	1	7.85	4.22	0.49	0.69	0.29	0	1	10
<i>P. dubia</i>	Pop5	1pd	26	0	9.23	4.82	0.44	0.75	0.40	0	2	12
Mean			18.45	0.5	6.59	3.82	0.48	0.65	0.25	0.9	1.5	6.6

Table 1. Continued

Species	Pop ID	Col ID	N	C	Na	Ne	Ho	He	F	LD	PA	NL
<i>P. glauciflora</i>	Pop14	2pg	27	0	7.69	4.05	0.42	0.69	0.37	0	2	9
<i>P. glauciflora</i>	Pop23	3pg	30	0	8.62	4.48	0.43	0.71	0.39	0	2	10
<i>P. glauciflora</i>	Pop6	1pg	28	4	4.15	2.15	0.35	0.47	0.28	27	1	6
Mean			28.33	1.3	6.82	3.56	0.40	0.63	0.35	9	1.7	8.3
<i>P. schizantha</i>	Pop47	7ps	30	9	3.85	2.20	0.53	0.47	-0.11	1	1	5
<i>P. schizantha</i>	Pop51	8ps	24	0	5.92	2.82	0.38	0.59	0.31	0	0	7
<i>P. schizantha</i>	Pop8	1psi	30	8	5.15	2.85	0.38	0.55	0.39	39	3	6
<i>P. schizantha</i>	Pop42	6ps	29	10	5.15	2.87	0.48	0.56	0.14	39	2	6
<i>P. schizantha</i>	Pop32	4psi	16	2	5.38	3.31	0.50	0.65	0.22	11	0	4
<i>P. schizantha</i>	Pop27	3pv	28	1	7.85	3.66	0.39	0.69	0.44	0	2	12
<i>P. schizantha</i>	Pop16	2psi	30	0	7.85	3.77	0.43	0.65	0.37	0	2	8
<i>P. schizantha</i>	Pop2	11ps	27	0	8.15	4.11	0.43	0.69	0.36	0	2	10
<i>P. schizantha</i>	Pop25	3psi	30	0	9.23	4.30	0.46	0.73	0.37	0	4	10
<i>P. schizantha</i>	Pop52	9ps	26	4	7.62	4.37	0.44	0.69	0.31	7	0	9
<i>P. schizantha</i>	Pop1	10ps	33	0	9.77	5.05	0.52	0.77	0.33	0	2	8

Table 1. Continued

Species	Pop ID	Col ID	N	C	Na	Ne	Ho	He	F	LD	PA	NL
<i>P. schizantha</i>	Pop37	5ps	31	2	10.69	5.39	0.44	0.75	0.39	0	4	10
Mean			26.32	3	7.22	3.73	0.45	0.65	0.29	8.0	1.8	7.9
<i>P. tunguraguae</i>	Pop43	6pt	28	1	5.00	2.45	0.40	0.48	0.19	0	0	0
<i>P. tunguraguae</i>	Pop48	7pt	28	3	5.15	2.95	0.37	0.50	0.34	0	1	0
<i>P. tunguraguae</i>	Pop17	2pt	10	0	5.69	3.98	0.54	0.62	0.12	0	0	0
<i>P. tunguraguae</i>	Pop26	3pt	26	0	6.92	4.11	0.57	0.66	0.10	0	1	0
<i>P. tunguraguae</i>	Pop38	5pt	18	0	6.62	4.18	0.47	0.60	0.24	0	0	0
<i>P. tunguraguae</i>	Pop33	4pt	15	0	6.77	4.67	0.51	0.68	0.21	0	1	0
<i>P. tunguraguae</i>	Pop9	1pt	31	0	9.85	4.75	0.51	0.67	0.32	0	1	0
Mean			22.28	0.5	6.57	3.87	0.48	0.60	0.22	0	0.57	0
<i>P. viridiflora1</i>	Pop7	1ps	31	26	1.92	1.29	0.25	0.14	-0.45	0	1	0
<i>P. viridiflora1</i>	Pop18	2pv	29	18	2.31	1.41	0.33	0.21	-0.22	0	0	2
<i>P. viridiflora1</i>	Pop31	4ps	30	12	2.77	1.48	0.31	0.22	-0.12	0	0	7
Mean			30	18.67	2.33	1.39	0.30	0.19	-0.27	0	0.33	3
<i>P. viridiflora2</i>	Pop19	2px	30	28	1.54	1.32	0.31	0.17	-0.41	0	0	1

Table 1. Continued

Species	Pop ID	Col ID	N	C	Na	Ne	Ho	He	F	LD	PA	NL
<i>P. viridiflora2</i>	Pop34	4pv	29	19	2.15	1.34	0.24	0.16	-0.01	0	0	3
<i>P. viridiflora2</i>	Pop10	1px	30	6	3.54	1.66	0.33	0.31	0.14	0	0	6
Mean			29.67	17.67	2.41	1.44	0.29	0.21	-0.09	0	0	3.3
<i>P. viridiflora3</i>	Pop44	6pv	31	14	4.92	2.10	0.51	0.47	0.08	22	2	4
<i>P. viridiflora3</i>	Pop39	5pv	27	2	6.85	2.90	0.20	0.59	0.66	19	1	12
Mean			29	8	5.88	2.50	0.36	0.53	0.37	5.1	1.5	8
Total Mean			25.01		6.31	3.38	0.42	0.57	0.26		1.4	
SE			0.26		0.14	0.08	0.01	0.01	0.02			

Table 2. Test for recent bottleneck events in seven populations of *Phaedranassa* spp. on the basis of three tests. In bold are evidence of bottleneck cases

Species	Pop ID	(1)Sign test		(2)Wilcox test		(3)Mode-shift
		IAM	SSM	IAM	SMM	
<i>P. brevifolia</i>	Pop11	0.32	0.11	0.23	0.86	
<i>P. brevifolia</i>	Pop20	0.53	0.25	0.21	0.92	
<i>P. brevifolia</i>	Pop28	0.31	0.04	0.05	0.98	
<i>P. brevifolia</i>	Pop3	0.05	0.01	0.03	0.97	
<i>P. cinerea1</i>	Pop21	0.51	0.00	0.34	1.00	
<i>P. cinerea1</i>	Pop29	0.01	0.00	1.00	1.00	
<i>P. cinerea1</i>	Pop4	0.44	0.00	0.53	1.00	
<i>P. cinerea1</i>	Pop45	0.27	0.00	0.86	1.00	
<i>P. cinerea1</i>	Pop49	0.44	0.04	0.08	0.99	
<i>P. cinerea2</i>	Pop12	0.01	0.00	0.98	1.00	
<i>P. cinerea2</i>	Pop15	0.23	0.02	0.92	1.00	
<i>P. cinerea2</i>	Pop35	0.33	0.04	0.39	0.98	
<i>P. cinerea2</i>	Pop40	0.16	0.00	0.00	1.00	
<i>P. dubia</i>	Pop13	0.33	0.00	0.15	0.99	
<i>P. dubia</i>	Pop30	0.28	0.12	0.07	0.92	
<i>P. dubia</i>	Pop36	0.54	0.04	0.11	1.00	
<i>P. dubia</i>	Pop41	0.15	0.00	0.05	1.00	
<i>P. dubia</i>	Pop5	0.23	0.00	0.19	1.00	
<i>P. glauciflora</i>	Pop14	0.56	0.01	0.12	1.00	
<i>P. glauciflora</i>	Pop23	0.35	0.00	0.11	1.00	

Table 2 Continued

Species	Pop ID	(1)Sign test		(2)Wilcox test		(3)Mode-shift
		IAM	SSM	IAM	SMM	
<i>P. schizantha</i>	Pop1	0.17	0.01	0.02	1.00	
<i>P. schizantha</i>	Pop16	0.31	0.00	0.27	1.00	
<i>P. schizantha</i>	Pop2	0.57	0.00	0.45	1.00	
<i>P. schizantha</i>	Pop25	0.44	0.00	0.25	1.00	
<i>P. schizantha</i>	Pop27	0.46	0.01	0.27	1.00	
<i>P. schizantha</i>	Pop37	0.58	0.01	0.34	1.00	
<i>P. schizantha</i>	Pop42	0.25	0.29	0.12	0.83	
<i>P. schizantha</i>	Pop47	0.23	0.53	0.08	0.66	
<i>P. schizantha</i>	Pop51	0.30	0.00	0.42	1.00	
<i>P. schizantha</i>	Pop52	0.05	0.04	0.00	0.98	
<i>P. schizantha</i>	Pop8	0.44	0.13	0.07	0.96	
<i>P. tunguraguae</i>	Pop26	0.30	0.14	0.05	0.93	
<i>P. tunguraguae</i>	Pop43	0.53	0.01	0.35	1.00	
<i>P. tunguraguae</i>	Pop48	0.45	0.04	0.18	0.97	
<i>P. tunguraguae</i>	Pop9	0.45	0.00	0.66	1.00	
<i>P. viridiflora1</i>	Pop18	0.53	0.20	0.63	0.85	
<i>P. viridiflora1</i>	Pop31	0.13	0.08	0.68	0.97	
<i>P. viridiflora1</i>	Pop7	0.39	0.26	0.58	0.95	
<i>P. viridiflora2</i>	Pop10	0.10	0.00	0.93	1.00	
<i>P. viridiflora2</i>	Pop19	0.35	0.45	0.05	0.05	+
<i>P. viridiflora2</i>	Pop34	0.22	0.04	0.81	0.95	
<i>P. viridiflora3</i>	Pop44	0.50	0.00	0.77	1.00	
<i>P. viridiflora h</i>	Pop39	0.26	0.00	0.88	1.00	

Table 3. Analysis of molecular variance (AMOVA) results as average over 13 loci

Source of variation	Sum of squares	Variance components	Percentage variation
Among groups	1479.05	0.50	9.34
Among populations within groups	2753.63	1.16	21.54
Within populations	9519.23	3.74	69.12
Total	13751.90	5.40	

Table 4. Number of migrants (Nm) using private alleles method (after size correction)

Species	Mean frequency private alleles	Nm
<i>P. brevifolia</i>	0.03	4.19
<i>P. cinerea</i>	0.03	3.2
<i>P. cinerea</i> 1	0.04	1.93
<i>P. cinerea</i> 2	0.04	2.74
<i>P. cinerea</i> 2 (no Pop35)	0.06	1.44
<i>P. cinerea</i> (no Pop35)	0.05	1.74
<i>P. dubia</i>	0.04	3.73
<i>P. glauciflora</i>	0.05	1.74
<i>P. schizantha</i>	0.05	1.95
<i>P. tunguraguae</i>	0.05	2.45
<i>P. viridiflora</i>	0.07	0.97
<i>P. cinerea</i> – <i>P.</i> <i>glauciflora</i>	0.03	3.4
<i>Phaedranassa</i> all	0.04	2.3

Table 5. Comparison of the number of collecting sites and the population number (k), inferred genetically by Evanno method (2008). The average pairwise similarity (H') among 20 runs

Species	Collecting sites	k	H'
<i>P. brevifolia</i>	4	2	0.99
<i>P. cinerea</i>	10	4	0.85
<i>P. dubia</i>	8	2	0.99
<i>P. glauciflora</i>	3	3	0.68
<i>P. schizantha</i>	12	3	0.88
<i>P. tunguraguae</i>	7	2	0.99
<i>P. viridiflora</i>	3	2	0.61
<i>P. cinerea</i> – <i>P. glauciflora</i>	13	10	0.74
<i>P. cinerea</i> - <i>P. viridiflora</i> South (case 1)	6	3	0.93
<i>P. schizantha</i> – <i>P. viridiflora</i> Pelileo (case 2)	3	2	0.99
<i>P. dubia</i> – <i>P. viridiflora</i> Puluahua (case 3)	1	3	0.99

Table 6. Percentages of Species Distribution Model overlap

Species pair comparison	Kappa
<i>P. glauciflora</i> – <i>P. viridiflora</i>	0.693
<i>P. glauciflora</i> – <i>P. schizantha</i>	0.451
<i>P. schizantha</i> – <i>P. viridiflora</i>	0.451
<i>P. dubia</i> – <i>P. viridiflora</i>	0.188
<i>P. dubia</i> – <i>P. glauciflora</i>	0.165
<i>P. dubia</i> – <i>P. schizantha</i>	0.099
<i>P. tunguraguae</i> – <i>P. viridiflora</i>	0.038
<i>P. glauciflora</i> – <i>P. tunguraguae</i>	0.032
<i>P. cinerea</i> – <i>P. viridiflora</i>	0.013
<i>P. brevifolia</i> - <i>P. glauciflora</i>	0.005
<i>P. brevifolia</i> – <i>P. viridiflora</i>	0.004
<i>P. cinerea</i> – <i>P. dubia</i>	0.004
<i>P. brevifolia</i> – <i>P. schizantha</i>	0.003
<i>P. brevifolia</i> - <i>P. cinerea</i>	0.002
<i>P. brevifolia</i> – <i>P. tunguraguae</i>	-0.002
<i>P. brevifolia</i> - <i>P. dubia</i>	-0.003
<i>P. schizantha</i> – <i>P. tunguraguae</i>	-0.004
<i>P. cinerea</i> – <i>P. tunguraguae</i>	-0.005
<i>P. dubia</i> – <i>P. tunguraguae</i>	-0.005
<i>P. cinerea</i> – <i>P. schizantha</i>	-0.019
<i>P. cinerea</i> – <i>P. glauciflora</i>	-0.23

Table 7. New proposed conservation status of *Phaedranassa* (Amaryllidaceae) in Ecuador

Species	IUCN 2010 status	2011 status proposed
<i>P. brevifolia</i>	Endangered B1ab(iii); C2a(i)	Endangered B1ab(iii)
<i>P. cinerea</i>	Vulnerable B1ab(iii)	Endangered B1ab(iii)
<i>P. glauciflora</i>	Endangered B1ab(iii)	Endangered B1ab(iii)
<i>P. schizantha</i>	Vulnerable B1ab(iii)	Endangered B1ab(ii,iii)
<i>P. tunguraguae</i>	Endangered B1ab(iii)	Endangered B1ab(iii)
<i>P. viridiflora</i>	Endangered B1ab(iii)	Endangered B1ab(iii)

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CHAPTER V.

CONCLUSIONS AND FUTURE RESEARCH DIRECTIONS

In this research, I developed eight newly designed microsatellite primers for *P. schizantha*. These primers and the results of cross-amplification with other *Phaedranassa* species are presented in Chapter II in this dissertation. Because of their high level of polymorphism and the amplification success with all other *Phaedranassa* species in Ecuador, these loci were used for the investigation of genetic variation of the genus (Chapter IV).

Another difficulty in working with tropical plants is the general deficiency of species records. Before this study, three of the seven *Phaedranassa* species were known by less than two populations each. The use of Species Distribution Models (SDMs), as addressed in Chapter III, is a valuable tool to identify areas where the species can potentially be found. Using the models designed as part of this research, I was able to find six new populations. However, as documented in Chapter III, species records used for SDMs require exhaustive prior verification in order to avoid misinterpretations caused by errors in the data. I choose to exemplify this problem by developing SDMs with three different dataset sources: (1) species records from databases available online only, (2) species records which taxonomy was verified and (3) species records confirmed at the field. My study showed that considerable differences were found between models obtained from different data sources.

Finally, genetic diversity is not randomly distributed across the landscape. The complexity of the demography with real species is not fully explained by simple models of speciation. Furthermore speciation processes in a genus do not necessarily follow the

same path for all the species. My study provides empirical evidence of spatial divergence of *Phaedranassa* species in Ecuador, a pattern that is evident in the majority of the species in the genus. The mechanism of speciation in *Phaedranassa* resembles a parapatric speciation model. My data suggests that the speciation process is occurring at the periphery of the species distributions, probably by migration and colonization along the Andes topography. At the same time, I provided the first natural hybridization support in the genus, between *P. viridiflora* and *P. dubia* in Pululahua crater. Moreover, two putative sympatric speciation cases were also described in this research.

Molecular studies of adult individuals in long-lived perennial plants such as *Phaedranassa* might still show an historical condition in which the genetic erosion as a result of habitat fragmentation is less severe (Tomimatsu and Ohara 2003, Van Geert et al. 2008). Further studies should included analysis of seedlings which will be affected by present processes (Van Geert et al. 2008). Also, the findings of this research need to be corroborated with other molecular markers, in order to overcome the violation of assumptions in the models analyzed (i.e., Non Hardy-Weinberg equilibrium, Linkage disequilibrium, non-random reproduction, selfing and null alleles).

The results of this study need to be taken as a first step towards understanding the genetic structure of *Phaedranassa* species. Pursuing these kinds of studies on non-model species in the tropics is challenging. Even for species with a relatively recent taxonomic review, species identification is not always clear-cut. In the process of this investigation I change the species names of seven out of the 52 populations collected (13%), using molecular data and additional field work. My results point out the need for a taxonomic review of the genus, especially for *P. cinerea* and *P. glauciflora* and *P. viridiflora*. For

this, additional collections are needed, especially for the southern taxa, because the species delimitations in this group require a taxonomic revision. New occurrence records can then be incorporated in the SDM for a better model of species range.

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PUBLICATIONS AND PRESENTATIONS

Oleas, N, Meerow, A, Francisco-Ortega, J 2005. Isolation and characterization of eight microsatellite loci from *Phaedranassa tunguraguae* (Amaryllidaceae). *Molecular Ecology Notes* **5**: 791-793.

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