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EFFECT OF TOPOGRAPHY ON GENETIC DIVERGENCE AND PHENOTYPIC TRAITS IN TROPICAL FROGS

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EFFECT OF TOPOGRAPHY ON GENETIC DIVERGENCE AND PHENOTYPIC TRAITS IN TROPICAL FROGS

by

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Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

August 2011

Dedication

To Isabel, Enrique, Marcela, and Carolina

Acknowledgements

First I want to thank my advisor David Cannatella, who gave me the opportunity to choose my own dissertation topic and also provided excellent advise in all aspects of my dissertation. I also thank my committee members Jim Bull, Claus Wilke, Tom Juenger, and Francisco Perez, who always were helpful every time I needed advise.

Funding was provided by the NSF-DDIG grant 0910313 and the L. I. Stengl and Hamilton Graduate fellowships at UT Austin. I want to thank Santiago Ron and Luis Coloma for their help with the logistics in Ecuador. Numerous people helped me during fieldwork. In Ecuador I specially thank Francisco Ayala. In Colombia I thank Adolfo Amézquita, Oscar Ramos, Alexandra Delgadillo, Onil Ballestas, Rafael Guerrero, and Vicky Flechas.

I thank Patricia Salerno, Monica Guerra, Patricia Jones, Laura Dugan, Vicky Huang and the people from Cannatella-Hillis-Bull labs for their insightful comments on this dissertation. Mark Helper provided advice for the calculation of contour and LCP distances. Finally, I thank my coauthors of chapter three, Camilo Escallón and Adolfo Amézquita, who provided part of the acoustic and morphological data used.

Samples were obtained from Colombia with exportation permit number 01187 (15 November 2007) by the Ministerio de Medio Ambiente, Vivienda, y Desarrollo Territorial. Samples were obtained in Ecuador through the permit granted to the Museo de Zoologia de la Universidad Católica del Ecuador.

EFFECT OF TOPOGRAPHY ON GENETIC DIVERGENCE AND PHENOTYPIC TRAITS IN TROPICAL FROGS

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Complex interactions between topographic heterogeneity and steep gradients in climate and environmental conditions are commonly assumed to promote biotic diversification. Using tropical frogs as a model, I investigate the nature of these interactions that disrupt migration between populations, causing genetic divergence and speciation. I determine the role of several putative factors that affect gene flow (Euclidean distances, Least Cost Path (LCP) distances, topographic complexity, and elevation difference) and promote genetic structure (FST) between populations of three tropical Andean frog species. Moreover, I investigate, from an intraspecific perspective, whether montane frog species display on average larger genetic distances per kilometer relative to lowland species. Finally, I test if recent genetic divergence caused by topographic barriers to gene flow is paralleled by independent character systems such as acoustics and morphological traits in the high Andean frog *Dendropsophus labialis*.

Even though the effect of geographic features on migration (and conversely, FST) was species-specific, LCP and Euclidean distances had the

strongest effect on migration rate. Topographic complexity also reduced migration rate whereas elevation difference did not have an effect. I found that indeed highland species show larger genetic distances per kilometer between haplotypes than do lowland species. Also, genetic divergence is strongly associated with topographic heterogeneity, which is an intrinsic characteristic of montane regions. Finally, I found that acoustic variation in *D. labialis* diverges according to genealogical history, but external morphology does not follow this relationship. Stochastic processes due to genetic drift appear to be a better explanatory mechanism for the divergence in calls than adaptive variation. The strong and congruent divergence observed in acoustic and genetic characters indicates that these two groups correspond to morphologically cryptic parapatric species.

Overall, the results of this study suggest some of the mechanisms that allow tropical mountains to promote intraspecific genetic divergence. The combined effect of ridges (promoting allopatric differentiation) and environmental gradients across elevation (promoting parapatric differentiation) are effective forces that are present mostly in highland biomes. Unfortunately, such biomes are critically threatened by habitat destruction and climate change, possibly more than any other biome on earth.

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Chapter 1: Geographic determinants of migration in tropical Andean frogs

ABSTRACT

Complex interactions between topographic heterogeneity and steep gradients in climate and environmental conditions are commonly assumed to promote biotic diversification. Here I investigate the nature of these interactions that disrupt migration between populations, causing genetic divergence and speciation. I determine the role of several putative impediments to gene flow (Euclidean distances, Least Cost Path distances, topographic complexity, and elevation difference) in restricting migration rate and promoting genetic structure (F_{ST}) between populations of three tropical Andean frog species. Even though the effect of geographic features on migration (and conversely, F_{ST}) was species-specific, overall, LCP and Euclidean distances had the strongest effect on migration rate. Topographic complexity also reduced migration rate whereas elevation difference did not have an effect. I suggest the former is a consequence of the two-fold effect of topography on species diversity. Finally, I developed a method that uses Least Cost Path distances to determine whether a pair of populations is likely to genetically diverge as a result of geographic isolation.

INTRODUCTION

That biological diversity increases at lower latitudes is a well-known biogeographic pattern (Pianka 1966; Rohde 1992). A closer look reveals, however, that tropical hotspots of biodiversity are not randomly distributed, but are mainly associated with continental highlands (Myers et al. 2000; Orme et al. 2005; Jansson and Davies 2008). Given that highlands encompass far less area than lowlands, montane regions contain exceptionally high diversity relative to their area (Korner 2007).

Many hypotheses have attempted to explain the role of tropical mountains in promoting diversity. Vicariance (Veith et al. 2003; Rull 2005) and ecological speciation across elevation gradients (Endler 1977; Moritz et al. 2000; Hall 2005) are commonly mentioned as contrasting factors that promote highland speciation; these processes are more likely to operate in heterogeneous montane habitats than in the more homogeneous lowlands (Garrick 2010).

Most studies that have tested hypotheses for highland speciation use phylogenetic approaches, comparing either rates of speciation (Wiens et al. 2007), or the association of tree topology with highland colonization and/or diversification (Hall 2005; Roberts et al. 2006). Even though phylogenetic approaches are useful to answer questions related to broad biogeographic processes, population genetics and phylogeography answer questions related to early stages of speciation.

Migration, the long-term movements of individuals from natal sites that produce patterns of gene flow (Vignieri 2005) is a relevant population parameter to estimate given its inverse relationship to population differentiation and subsequent speciation (Latter 1973; Wiens and Donoghue 2004). Determining how migration is associated with particular features of the landscape can provide fine grain information regarding why mountains promote diversification.

For organisms that live in mountains, moving 100 m along a flat terrain is not the same as moving 100 m up a steep terrain. Even though the thermal physiology of some ectotherms is conservative relative to other groups (Angilletta 2002), the steepness in temperature gradients across elevation strata is so strong that even the slightest changes in elevation expose individuals to extremely different energy consumption and environmental regimes. Therefore, in complex topographies an organism must traverse environmental gradients

ascending and descending several times to go from point A to point B; which can potentially restrict the long-term rate of movement.

The direction of migration may be influenced by steep elevational gradients. Asymmetric migration has been reported in several species. For example, water currents have been associated with asymmetric migration patterns in bull-kelp (Collins et al. 2010), salmon (Consuegra et al. 2005), and sticklebacks (Bolnick et al. 2008). Given that mountains have not only gradients in climate, but also unidirectional flow of water, we also expect to find asymmetric migration patterns in montane organisms associated with water.

Tropical Andean frogs are an ideal group for studying diversification in montane regions because a) Anurans generally have restricted migration abilities (Smith and Green 2005), b) Their metabolism is under the direct influence of elevational gradients (Feder and Burggren 1992), c) Montane clades of frogs tend to have higher rates of diversification (Smith et al. 2007), and d) The preeminent global hotspot of terrestrial vertebrate diversity is the tropical Andes (Myers et al. 2000; Orme et al. 2005). We used the sister-species *Dendropsophus labialis* and *Dendropsophus sp.* (Hylidae), and *Pristimantis achatinus* (Strabomantinae), all of which have a wide elevational distribution in the Andes, to determine how topography influences the amount and direction of migration. *Dendropsophus sp* corresponds to the northern clade of *D. labialis* as described in Guarnizo et al. (2009).

I test the relative effect of vertical and horizontal distances on the amount and direction of gene flow. Our questions are a) What are the relative effects of Euclidean and ecologically based distances (Least Cost Path) on migration? b) Is topographic complexity associated with migration? c) Does the difference in

elevation between populations affect migration? d) Do elevation differences promote migration asymmetry?

MATERIALS AND METHODS

Sampling and DNA sequencing

Tissues were obtained from *D. labialis* and *D. sp.* captured around small ponds (~ 10-15 m in diameter) along the western slope of the Colombian Eastern Andes. *Pristimantis achatinus* was collected in forested areas along the western slope of the Ecuadorian Andes (see Fig. 1.1; and Table 1.1). High abundance and elevational ranges of at least 2000 m characterize these three species, whereas most Andean species of frogs have narrower elevational ranges between 400 and 600 m (Navas 2002). I obtained tissues by clipping the toe of adult frogs or the tail tips of larvae (IACUC protocol number 07101901). To avoid siblings (which will bias allelic diversity), adults were preferred over larvae. In cases where larvae were collected, different larval sizes from different regions in the pond were used. After recording the geographic coordinates of each individual, tissues were immediately transferred to plastic tubes of 97% ethanol and were stored at -80ºC until DNA extraction.

I estimated migration rates and genetic structure using DNA sequences from the fast-evolving control region of mitochondrial DNA (600 bp), together with the nuclear proopiomelanocortin (POMC) gene (480 bp) and the exons 2, 3 of the cellular myelocytomatosis (c-myc) gene (800 bp). Genomic DNA was extracted using the Viogene Blood and Tissue Genomic DNA Extraction Miniprep following the manufacturer protocols. Three DNA fragments were amplified using published primers for the control region (controlJ2-L: 5'GCATTACGTTCACGAAGWTGG3', controlP-H: 5'GTCCATAGATTCASTTCCGTCAG-3'; (Goebel et al. 1999)), POMC (PomcR1: 5'GGCRTTYTTGAAWAGAGTCATTAGWGG-3', PomcF1:

5’ATATGTCATGASCCAYTTYCGCTGGAA-3’; (Vieites et al. 2007)), and c-myc (cmyc1U: 5’ GAGGACATCTGGAARAARTT-3’; cmyc3L: 5’GTCTTCCTCTTGTCRTTCTCYTC-3’; (Crawford 2003)). Amplifications were performed with a reaction mix containing 2.5 µl of 10X PCR buffer (500 mM KCl, 100mM Tris-HCl, pH 8.5), 2.5 µl of 8 µM dNTP’s, 13.875 µl of dH2O, 1.0 µl of MgCl2, 0.125 µl of DNA polymerase, 1.25 µl of each primer (10µM), and 2.5 µL of the DNA extract for a final volume of 25 µL.

The PCR cycle for the control region included an initial denaturing step of 2 of min at 94ºC, followed by 35 amplification cycles (30 s at 94ºC, 30s at 48ºC, 1 min at 72ºC), and a final extension of 7 min at 72ºC. For POMC we used the PCR cycle protocol from Guarnizo (2009). For c-myc I used the PCR cycle protocol from Crawford (2003). PCR products were purified using a Viogene Gel Purification Kit. Clean PCR products were sequenced in the ICMB Core Research Facility at the University of Texas using capillary-based ABI 3130 and ABI 3730 DNA sequencers. Each fragment was sequenced in both directions to confirm base calls. Nucleotide sequences were aligned using Sequencher 4.8 (Gene Codes Corporation Inc., 1998), and edited by eye. To determine the two alleles present on each heterozygous individual (identified as having two chromatogram peaks of the same intensity at a base position) for POMC and c-myc, I used the PHASE algorithm (Stephens et al. 2001) in the program DNAsp 5.1 (Librado and Rozas 2009). I used 100 iterations, and a confidence probability threshold larger than 90%.

Given that the Isolation with Migration model used to estimate migration rates assumes no intragenic recombination, I examined the nuclear genes POMC and c-myc for evidence of recombination using IMgc (Woerner et al. 2007), which uses the four-gamete test (Hudson and Kaplan 1985) to identify the

largest recombination-free block of sequences for each locus. I then used the longest contiguous non-recombining block for all further analyses.

Estimation of migration rate

I estimated migration with a bayesian approach under a coalescent framework using IMa (Hey and Nielsen 2004; Hey 2005). Preliminary runs with large priors were conducted to determine prior maxima on the migration parameter. Priors were reduced in subsequent runs until their distribution was optimized, minimizing 0-probability tail lengths. I estimated the parameters m_1 (maximum migration rate from population 1 to population 2), m_2 (maximum migration rate from population 2 to population 1), θ_1 and θ_2 (the product of four times the effective population size and mutation rate of populations 1 and 2). I used two models, one maintaining $m_1 = m_2$ (to estimate a single migration rate parameter), and the other allowing m_1 and m_2 to vary (to estimate asymmetric migration). Migration rate estimates were based on a Markov Chain Monte Carlo of 10^7 generations until the effective sample size (ESS) values of parameter correlations were greater than 200, following a 100,000 step burn-in. I used the HKY model of sequence evolution and an inheritance scalar of 0.25 for the mitochondrial control region and 1.0 for the two nuclear loci (given that the mitochondrial genome effective size is one fourth that of the nuclear genome). I used 10 coupled chains with a linear heating increment of 0.05. Three independent runs with different random seed numbers were used to evaluate congruency of results. The Bayesian confidence interval was estimated with the 90% highest posterior density, estimated from the shortest span on the X-axis that contains 90% of the posterior probability for each parameter. Asymmetry in population migration rates ($2N_1m_1$ and $2N_2m_2$) was evaluated by determining the proportion of times one parameter was larger than the other over the course of the run.

I also indirectly estimated migration rates with a complementary approach. I estimated F_{ST} (modified for DNA sequences; (Hudson et al. 1992)) between all the pairwise populations using the mitochondrial dataset with DNAsp 5.1 (Librado and Rozas 2009). Even though migration rates and F_{ST} are estimated under different assumptions (i.e. the inclusion or not of incomplete lineage sorting, and the potential differences in timescale), the expectation is that F_{ST} values will be inversely correlated with the migration estimates obtained from IMa because lower levels of dispersal produce larger differences in genetic structure between geographically distinct populations.

Least Cost Path distances and elevation difference

I tested the effect of geographic distance metrics with different landscape components on migration rate. Least Cost Path distances (LCP) were estimated with the Spatial Analyst Extension in ARCGIS V9.2 (ESRI). LCP accounts for variation across the landscape in the climatic properties that potentially affect species distributions. A user-defined cost matrix is used to assign relative costs to different landscape features, and then the LCP analysis identifies the path that minimizes the total costs between each pair of populations. The costs were estimated with the approach of Wang and Summers (2009), which assumes that regions in which the species has low probability of occurrence have high costs to migration. I performed the distribution probability analysis for each species based on the 19 bioclimatic layers with 30 arc-second resolution (Hijmans et al. 2005) using MAXENT V3.3.1 (Phillips et al. 2006). The inverse of the distribution probability analysis is used as the cost matrix. Because the 19 bioclimatic variables are associated with precipitation and temperature, LCP distances contain some ecological information.

To estimate occurrences of each species, I included 21 localities for *D. sp.,* 20 for *D. labialis*, and 15 for *P. achatinus*, based on our own sampling and

data from the museum of the Instituto de Ciencias Naturales of the Universidad Nacional de Colombia. I assessed the accuracy of the distribution probabilities generated by MAXENT with a receiver operating characteristic (ROC) analysis (Phillips et al. 2006), randomly selecting 25% of occurrence records and 10,000 cells as background points. I confirmed the model by comparing its output with data from museum distribution.

I also estimated Euclidean distances (straight line distance across the horizontal plane) and altitudinal difference (straight line distance across the vertical plane) between all the pairwise population comparisons. These distances do not include any information about topographic or climatic components.

Topographic complexity ratio

To determine how accumulated changes in elevation alone between all pairwise population comparisons affect migration rate and genetic structure, I modified a technique commonly used in oceanography to estimate the surface complexity of coral reefs called rugosity (Zawada et al. 2010). I estimated all possible pairwise contour distances (which take into account relief; Fig. 1.2) among populations using the 3D analyst of ARCGIS 9 (ESRI) based on a 250m resolution STRM digital elevation model (http://srtm.csi.cgiar.org/). I then divided each contour distance by the corresponding Euclidean distance between each pair of populations (Fig 1.2). The ratio is always ≥ 1. The larger the ratio, the more complex is the landscape as a result of its divergence from the flat Euclidean distance. A smaller ratio indicates a less complex (flatter) terrain.

Causal modeling

I used causal modeling (Legendre and Troussellier 1988; Cushman et al. 2006; Richards Zawacki 2009; Cushman and Landguth 2010) to determine which combination of spatial factors drives the patterns of migration and genetic

structure between populations. Causal modeling is more effective at correctly identifying contributing factors and rejecting spurious correlations than simple correlation analysis (Legendre and Troussellier 1988; Cushman and Landguth 2010). I formulated seven models that describe the effect of alternative geographic combinations on migration rate and genetic structure: a) LCP distances only, b) Topographic complexity only, c) Elevation difference only, d) LCP and topographic complexity, e) Topographic complexity and elevation difference, f) LCP and elevation difference, and g) LCP, topographic complexity, and elevation difference (Table 1.2). Each model is characterized by a unique combination of partial correlations, and therefore, each model is supported only if all its conditions are met.

To test the causal models I used zt version 1.0 (Bonnet and Van de Peer 2002) to perform partial Mantel tests, which detect correlations between two matrices while controlling for the effect of a covariate third matrix.

Migration asymmetry

To measure up versus down-slope migration I used IM analyses in which m was allowed to vary in both directions and used a paired t-test to compare the proportion of the MCMC samples in which the product between the effective number of gene copies and the per gene copy migration rate ($2Nm$) was larger in the upwards direction ($2N_1m_1 > 2N_2m_2$) than the downwards direction ($2N_2m_2 > 2N_1m_1$). The null hypothesis is that there are no significant differences between the proportion of migration upwards and downwards (symmetric migration) within each population pair. In order to perform the previous analysis I discarded pairwise comparisons with the same elevation.

RESULTS

I sequenced 114 individuals of *D. sp.*, 74 of *D. labialis*, and 61 of *P. achatinus* (see Table 1.5). Not surprisingly, for each species, the nucleotide diversity per site (π) of the two nuclear fragments was on average an order of magnitude lower relative to the mitochondrial control region (*D. sp.*: control region π = 0.0450, POMC π = 0.0032, c-myc π = 0.0023; *D. labialis*: control region π = 0.0234, POMC π = 0.0025, c-myc π = 0.0015; *P. achatinus*: control region π = 0.0361, POMC π = 0.0047, c-myc π = 0.0013).

I performed 37 IMa runs with all pairwise combinations of populations (Table 1.3). Given that I were interested in comparing relative and not absolute migration rates within each species I did not scaled parameters with mutation rate or generation time. Migration rate within each of the three species was relatively low and genetic structure was high. Average migration rates (based on the symmetrical estimates) were highest in *D. sp.* (0.851), followed by *P. achatinus* (0.786), and *D. labialis* (0.484). Average F_{ST} values were highest in *D. labialis* (0.739), followed by *D. sp.* (0.601), and *P. achatinus* (0.577).

To estimate the cost matrix for the LCP distances I generated a distribution model for each species using MAXENT based on the 19 bioclimatic layers. The average area under the curve for the ROC (10 replicates) was 0.915 ± 0.053 (s.d.) for *D. sp.*, 0.931 ± 0.044 for *D. labialis*, and 0.904 ± 0.052 for *P. achatinus*. The estimated model indicated a good fit to the distribution of each species according to our own and museum data.

Causal models and the effect of geography on migration rate and genetic structure

Causal model A (based on migration rate) in *D. sp.* was fully supported after Bonferroni correction (critical P < 0.0083; Table 1.4). Causal model E (based on F_{ST}) in *D. labialis* was marginally supported, however, it did not pass the filter of Bonferroni correction (Table 1.4). Therefore, the highly correlated LCP and Euclidean distances were the factors most strongly associated with migration rate in *D. sp* and topographic complexity and altitudinal difference had marginal impacts on the genetic structure of *D. labialis.* In *P. achatinus* no models were fully supported, neither were there significant correlations between geographic variables and migration rate or F_{ST}.

Migration asymmetry

I performed a paired t-test to determine if there were significant differences between upwards and downwards posterior probability migration parameters based on asymmetrical estimates (Fig. 1.3). Downwards directionality was statistically supported in *D. sp.* (t = 2.126, df = 18, P = 0.023), marginally supported in *D. labialis*, (t = 1.634, df = 9, P = 0.060), while there was no evidence of directionality in *P. achatinus* (t = -0.032, df = 5, P = 0.487).

DISCUSSION

The fact that proportionally most of the DNA sequence polymorphisms were found in the mitochondrial control region suggests our migration rate estimates might be female-biased. Nevertheless, there is evidence in *D. sp* and *D. labialis* that both males and females are highly phylopatric (Lüddecke and Amézquita 2010), decreasing a possible discrepancy between female and population migration rate estimations. In *P. achatinus* I do not have information regarding sex-dependent bias in movements associated to reproductive activity,

and therefore female and population migration rate estimations might be decoupled.

The effect of LCP distances

As expected under Wright's (1943) isolation by distance model, I found that Euclidean and LCP distances had a significant effect on the reduction of migration rate between populations of *D. sp.* Even though I expect the two metrics to diverge in heterogeneous landscapes (where divergence is measured as the sum of the absolute value of the deviations of the LCP distance from the Euclidean distance) I found little divergence. This is interesting because the environmental characteristics are expected to change more in montane regions.

Euclidean and LCP distances decouple when climatic conditions not suitable for the species appear in-between pairs of populations, in which case LCP distances become larger than Euclidean distances. The fact that I obtained a strong correlation between LCP and Euclidean distances might be the consequence of including species with wide elevational distributions that presumably are physiological generalists (in terms of the variables used to build the cost matrix dependent on temperature and precipitation). Species with narrow elevational ranges, however, should display larger discrepancies between LCP and Euclidean distances, as they are more likely to be physiological specialists (Navas 2006). In other words, LCP distances would more accurately resemble migration pathways in species with narrow elevation ranges, which is the case for most montane species in tropical regions (Ghalambor et al. 2006).

In landscapes where the ratio between LCP and Euclidean distance is > 1 the intervening landscapes are potentially restricting migration. Conversely, ratios ≈ 1would indicate either homogeneous intervening regions (ignoring potential barriers not taken into account by climatic variables, such as a river), or

heterogeneous regions in which the species have already been adapted. The average of the ratios for all pairwise comparisons would describe the species' potential to differentiate (Fig. 1.4).

Using this approach, I observed that the pairs of populations most geographically close were indeed the least likely to differentiate (*D. sp.*: C vs. B, ratio 1.0; *D. labialis*: J vs. I, ratio 1.0; *P. achatinus*: N vs. O, ratio 1.01). However, for the three species tested, the populations more likely to be genetically isolated from each other were not the most geographically distant (*D. sp.*: C vs. D, ratio 1.10; *D. labialis*: K vs. J, ratio 1.08; *P. achatinus*: P vs. N, ratio 1.11). Thus, even though geographic distance importantly predicts population differentiation, specific features associated with the landscape (i.e. topographic complexity) might have a greater effect on the levels of migration than Euclidean distance. Of course, the temporal scale of our LCP estimations is as narrow as the resemblance of historical temperature and precipitation values to present day estimations.

Topographic complexity ratio and its cumulative effect

I explain the positive effect of topographic complexity on the genetic structure of *D. labialis* as a result of the close association between topography and climate. Such additive effect, also called "the twofold effect", have been used to explain why topography stimulates mountain high levels of species diversity because topographic complexity generates both, local geographic isolation as well as complex climatic gradients that might promote speciation through phenotypic divergence or ecological differentiation of parapatric populations (Ruggiero and Hawkins 2008).

I expected topographic complexity to have a stronger effect reducing population connectivity, given the accumulated evidence from other amphibians

such as *Ambystoma macrodactylum* (Giordano et al. 2007); cophyline frogs (Wollenberg et al. 2008); *Bufo boreas* (Murphy 2010a), and *Rana luteiventris* (Funk, 2005; Murphy 2010b). The reason why I believe LCP distances (and not topographic complexity) were more closely associated with migration rates is that the maximum LCP distance observed is orders of magnitude larger than cumulative change in elevation across the vertical axis, and therefore, comparatively, more genetic variation would be observed across the horizontal axis.

The lack of effect of elevation difference

If local adaptation to elevation is strong, then altitudinal difference per se would decrease intraspecific migration patterns, as it would be easier for individuals to move within similar elevation belts (lower elevation ranges), than between different elevation belts (higher elevation ranges). Our results show, however, that elevation difference does not play a significant role in the patterns of migration and/or genetic differentiation of populations when the effects of geographic distance and topographic complexity were statistically controlled. This suggests that even if adaptations to elevation exist, these do not drastically restrict migration along the elevation axis.

Multiple authors (Angilletta 2002; Navas and Chauì-Berlinck 2007) indicate that frogs, as opposed to heliothermic lizards, are very labile to temperature change across elevation, and that the generalization “jack of all temperatures is a master of none” (Huey and Hertz 1984) does not hold in most cases; in other words, frogs may be environmental generalists. I suggest, then, that the absolute altitudinal difference per se is not a good predictor of genetic differentiation of frog populations.

Migration asymmetry and life history

Our results indicate migration seems to favor downwards directionality in both *Dendropsophus* species, while there is no evidence for asymmetry in *P. achatinus*. Why would this be? Frogs with free-living larvae usually lay eggs in water (as opposed to *P. achatinus*, which has direct development and lays terrestrial eggs in concealed, moist places). Given that water flows downhill, there is a higher probability that high-elevation individuals would be passively migrating to low elevations than the reverse. Alternatively, the reason why I do not find evidence of asymmetric migration in *P. achatinus* might be the limited number of populations used in this species.

If indeed highland populations behave as sources and lowland ones as sinks, the strongest effect of global climate change on the highland populations will have significant effects on population dynamics. For example, the fact that some species are more likely to migrate down elevation gradients may introduce to local populations alleles that have been under selection at other elevations and are potentially locally maladaptive. Also, it increases the probability of metapopulation extinction (Kawecki and Holt 2002; Vuilleumier and Possingham 2006).

Although population sampling is limited in this study, the results reveal the important effects of horizontal distance and the accumulated changes in topography/climate across geographic distance on the amount and direction of population migration rate. Identifying the intraspecific mechanisms that promote diversity in tropical mountains should be a priority, as this is the most vulnerable biome in terms of mass extinctions due to climate change.

Chapter 2: Topographic heterogeneity and intraspecific genetic divergence in species of tropical frogs

ABSTRACT

The richest global hotspots of biodiversity and endemism are located in montane regions. One proposed explanation is that montane regions are characterized by intrinsically higher rates of speciation compared with the lowlands because complex mountain topography and climate variation facilitate genetic isolation among populations. Here we investigate, from an intraspecific perspective, whether montane species of frogs display strong signatures of genetic isolation with larger average genetic distances per kilometer relative to species from the lowlands. We analyzed published DNA sequences for the mitochondrial genes cob and cox1 of multiple frog species from Central America and tropical South America. We found that indeed highland species show larger genetic distances between haplotypes per kilometer than do lowland species. Also, genetic divergence is strongly associated with topographic heterogeneity, an intrinsic characteristic of montane regions. Montane frog species, then, seem to have a greater potential to speciate than lowland species.

INTRODUCTION

Regions of very high species diversity may reveal general mechanisms that promote genetic divergence. One such region is the tropical Andes, which is recognized as a global hotspot of species richness and endemism for plants and vertebrates (Myers et al., 2000, Orme et al., 2005). Historically, researchers have compared Andean diversity patterns with those of the surrounding lowlands for a multitude of organisms. Duellman (1999) found beta diversity levels for amphibians in the tropical Andes (753 species, 95% endemic) much higher than

the surrounding Amazon-Guyana lowlands (335 species, 82% endemic), or the Chocó (126 species, 42% endemic).

Two alternative hypotheses may explain this observation. The first is that Andean diversity exists as a consequence of multiple and independent dispersals from the lowlands. In contrast, the second states that montane regions are characterized by intrinsically higher rates of speciation (or lower rates of extinction) compared with the lowlands. Currently, multiple lines of evidence suggest that the latter is better supported, as the vast majority of Andean diversity was formed in situ (Hughes & Eastwood, 2006, Kroner et al., 2005, Santos et al., 2009, Sedano & Burns, 2010).

More recent alternative explanations for local diversity patterns within tropical lowlands are based on accumulation of species through time. Wiens (2011) suggested that the variation in local richness of Amazonian treefrogs is explained more by the timing of colonization, than by variation of particular climatic conditions. In contrast, Gonzales-Voyer (2011), suggested that within tropical highlands, it is not clade age that explains among-clade variation in species richness, but rather a combination of morphological and ecological traits.

All studies exploring the specific mechanisms that promote tropical diversification have been performed at the species level. Another way of determining what factors promote diversification in the tropics is to compare intraspecific patterns in genetic variation across multiple species (comparative microevolutionary studies). Even though the connection between microevolution and speciation has been debated terms of the roles of local adaptation and isolation by distance in promoting speciation in spite of gene flow (Irwin et al., 2001), the ring species example has indicated that when geographic divergence is reinforced by ecological divergence, it is more likely that speciation will occur in

spite of various degrees of gene flow (Irwin et al., 2001). Therefore, regions that are more likely to display a gradient of ecological conditions, such as mountainous regions, would be more prone to generate reproductive isolation among populations relative to more homogeneous regions such as the lowlands.

Some evidence, at least in amphibians, supports this scenario, where topographic heterogeneity (and its associated ecological variation) is one of the major factors isolating populations. For example, studies of frogs such as Hypsiboas andinus (Koscinski et al. 2009), narrow-mouthed frogs (Cophylinae) (Wollenberg et al. 2008), and Dendropsophus labialis (Guarnizo et al. 2009), have confirmed the role of topographic heterogeneity in genetically structuring populations and/or reducing gene flow.

If complex topographies indeed are an important factor promoting genetic divergence in tropical regions, then it is expected that maximum genetic differentiation per unit geographic distance would be larger in species from regions under high topographic heterogeneities (i.e., montane species) than in species from more homogenous topographies (i.e., lowland species). Here we use published DNA sequences from multiple frog species to determine whether intraspecific genetic distances are correlated with topographic heterogeneity.

MATERIALS AND METHODS

DNA sequences

DNA sequences from six families of tropical frogs from Central and Northern South America were obtained from Genbank (Carnaval & Bates, 2007, Crawford et al., 2007, Elmer et al., 2007, Elmer & Cannatella, 2008, Guarnizo et al., 2009, Noonan & Gaucher, 2005, Richards-Zawacki, 2009, Weigt et al., 2005) (Supplemental Table 1). We focused on phylogeographic studies that explicitly indicated the geographic coordinates for each sequence, or provided a map with

sampling localities that could be georeferenced. Genbank sequences of the mitochondrial genes Cytochrome b (cob) and Cytochrome Oxidase I (cox1) were selected because many studies have used these two loci, allowing for comparative analyses. Sequences from the mitochondrial control region were also available from multiple species, but the hypervariable sequences of this region prevented alignment. The sequences were from Central America (Craugastor, Physalaemus, and Atelopus), Northern South America (Dendropsophus, Pristimantis, Physalaemus, and Atelopus), and Southeastern Brazil (Proceratophrys) (Fig. 1). Sequences were aligned with MUSCLE (Edgar, 2004), and then were trimmed to the same length to analyze only homologous sites in all species.

Testing for rate heterogeneity

A positive correlation between topographic heterogeneity and intraspecific genetic divergence might be found because a) higher levels of topographic heterogeneity produce higher levels of genetic isolation, or b) species at higher topographic heterogeneities are characterized by faster rates of mtDNA evolution. To differentiate these scenarios, we used a relative rate test (rrlike) based on the Langley-Fitch method (Langley & Fitch, 1974), implemented in the program r8s, (Sanderson, 2006) to determine if the rates of evolution for cob and cox1 were uniform across taxa. Significance was assessed with a chi-square test with 1 degree of freedom. The phylogeny for the relative rate test was generated as a maximum likelihood (ML) tree using RAxML-VI-HPC (Stamatakis, 2006) with a GTRGAMMA model of evolution and a standard heuristic search with 1,000 replicates. To root the trees we used Microhyla rubra (accession number: AB201224) for cob, and Microhyla heymonsi (accession number: AY458596) for cox1.

Estimating intraspecific genetic divergence

Genetic distances between all haplotype pairs were estimated with the Kimura-2 parameter (K2P) model (Kimura, 1980) using MEGA 4.0 (Tamura et al., 2007). The K2P model was chosen to facilitate comparison with other studies performed with frogs (Crawford et al., 2010, Vences et al., 2005). Pairwise geographic distances were estimated with the R-Package (Legendre & Vaudor, 1991), which estimates great circle distances (along the Earth's curvature). Both the average and the maximum genetic distance per kilometer were used to describe genetic variation in each species.

To determine what proportion of the intraspecific genetic variation was explained by geographic distance alone, we tested for isolation-by-distance by assessing correlations between geographic (Great Circle) and genetic (K2P) distances with simple Mantel tests implemented in the program zt (Bonnet & Van de Peer, 2002).

Accounting for cryptic species

Morphologically indistinguishable (cryptic), yet genetically divergent, species are well-known in frogs (Elmer & Cannatella 2008; Vences et al. 2005). Given that sequences were obtained from studies of different geographic scope, cryptic species might exist within the dataset. To prune highly divergent sequences that might correspond to cryptic variation, we generated multiple scatterplots of genetic distance vs. geographic distance. Cases characterized by a smooth pattern of isolation by distance (no evident gaps in geographic distance) were assumed to correspond to a single species. If, however, two well-defined clusters of points overlapping on the x-axis were observed (Fig. 2), then a ML gene tree was estimated with the software MEGA 4.0 to calculate genetic divergence. If the deepest genetic divergence was larger than ~5% (Fig. 2), we selected the clade with more geographic localities and discarded the other one.

This is conservative limit, since the proposed threshold to identify candidate species in frogs using cox1 is ~10% (Vences et al., 2005).

Estimating topographic heterogeneity

Topographic heterogeneity was estimated from rugosity, an approach commonly used in oceanography to characterize surface complexity in coral reefs (Zawada et al., 2010). We divided the surface area (which takes into account three-dimensional relief) by the planar area of the minimum convex polygon that encompasses all sampled localities for each species. Values close to 1 indicate low heterogeneity and values higher than 1 indicate high heterogeneity. The minimum convex polygons were estimated with the Hawths tools extension (www.spatialecology.com) for ArcGis 9.0 (ESRI). Rugosity was calculated with the 3D analyst of ArcGis 9.0 (ESRI), based on a 250m resolution STRM digital elevation model (http://srtm.csi.cgiar.org/).

Finally, to test whether topographic heterogeneity was associated with intraspecific genetic distances/km across multiple species, we calculated a Pearson correlation coefficient.

RESULTS

In total, 145 cox1 (488 bp) haplotypes were found in 7 species and 350 cob (337 bp) haplotypes in 12 species (Table 1). Genbank accession numbers and associated geographic coordinates are in Supplemental Table 1 (because of the possibility of poaching, geographic coordinates for A. varius are not included, as requested by C. Richards-Zawacki). Atelopus were characterized by lowland distributions and small elevation ranges, except for A. varius, which had an elevational range of at least 1000 meters. Craugastor and Physalaemus had on average larger elevational ranges than Atelopus, but they still were restricted to

the lowlands. Both Dendropsophus species had wide elevation ranges and by far reached the highest elevations (Fig. 3; Table 4).

When applied to the entire tree, the relative rate test rejected the hypothesis that cox1 and cob were each evolving at similar rates across taxa (Table 2). Rate homogeneity was not rejected in less inclusive clades (Table 2, Supplemental Fig. 2).

Topographic heterogeneity and elevation were positively correlated (cob: $r = 0.685$, $P = 0.014$, $N = 12$; cox1: $r = 0.6494$, $P = 0.107$, $N = 7$, $\alpha = 0.05$; Supplemental Fig. 1). We found evidence for isolation by distance in 4/7 species for cox1 (57% of the species) and 9/12 species for cob (75% of the species; Fig. 4, Table 3).

The largest K2P distances (not scaled by geographic distance) were found in A. varius (cob) and C. fitzingeri (cox1), and the smallest were found in A. s. barbotini (cob) and C. crassidigitus (cox1; Table 4). The species category was not a good predictor of intraspecific genetic variation, as the standard deviation within species of average K2P distances was large (Supplemental Table 2). A positive relationship between rugosity and maximum K2P distances (Fig. 5) was found; rugosity explained 52% of the variation in maximum K2P distances across species for the cox1 gene, and 38% of the variation for cob. The correlation was marginally significant for cob ($r = 0.613$; $P = 0.034$; $N = 12$), and marginally not significant for cox1 ($r = 0.724$, $P = 0.066$; $N = 7$).

When using average genetic distances/km instead of maximum genetic distances/km, we found a significant correlation for cox1 ($r = 0.795$, $P = 0.033$, $N = 7$), but not for cob ($r = 0.047$, $P = 0.885$, $N = 12$). The rationale for preferring maximum over average genetic distances is based on the variety of sampling

designs that characterize the sequences retrieved from Genbank. For instance, if there is isolation by distance, a study that samples multiple individuals per geographic locality would display a higher proportion of 0.0 genetic distances compared to a study that samples specimens scattered across the landscape. Thus, the average estimates for genetic divergence are biased if multiple individuals per population were sequenced. Maximum genetic distances do not suffer from this bias since they indicate the highest genetic divergence that occurs within each species independently of sampling design.

DISCUSSION

Genetic divergence, topographic heterogeneity and the potential for speciation

That topographic heterogeneity was positively correlated with maximum genetic divergence provides good evidence that the more complex topographies in montane regions restrict gene flow more than the less complex topographies of the lowlands. That subsets of species under a uniform rate of molecular evolution replicate this positive correlation indicates that it is not the differences in rate of molecular evolution among clades, but topographic heterogeneity itself, that is positively associated with intraspecific genetic divergence.

The long-term consequence of high genetic divergence per unit distance in mountains is presumably an increase in potential for speciation (Jennings et al., 2011, Martin & McKay, 2004), because larger genetic divergences and associated lower gene flow levels, which may facilitate reproductive isolation (Martin & McKay, 2004). The latter is supported by simulation data that suggest genetic divergence combined with some level of outbreeding depression, in the absence of geographic barriers to gene flow, can result in parapatric speciation (Hoelzer et al., 2008).

One factor that explains why the maximum genetic distances per unit geographic distance are on average larger in mountains may be related to the two-fold effect of topography on species diversity (Ruggiero & Hawkins, 2008); a change in elevation is accompanied by a steep change in climate. It is well known that amphibians characterized by wide elevational distributions contain populations physiologically adapted to their particular elevation ranges (Navas, 2006). Therefore, mountains, compared to lowland regions, would not only provide more opportunities for parapatric speciation caused by isolation by distance (Hoelzer et al., 2008), but also to ecological selection and parapatric reproductive isolation as a result of steep climatic gradients. Moreover, allopatric speciation would be promoted as well due to the cumulative effect of hills and valleys acting as geographic barriers.

The apparent effectiveness of mountains in promoting genetic divergence in frogs might strongly depend on latitude because the seasonally-determined temperatures depend on the latitudinal gradient. According to the Janzen hypothesis (Janzen, 1967), tropical organisms should be adapted to narrow physiological tolerances in temperature as a consequence of low temporal overlap in thermal regimes along elevation. Accordingly, in temperate regions organisms should be adapted to wider temperature tolerances as a result of the greater temporal overlap in temperatures along elevation. Therefore, we predict that the relationship between topographic heterogeneity and genetic divergence would be less pronounced in temperate regions when comparing multiple species across different elevations.

The mechanisms that explain Neotropical diversity patterns have been studied at the species level, using phylogenies to compare speciation rates among regions or broad biogeographic patterns based on isolation and dispersal (Rull, 2005, Santos et al., 2009, Wiens et al., 2011). Our study, however, focused

on how landscapes influence genetic variation across different species, which might increase or decrease the potential for future speciation. As massively parallel sequencing becomes more common in phylogeographic studies, a combination of species-level and population-level studies will enlighten the very complex diversification processes in the Neotropics.

To conclude, comparative analysis of multiple species supports the hypothesis that the complex topographies that characterize highlands facilitate intraspecific genetic divergence, and likely, the potential for future speciation. Mountains, which are commonly recognized as cradles of speciation (Dick & Wright, 2005), should be intensively studied, not only because they might yield important clues for understanding latitudinal gradients in species diversity (Wiens et al., 2006), but also because montane regions are the most threatened ecosystem in terms of habitat destruction and climate change (Lips, 1998).

Chapter 3: Congruence between acoustic traits and genealogical history reveals new parapatric species of *Dendropsophus* (Anura: Hylidae) in the high Andes of Colombia

ABSTRACT

The high Andean frog *Dendropsophus labialis* is continuously distributed along the Eastern Andes of Colombia between 1900 and 4100 m. In this paper I conducted a multi-trait analysis to determine if acoustics and morphology co-varied with genealogical history or if they evolved independently of each other. I further tested if the pattern of phenotypic differentiation could be explained by either selection or drift. I generated a phylogeny of *D. labialis* with mitochondrial (12s-16s) and nuclear (POMC) DNA sequences and obtained two well-supported clades. The phylogeny showed that acoustic variation diverges according to genealogical history, but external morphology does not follow this relationship. Stochastic processes due to genetic drift appear to be a better explanatory mechanism for the divergence in calls than adaptive variation. The strong and congruent divergence observed in acoustic and genetic characters indicates that these two clades correspond to morphologically cryptic parapatric species. I therefore described the northern clade as a new species (*Dendropsophus luddeckei*), maintaining the southern clade as *D. labialis*. I included in our phylogenetic analysis the species *Dendropsophus meridensis* (which has not been included in a molecular phylogeny before) and *Dendropsophus pelidna,* both collected in Venezuela. Our data suggests these two species are synonyms and their geographic range reaches Serrania del Cocuy in Colombia, which is a new record for both lineages.

INTRODUCTION

The neotropics are characterized by a particularly high endemic diversity (Myers et al. 2000; Orme et al. 2005). Historically, most of this diversity has been described using exclusively a subset of morphological traits, a phenomenon that is evidenced by the fact that most published species descriptions include a list of diagnostic morphological characteristics (Wiens and Servedio 2000). One of the problems of inferring species boundaries using morphology alone can be observed when genealogical history and morphology do not evolve in a coupled fashion, as can happen when selection breaks correlation patterns among different phenotypic traits (Lougheed et al. 2006). Therefore, using morphology alone can be problematic in recovering the genealogical history and/or delineating species if external forces such as natural or sexual selection have shaped the phenotype. Even if phenotypes evolve neutrally according to genealogical history, problems with species delimitation still can occur when there is not enough time for traits that evolve at slow rates to fully diverge after a recent speciation event (De Queiroz 2007). As a result, regions in the world where diversification has occurred very recently are prone to display incongruence between the number of species described and the true species diversity.

The rapid uplift of the tropical Andes (Gregory-Wodzicki 2000; Garzione et al. 2008), exemplifies a case where the prevalent use of morphological traits in taxonomic analyses may have produced an underestimation of biological diversity. This underestimation is evidenced by the increasing rate of discovery of new cryptic species in phylogeographic analyses (Cadena et al. 2007; Fouquet et al. 2007; Solari 2007). Therefore, the tropical Andes are likely to be more diverse than current estimates predict, and this underestimation is probably due to the combination of its recent formation as a biogeographic region, and the

predominant use of traits under putatively slow rates of evolution (Cherry et al. 1982) for species characterizations.

Multi-trait analyses that include both genetic and phenotypic information should be more accurate in determining how much diversity actually exists in places where recent diversification has occurred (e.g., recently uplifted mountains or recently formed islands). Additionally, these analyses, especially in sister species, can be useful in determining the evolutionary forces driving allopatric divergence (Amézquita et al. 2009). For these reasons, I used the high Andean frog *Dendropsophus labialis* (Peters 1863) to contrast patterns of evolution of independent character systems (mitochondrial and nuclear DNA, acoustics and morphology) following recent divergence, and to test for the possible existence of cryptic diversity that has not been previously detected with morphological analyses.

Dendropsophus labialis is continuously distributed along the Eastern Andes of Colombia between 3.5º to 6.3º in latitude north, and 1900 to 4100 m in elevation (Ruiz-Carranza (1996); Escallon and Guarnizo, Personal Observation). Mitochondrial and nuclear DNA sequences indicate that within *D. labialis* there are two strongly differentiated clades with parapatric distribution (Guarnizo et al. 2009). Despite such divergence, morphological analyses have historically characterized these two clades as a single species (Cochran and Goin 1970). Even though some studies have analyzed the effect of elevation on the phenotype, physiology, and life history of populations within the *D. labialis* southern clade (Amézquita and Lüddecke 1999; Lüddecke 2002; Lüddecke and Sánchez 2002), there are no studies that have contrasted the phenotypic variation between the northern and southern genetic clades.

In this paper I conducted a multi-trait analysis to determine if acoustics

and morphology co-varied with genealogical history (revealed by the tree topology obtained with the mitochondrial 12s-16s region and the nuclear POMC gene) or if they evolved independently of each other. I also tested if the climatic characteristics associated with divergent genes or phenotypes were statistically different. The analyses utilized in this study allowed us to determine the degree to which independent traits can converge on measurable differentiation after a recent reproductive isolation event that occurred in the very young northern Andes (Fjeldsaå and Lovett 1997; Young et al. 2002).

MATERIALS AND METHODS

DNA sequencing

Dendropsophus labialis DNA was obtained from 10 populations across the Eastern Andes of Colombia (Fig. 3.1). Three of such populations geographically correspond to the southern clade revealed by Guarnizo et al. (2009), and seven to the northern clade. Tissues were obtained by clipping the toe of adult frogs (IACUC protocol number 07101901). Total genomic DNA was extracted using a Viogene Blood and Tissue Genomic DNA Extraction Miniprep System (Viogene, Inc., Taipei, Taiwan). Four primer pairs were used to amplify the complete 12s-16s rRNA region of the mitochondrial DNA (see primers sequences in Symula (2008)). One primer pair was used to amplify the nuclear gene proopiomelanocortin A (POMC) (see primer sequences in Guarnizo et al., 2009). For POMC I sequenced additional populations that were not included in the Guarnizo et al, (2009) study. Amplifications were performed with a reaction mix containing 2.5 µl of 10X PCR buffer (500 mM KCl, 100mM Tris-HCl, pH 8.5), 2.5 µl of 8 µM dNTP's, 13.875 µl of dH2O, 1.0 µl of MgCl2, 0.125 µl of DNA polymerase, 1.25 µl of each primer (10µM), and 2.5 µL of the DNA extract for a final volume of 25 µL.

The PCR cycle for the 12s-16s region included an initial denaturing step of

2 of min at 94ºC, followed by 35 amplification cycles (30 s at 94ºC, 30s at 48ºC, 1 min at 72ºC), and a final extension of 7 min at 72ºC. For POMC I used the PCR cycle protocol that appears in Guarnizo et al.(2009). PCR products were purified using a Viogene Gel Purification Kit. Clean PCR products were sequenced at the ICMB Core Research Facility at the University of Texas using capillary-based ABI 3730 DNA sequencers. Each fragment was sequenced in both directions to confirm base calls. Nucleotide sequences were aligned using Sequencher 4.8 (Gene Codes Corporation Inc., 1998) and edited by eye.

Phylogenetic analyses

Phylogenetic trees were constructed using both Bayesian and Maximum Likelihood (ML) methods. The Akaike Information Criterion (AIC) implemented in jModelTest 0.1.1 (Posada 2008) was used to select the optimal nucleotide substitution model for the Bayesian analysis. I used MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) to generate two independent runs of 5,000,000 generations implementing Metropolis-coupled Markov chain Monte Carlo (MCMC). Each run contained four incrementally heated Markov chains (temperature = 0.05), sampling every 1000 generations, and a burn-in of 25%. Convergence of the two runs was assumed when the average standard deviation of the split frequencies was less than 0.01. ML trees were generated with RAxML-VI-HPC (Stamatakis 2006) using a GTRGAMMA model of evolution (GTR + Γ) and implementing a standard bootstrap search with 1,000 replicates.

I included in the phylogenetic analysis two other members of the *labialis* group (Duellman 1989): *Dendropsophus meridensis* sampled in Merida Venezuela, and *Dendroposphus pelidna* sampled in Tachira Venezuela (Genbank accession number: AY819434). For POMC I only included *D. meridensis* because *D. pelidna* has no POMC sequences uploaded in Genbank.

I combined fieldwork and museum data to perform acoustic and morphological analyses respectively. Therefore, each individual was analyzed either for acoustic or morphologic variation, but not both. Table 3.1 indicates all the individuals, localities, and analyses performed.

Acoustic analyses

I analyzed the calls of 176 individuals distributed in twelve localities across most of the geographic range of the species (Fig 3.1; Table 3.1). I included four localities that correspond to the northern clade, seven to the southern clade, and one from a sympatric locality that contained haplotypes from both clades (Guarnizo et al. 2009). The call information of seven of the localities was obtained form Amézquita (2002) as averages from each population. Between 9 and 14 consecutive advertisement calls from 8-12 males per locality were taped by placing a Sennheiser ME67 directional microphone in the frontal plane of a calling male. The distance between the microphone and the calling male was never less than 50 cm. The calls were recorded on TDK MA90 metal bias tapes using a Sony WM D6C professional walkman. While each male call was recorded, ambient temperature was measured with a digital thermometer placed within 1 meter of the male.

Male calls were digitized at 44.5 KHz and 16-bit and analyzed with the software Cool Edit (Syntrillium Software Cooperation, USA). The peak frequency was measured in sonograms with a filter bandwidth of 133.3 Hz, a frequency resolution of 21.53 Hz, and a fast Fourier transformation size of 1024 points. Oscillograms were used to measure call duration and to count the number of pulses. Pulse rate was calculated as the number of pulses/call duration. Based on the study of Amézquita (2002) I measured three call traits that display the strongest levels of geographic variation between localities in *D. labialis*: call duration, number of pulses per call, and pulse repetition rate.

Localities from the same clade were analyzed as a group and the sympatric locality was analyzed separately. In order to reduce the number of dimensions needed to describe the variation in acoustic parameters, I performed a principal-component analysis (PCA) using the covariance matrix. The degree of acoustic divergence between the two genetic clades was directly estimated using ANOVA on the PCA factors.

Given that our phenotypic analyses include a spatial component they can suffer from spatial autocorrelation (Legendre and Fortin 1989), thus it is important to control for the possible confounding effect of geographic distance on acoustic differentiation. I performed a partial Mantel test in order to determine if acoustic distance (estimated as Euclidean distances between the first principal component PC1) was significantly correlated with clade correspondence (a binary matrix where 0 indicates intraclade and 1 interclade comparisons), controlling for the effect of linear geographic distance. Partial Mantel tests were performed with the program zt (Bonnet and Van de Peer 2002) based on 10,000 permutations.

Morphometric analyses

I performed morphometric analyses of 48 individuals distributed in twelve localities (Fig 3.1, Table 3.1). I included four localities that corresponded to the southern clade and eight to the northern clade. Measurements were performed with a digital caliper (precision 0.01 mm) on preserved frogs from the collections at the Instituto de Ciencias Naturales at the Universidad Nacional de Colombia. I collected 10 external morphometric variables (using the methodology that appears in Ron (2005)) on each of the 48 individuals analyzed: snout–vent length (SVL), tibia length (from knee to heel), foot length (from proximal border of inner metatarsal tubercle to tip of toe IV), head length, head width, inter-orbital

distance, eye-to-nostril distance, eye diameter, tympanum diameter, and eye-to-tympanum distance. All variables were obtained from the right side of the specimen and $\log_{10}$ transformed for scale adjustment. Acknowledging that morphological measurements vary continuously due to ontogenetic stage or sexual dimorphism, I removed this source of inter-individual body size variation by regressing each variable with SVL and thereafter working with the saved residuals.

To determine if morphometric variation can be differentiated according to clade correspondence, I performed a principal-component analysis (PCA) using the covariance matrix and then tested for differences between the PCA factors using ANOVA. Partial Mantel tests, as described earlier, were performed to determine if there was a correlation between morphological distance and clade correspondence controlling for the effect of linear geographic distance between populations. Morphological distance was estimated as the Euclidean distance between PC1 factors.

Climatic analysis

I wanted to determine if the climatic properties of the sampled localities (averaged across the last 50 years) could be differentiated according to their assigned clade. The existence of an association between genetic and climatic variation might indicate an effect of ecological mechanisms in the process of diversification (i.e. ecological speciation (Ogden and Thorpe 2002)). I used the program DIVA-GIS (Hijmans et al. 2001) to extract (from all the geographic localities used in this study) 30 arc-second resolution layers of annual temperature and precipitation averages (Hijmans et al. 2005). I then used ANOVA to determine if there were significant differences in precipitation and temperature accordingly to their assigned clade.

RESULTS

Phylogenetic analysis

I sequenced 2369 bp of the 12s16s region of the mitochondrial DNA and 490 bp of the nuclear gene POMC. The nucleotide diversity of 12s-16s was around an order if magnitude higher than POMC. The Bayesian (best-fit model GTR + Γ + I) and ML analysis confirmed the results obtained by Guarnizo et al. (2009), finding two clades with high posterior probabilities and bootstrap support values (Fig 3.2). The mean 12s-16s sequence divergence (based on p-distance) between the two clades was 2.1%. The Southern clade contains haplotypes from the geographical region southwest of the city Chiquinquira (code 17 in Figure 1), while the Northern clade contains individuals northeast of that city. In the 12s-16s tree, the outgropus *D. meridensis* and *D. pelidna* (both sampled in Venezuela) were joined in a single clade together with four individuals sampled in the Serrania del Cocuy in Colombia. POMC displays two main clades, one corresponds to the southern clade, and the other clusters within a single polytomy: the outgroup *D. meridensis* together with the northern clade individuals (Fig. 3.2).

Acoustic analyses

Call duration was positively correlated with number of pulses per call (Pearson's r = 0.78, P < 0.0001, N = 186 frogs) and negatively related to pulse rate (r = 0.86, P < 0.0001). These correlations were expected, because pulse rate was not directly measured, but calculated from the two other variables. Therefore, to avoid artifactual redundancies in posterior analyses I used pulse rate, pulses per call and peak frequency as call descriptors. Frogs calling at higher temperature produced calls with slightly fewer pulses (linear regression, r^2 = 0.06, F = 11.3, P = 0.009, N = 186 frogs) at a higher repetition rate (r^2 = 0.21, F = 50.0, P < 0.0001). Higher temperatures were also correlated with higher values of call peak frequency (r^2 = 0.06, F = 12.5, P = 0.0005). To remove the

confounding effect of body temperature in among-locality comparisons of call features (temperature ranged between 8.2 C and 20 C), I saved the residuals of these regressions as temperature-independent call descriptors to be used in subsequent analyses.

Two principal components summarized more than 99% of the acoustic variance (PC1: 77.5% and PC2: 21.9%; Table 3.2). The highest load for PC1 was pulses per call, while the highest load for PC2 was pulse rate. I found a partial overlap in the acoustic space between the two clades, having the sympatric locality placed in-between (Fig. 3.3). When using the PC1 as a descriptor of the acoustic variation I was able to identify significant differences between the two clades after controlling for temperature effects (ANOVA, F = 26.67, $P < 0.001$). Also, I found a significant correlation between acoustic distance and clade assignment when controlling for geographic distance (partial Mantel test, $r = 0.1856$, $P < 0.001$).

Morphometric analyses

Three components with eigenvalues higher than 1.0 were extracted from the PCA and account for 56% of the variance. The highest loadings for PC1 were head width and eyes to nostril distance, while the highest loadings for PC2 were foot length, head length and eye diameter (Table 3.2). I found a wide overlap in the morphological space between the two clades (Fig. 3.3). When the PC1was used as a descriptor of morphological variation I did not find statistical differences between both clades (ANOVA, $F = 1.8498$, $P = 0.1807$). I also did not find a significant correlation between morphological distance and clade assignment when controlling for geographic distance (partial Mantel test, $r = 0.07172$, P = 0.1976).

Climatic analyses

I did not find statistical differences in annual averages of temperature and precipitation across the sampled localities between the two clades (ANOVA, temperature: $F = 1.07$, $P = 0.320$; precipitation: $F = 2.220$, $P = 0.170$).

DISCUSSION

Genealogical history of *D. labialis*

The tree topology obtained by 12s-16s indicates *D. labialis* is composed of two highly divergent clades distributed across the Eastern Cordillera in Colombia, confirming the results obtained by an earlier study using faster evolving mitochondrial genes (Guarnizo et al. 2009). Even though POMC, relative to 12s-16s, had a shorter size (five times shorter) and slower rate of evolution (about an order of magnitude lower), it still was able to recover two major clades. The polytomy that groups the northern clade together with *D. meridensis* suggests that I do not have enough nuclear resolution to delineate how these two lineages are related.

Given the geographic distribution of the individuals that I sampled, it might first appear that the northern and southern clades form within *D. labialis* as a consequence of the spatial sampling gap that exists between them (Fig. 3.1). However, the relationship between geographic and genetic distances (Fig. 3.4) indicates that such sampling gap does not provide an explanation for the divergence between the two clades, because large inter-clade genetic distances (higher than 2%) completely overlap with low intra-clade genetic distances (lower than 1%) along the x-axis. A meaningful effect of a spatial sampling gap on genetic divergence would be observed if large inter-clade genetic distances did not overlap with low intra-clade genetic distances along this axis (Fig. 3.4).

Phenotypic divergence in *D. labialis*

I found that acoustic but not external morphological traits diverged according to the molecular phylogeny. Two alternative hypotheses might explain this result: a) acoustic traits are evolving according to population history and morphological traits are not (i.e. they are being constrained by selection), or b) both acoustic and morphological traits are evolving according to population history, but the rate of acoustic divergence is faster. Our relatively low sample size for morphological measurements does not allow us to support or reject either of these hypotheses. However, amphibians are generally characterized by slow rates of morphological evolution (Cherry et al. 1982), as exemplified by the fact that species from the genus *Dendropsophus* have shown acoustic inter-population variability three times as large, on average, as morphological variability (Lougheed et al. 2006). This phenomenon suggests to us that *D. labialis* morphology is diverging according to population history but I are unable to detect such slight divergence.

Why might acoustic traits be evolving faster in *D. labialis*? Even though it would appear that acoustic traits should display constrained evolution given their biomechanical correlation with morphology, their dependence on other factors such as physiology and behavior seem to accelerate their evolution rate. For instance, Cocroft and Ryan (1995) indicate that in hylids, characters whose variation is based on the morphological basis of call production are more conservative than characters whose variation is based on the behavior and physiology of calling. Thus, the fact that in our analysis the first principal component for acoustic variation included a variable dependent on physiology (pulses per call) indicates that in *D. labialis* physiology might be related to acoustic divergence.

I wanted to further test the extent to which the observed acoustic

divergence was a result of either selection or genetic drift. However, determining the relative effect of these two processes in natural populations is not a straightforward endeavor (Orr 1998). Nevertheless, the existence of congruence in spatial patterns of variation among independent traits might indicate that trait evolution has been shaped by similar microevolutionary forces (i.e. selection and/or genetic drift) (Lande 1976; Robertson and Zamudio 2009). The locus comparison approach (Gockel et al. 2001; Hoffman et al. 2006) can be used to determine if a particular phenotype has evolved through drift when the geographic variation in the phenotype correlates with genealogical history (i.e. the variation of putatively neutral loci). The role of selection, on the other hand, is commonly determined when phenotypic traits depart from genealogical history, and instead correlate with particular biotic and/or abiotic environmental factors (Endler 1977; Long and Singh 1995).

I provide supporting evidence that acoustic traits are evolving according to genealogical history as I found statistical differences when grouping frogs in agreement with their clade of origin. However, I did not find differences in the annual averages in temperature and precipitation between the two regions (two critical factors in anuran reproductive activity (Oseen and Wassersug 2002)). The combination of both sources of evidence suggests the acoustic divergence observed in *D. labialis* evolved through drift and not environmental selection. It is challenging to reject, however, the effect of environmental selection on the phenotypic divergence because of the intrinsic difficulties in calculating the fundamental niche of a species using geographic information systems (Kearney and Porter 2004). I am also aware that very large sample sizes are needed to detect selection in natural populations (Kingsolver et al. 2001), and that alternative methods based on QTL's can also measure the relative effects of drift and selection (Orr 1998). Therefore, I consider our mechanistic comparison as a first approximation and I encourage future research that includes different

techniques and larger sample sizes.

Multi-trait divergence as evidence of speciation

Dendroposphus labialis is characterized by an abrupt (~30 Km) but continuous change in allele frequencies and phenotypic traits (Fig. 3.5), which is a typical characteristic of a secondary contact zone (Barton and Hewitt 1985). It also displays statistical differences in the calls between two highly supported reciprocally monophyletic clades obtained with nuclear and mitochondrial loci. This evidence, together with the fact that the observed 2% sequence in 16s is close to the threshold proposed by some authors to identify candidate frog species with the same gene (Hebert et al. 2003; Vences et al. 2005b; Fouquet et al. 2007), indicates that the northern and southern clades are indeed two different morphologically cryptic species with parapatric distribution. For this reason, I designate the northern clade as *Dendropsophus luddeckei sp. nov.* (see below for species description). Given that the holotype of *D. labialis* (ZMB 4913) geographically corresponds to the southern clade ("vicinity of Bogota" in Cundinamarca), I maintain the southern clade with the name *D. labialis*.

What caused the divergence between *D. labialis* and *D. luddeckei*?

In their study, Guarnizo et al. (2009), suggest that *D. labialis* and *D. luddeckei* became geographically isolated during the late Miocene at the peaks of the highest mountains where warmer temperatures at low elevations acted as a geographic barrier. During the early Pliocene, the Northern Andes started to uplift very quickly (Garzione et al. 2008), becoming colder and removing the temperature barrier that previously existed. The rate of range expansion and the concomitant formation of a secondary contact zone depended on the ability of populations to respond to rapid environmental changes through acclimatization, adaptation, dispersal, and/or behavioral plasticity (Navas 1996; Angilletta et al. 2006). I expect that the effect of temperature change on population fragmentation

and reproductive isolation is higher in tropical regions relative to temperate ones, as the climatic conditions are less variable in the tropics, and species are therefore adapted to narrower temperature ranges (Ghalambor et al. 2006).

Many recent studies have predicted the future response of high elevation ectotherms to global warming (Deutsch et al. 2008; Wake and Vredenburg 2008; Sinervo et al. 2010). However, very few studies have determined how high elevation ectotherms have responded to temperature cooling (as a consequence of mountain uplift) in the past. The impacts of atmospheric cooling might be as dramatic as warming, and therefore its importance needs to be studied as a causal factor of the high endemic diversity observed today in the highland tropical Andes.

What is preventing *D. labialis* and *D. luddeckei* from exchanging alleles?

Barton and Hewitt (1985) indicate that it is hard to distinguish if parapatrically distributed forms remain distinct because they are adapted to different environments (ecological speciation) or because the hybrids between them are less fit. Because our data suggest there are no environmental differences between the two species, it would appear that the more likely scenario is that the hybrids are under selective disadvantage. Ulloa (2003) studied the effect of geographic distance on the reproductive isolation of populations of *D. labialis*. Based on the clades found by Guarnizo (2009), she found that southern clade individuals (*D. labialis)* can mate with northern clade ones (*D. luddeckei*) under laboratory conditions (which might indicate an absence of pre-zygotic barriers). To test for presence of post-zygotic barriers, she measured if there were differences in larval survival (% of living larvae x day) between and within the two clades crosses. Even though she did not find statistical differences, it appears that the hybrid F1 larval survival is reduced compared to intraspecific crosses.

If hybrid individuals are being selected against, I would expect to find acoustic reinforcement preventing maladaptive hybridization. Our partial data suggests, however, that the only population that contains haplotypes of both species in sympatry (San Carlos, code 18), contains individuals characterized by intermediate call properties (Fig. 3.2.), which does not support the existence of reinforcement. Having hybrid inviability between sister species calling at the same place does not mean, however, that acoustic reinforcement must occur. There is the possibility of niche partitioning (spatial or temporal) that could prevent females of one species to be in contact with the males of the other species. Our data does not contain fine grain spatial or temporal acoustic information; therefore I cannot further discuss this possibility. A more complete description of the genetic variation across the contact zone, together with experiments that can reveal an effect of pre-zygotic barriers (such as phonotaxis experiments), and/or the effect of post-zygotic barriers (proportion of fertilized eggs in interspecific crosses, and fitness assessment of the F1 and F2 generations), are fundamental to characterize the mechanisms that are keeping both lineages isolated.

Relationships within the *labialis* group

The *labialis* group is currently characterized by three highland species: *D. labialis* (Colombia), *D. pelidna* (Colombia and Venezuela), and *D. meridensis* (Venezuela) (Cochran and Goin 1970; Duellman 1989). Even though Duellman (1989) indicates *D. meridensis* is morphologically distinguishable from *D. pelidna* in color pattern, size and presence of dorsal tubercles, I only found a surprisingly low (0.9 %) sequence divergence between both species. This level of divergence is an order of magnitude lower than the divergence between *D. labialis* and *D. luddeckei*, which suggests *D. pelidna* is synonym to *D. meridensis*. Equally interesting is the fact that some of the specimens I collected syntopic with *D.*

luddeckei (at Serrania del Cocuy) clustered within the *D*. *meridensis* clade, suggesting *D. meridensis* distribution starts at Andes of Merida in Venezuela and expands to Serrania del Cocuy in Colombia.

If the *D. meridensis*-*D. pelidna* synonymy is correct, it provides an interesting biogeographic puzzle (Fig. 3.6), where *D. meridensis* would be found on both sides of the point where the Eastern Andes reaches its lowest elevation (Tachira depression: 900 m), which is below the altitudinal range of the species (1200-2400 m; (La Marca 2004)). Our explanation for such a distribution is that originally *D. meridensis* was found only west of the Tachira depression. Because of Pleistocene glaciations, populations of *D. meridensis* might have been pushed downwards, allowing them to cross the altitudinal barrier to the East. Following that, interglacial warmer periods might have, again, pushed up populations and isolated a small relict of *D. meridensis* at the Andes of Merida in Venezuela. That would explain why *D. meridensis* is currently listed as endangered, and *D. pelidna* is listed as very abundant (La Marca 2004). If *D. meridensis* is indeed synonym to *D. peldina,* the endangered status of *D. meridensis* should be revised.

This study shows that even a highly taxonomically studied organism such as *D. labialis* can contain cryptic diversity that has not been detected, likely due to the fact that a character system under a putatively slow rate of evolution has been used for species delimitation. We predict that the number of endemic species of the highland Andes will inevitably increase as multi-trait analyses that combine morphological characters with traits under faster rates of evolution become more common.

FIGURES

Figure 1.1. *a*) Map of Colombia and Ecuador showing the sampling regions for each species. *b*) Sampling region for *D. sp*. c) *D. labialis*. and d) *P. achatinus*. Below each map there is a contour view of a line that connects all the populations within each species. This contour view has a vertical exaggeration of 10x to facilitate the visualization of elevation differences. Notice that the Y-axis is not the same on each contour.

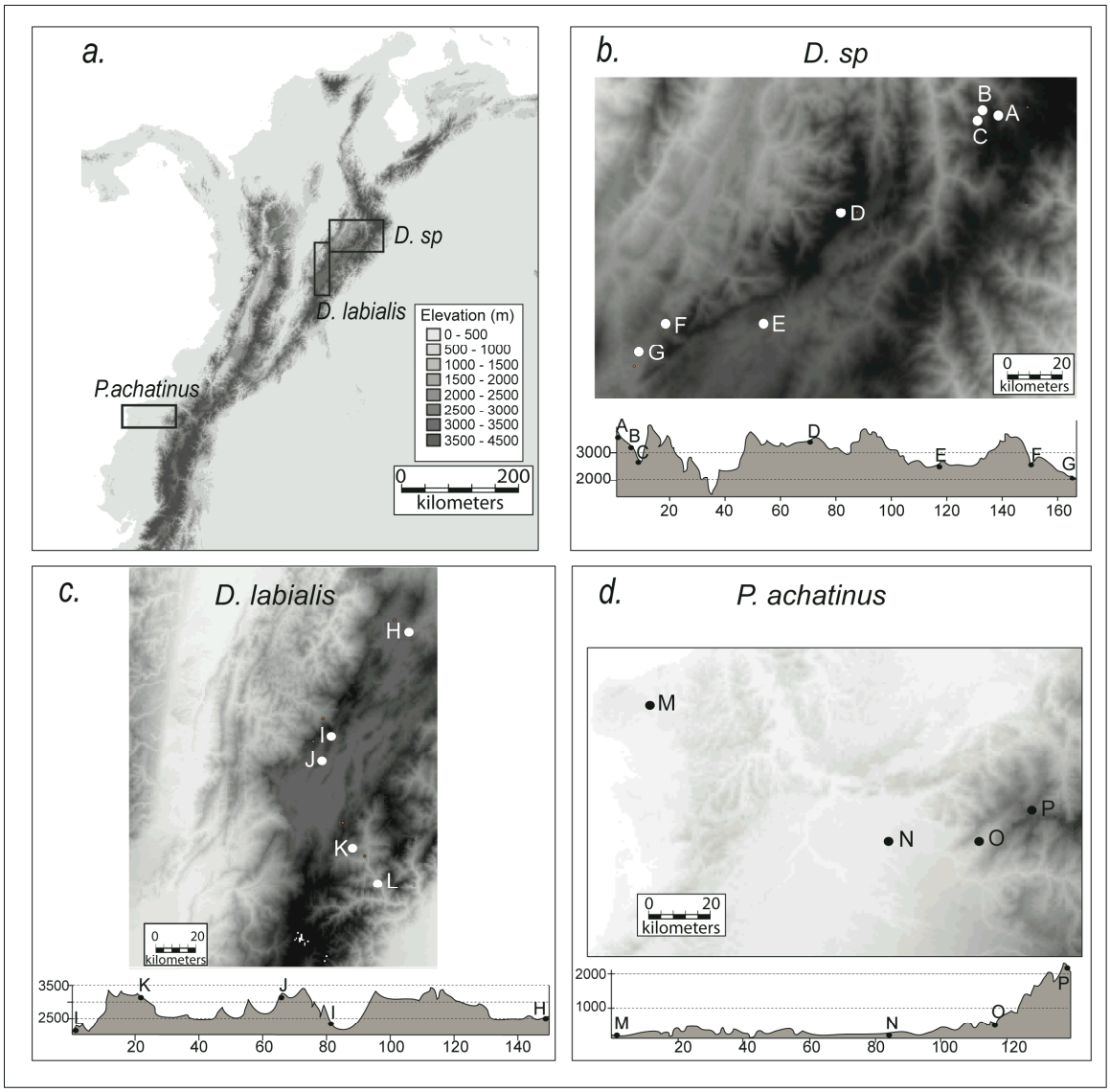


Figure 1.2. Left box: Hypothetical high topographic complexity scenario between the populations A and B, where the contour distance (dotted line) is larger than Euclidean distance (dashed line), making the ratio bigger than 1. Right box: Low topographic complexity scenario between populations B and C, where the contour distance is similar to the Euclidean distance, making the ratio close to 1. *h* = altitudinal difference between each pair of populations. Euclidean distance is determined as the hypotenuse of the right triangle whose sides are *h* and *g*.

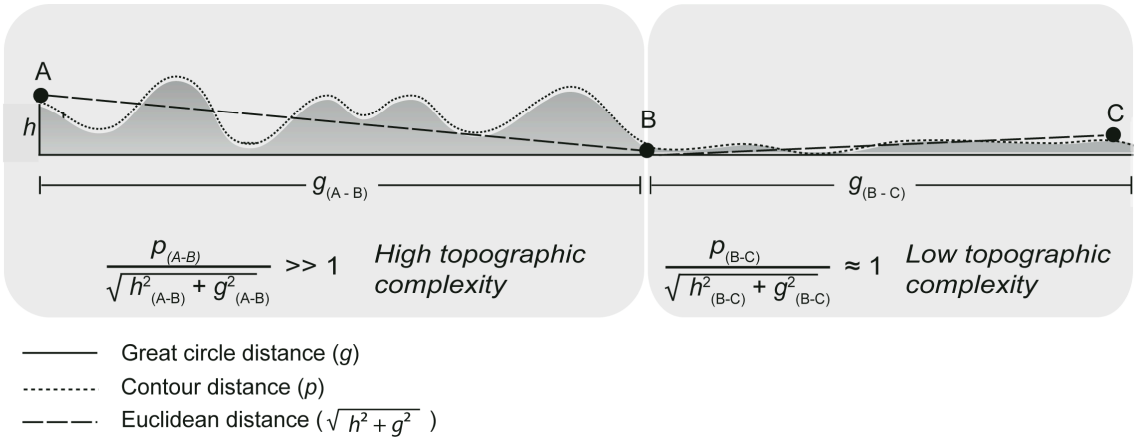


Figure 1.3. Directional migration patterns in *D. sp., D. labialis,* and *P. achatinus*. Gray bars indicate migration upwards ($2N_1m_1 > 2N_2m_2$), and black bars indicate migration downwards ($2N_2m_2 > 2N_1m_1$). The height of the bar indicates its posterior probability. Each bar corresponds to a single pairwise population comparison excluding pairs with the same elevation (all pairwise comparisons along the x-axis are depicted in Table 3). Dotted lines indicate migration rates with posterior probabilities above 60%.

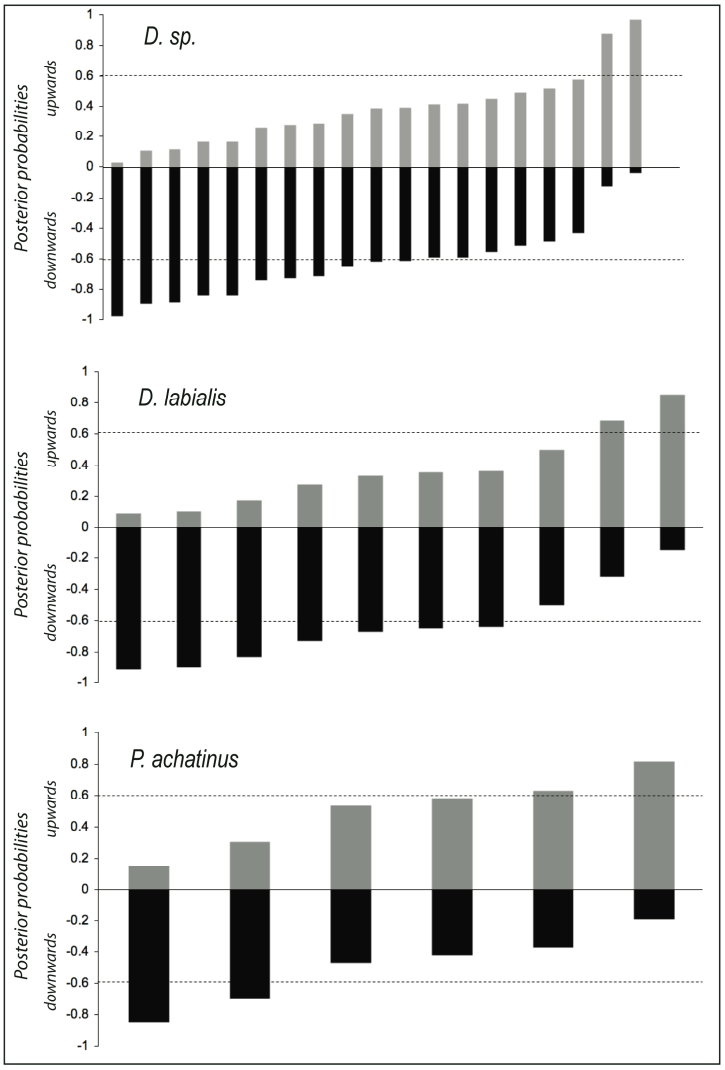


Figure 1.4. Hypothetical scenario that depicts migration between populations A and B (seen from above). Top: The two shaded areas indicate regions unsuitable for the species. If these areas are used to generate a cost matrix, Least Cost Path distances (dotted line) will pass around these regions, increasing their length relative to Euclidean distances (dashed line). Bottom: If the species is adapted to the shaded areas, then these would be estimated inside the species distribution range using species distribution modeling, making Least Cost Path and Euclidean distances to be similar. The higher the ratio between Least Cost Path Distances and Euclidean distances, the higher the probability that migration rate between A and B would be compromised.

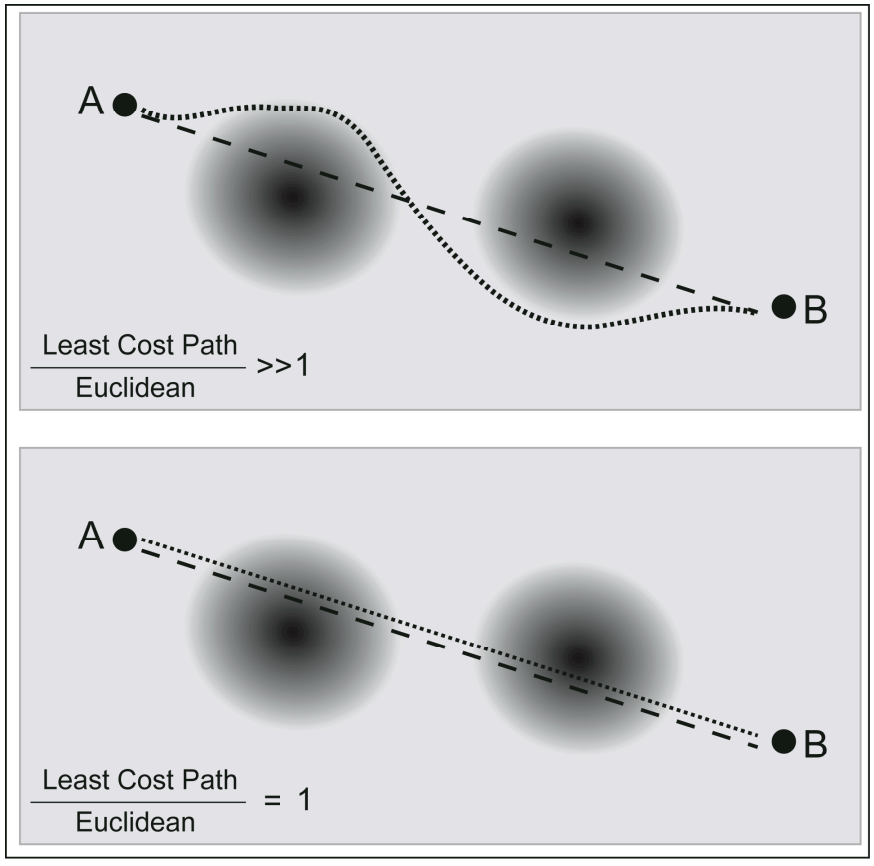


Figure 2.1. Geographic distribution of the species included on the analysis. Top: northern South America. Below left: Central America, below right: Southeastern Brazil.

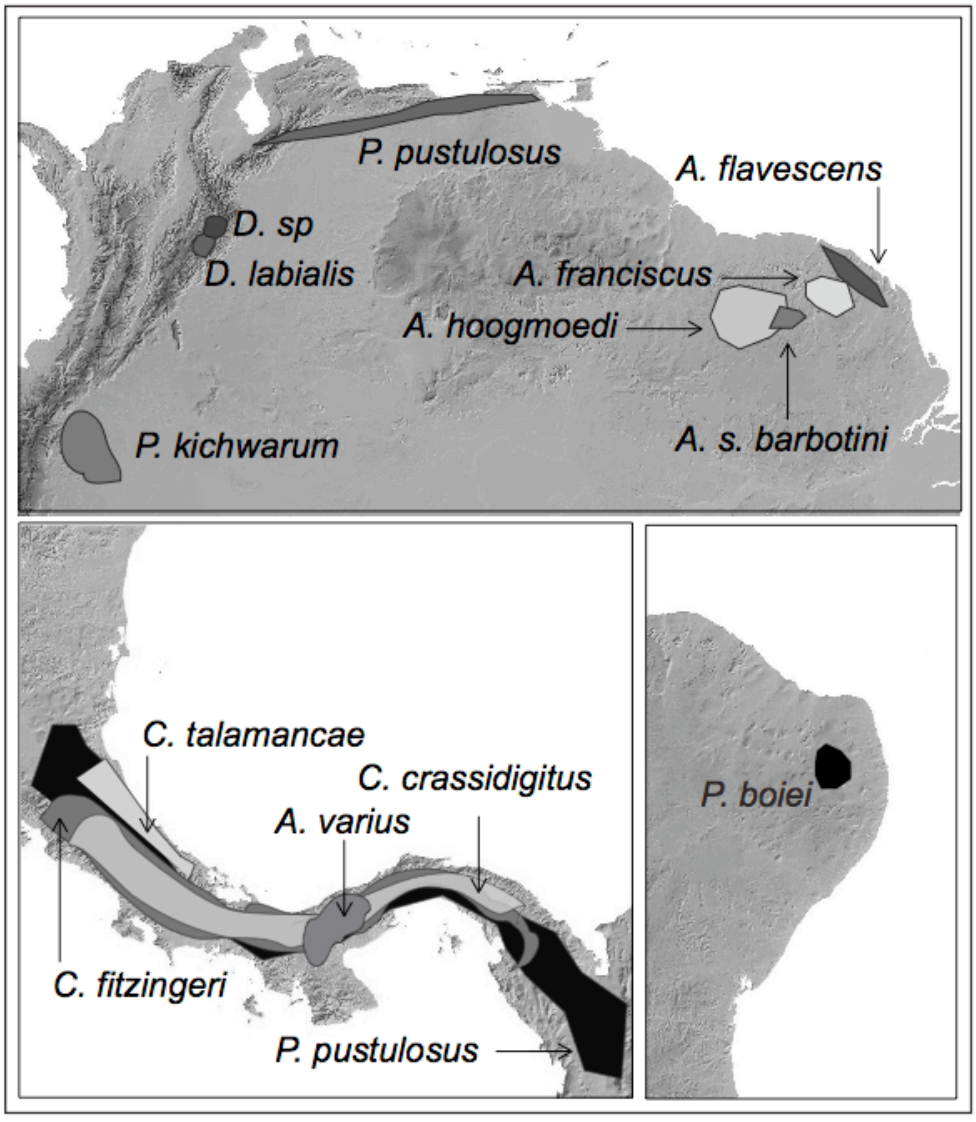


Figure 2.2. A hypothetical scenario where two cryptic species are identified using a scatterplot. If two clearly defined clusters of points are identified with some overlap across the x-axis and their phylogeny indicates K2P sequence divergences above 5%, then the clade with more samples is selected for further analysis.

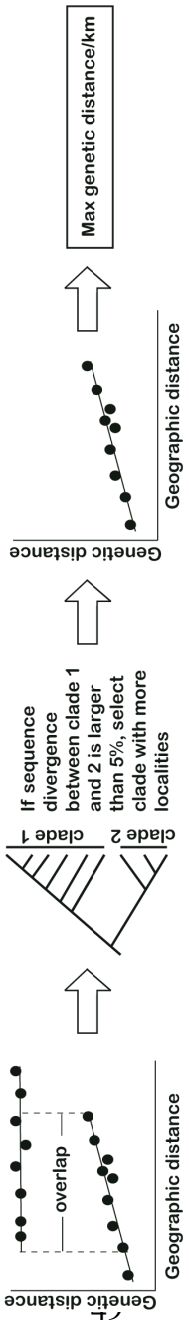


Figure 2.3. Elevational range of the species included in the analysis based on the samples retrieved from Genbank.

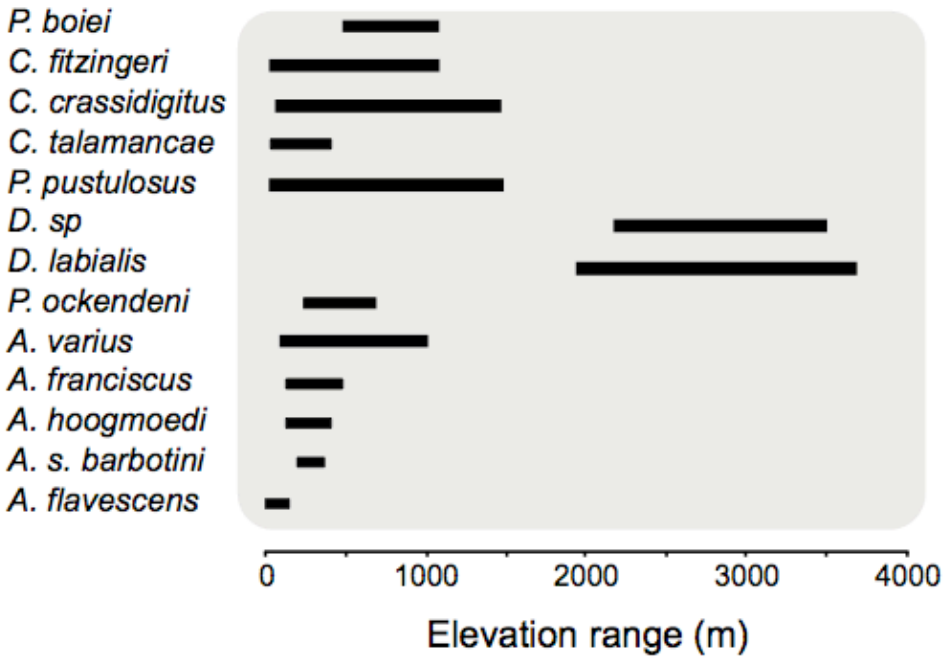


Figure 2.4. Scatterplots between geographic and genetic distances for all species included in the analysis. The top three rows correspond to *cob* and the bottom two to *cox1*.

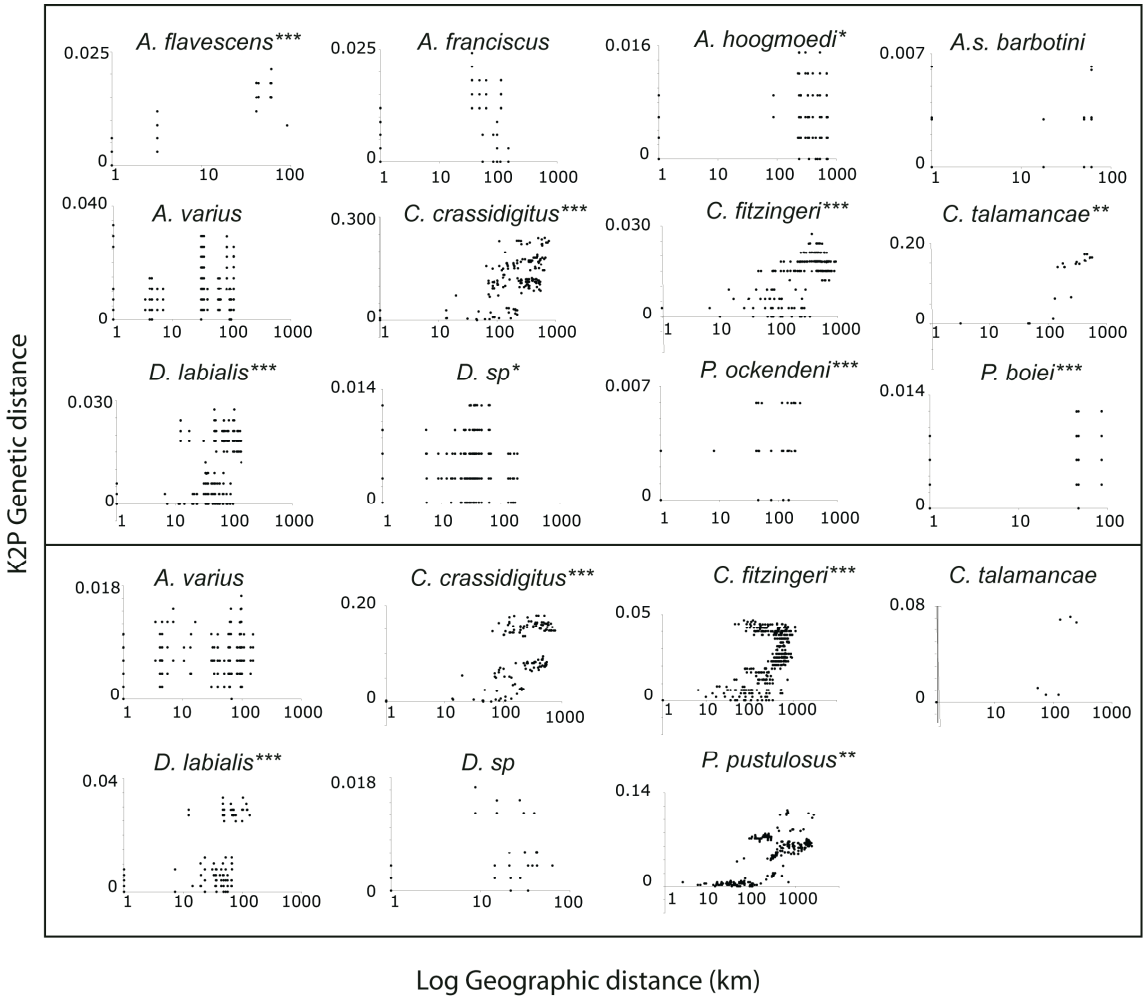


Figure 2.5. Relationship between rugosity and the maximum genetic distance (K2P) within the species included in the analysis. Top: *cob*, bottom: *cox1*.

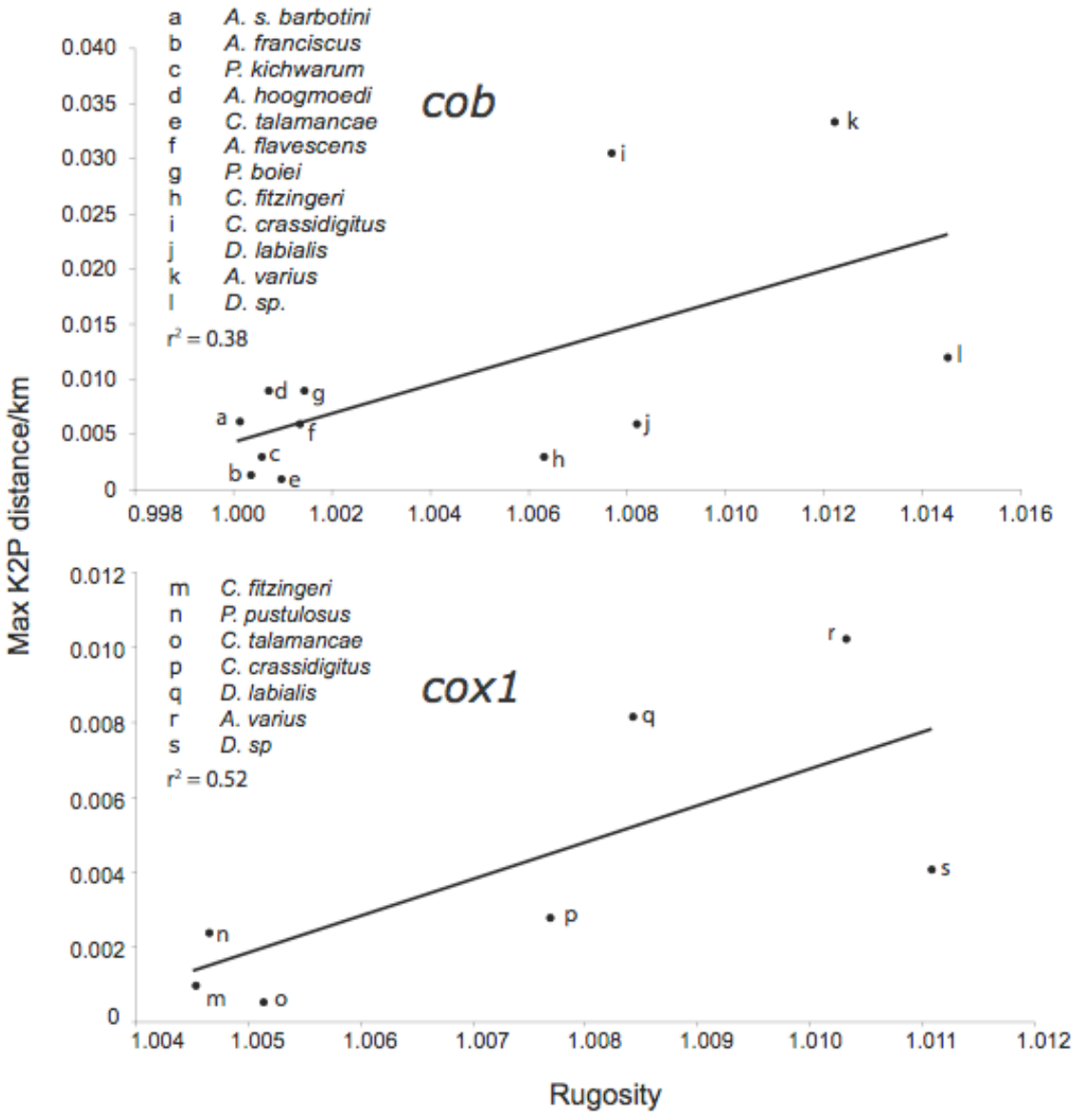


Figure 2.6. Relationship between rugosity and the average elevation of each species (estimated from sampled localities only).

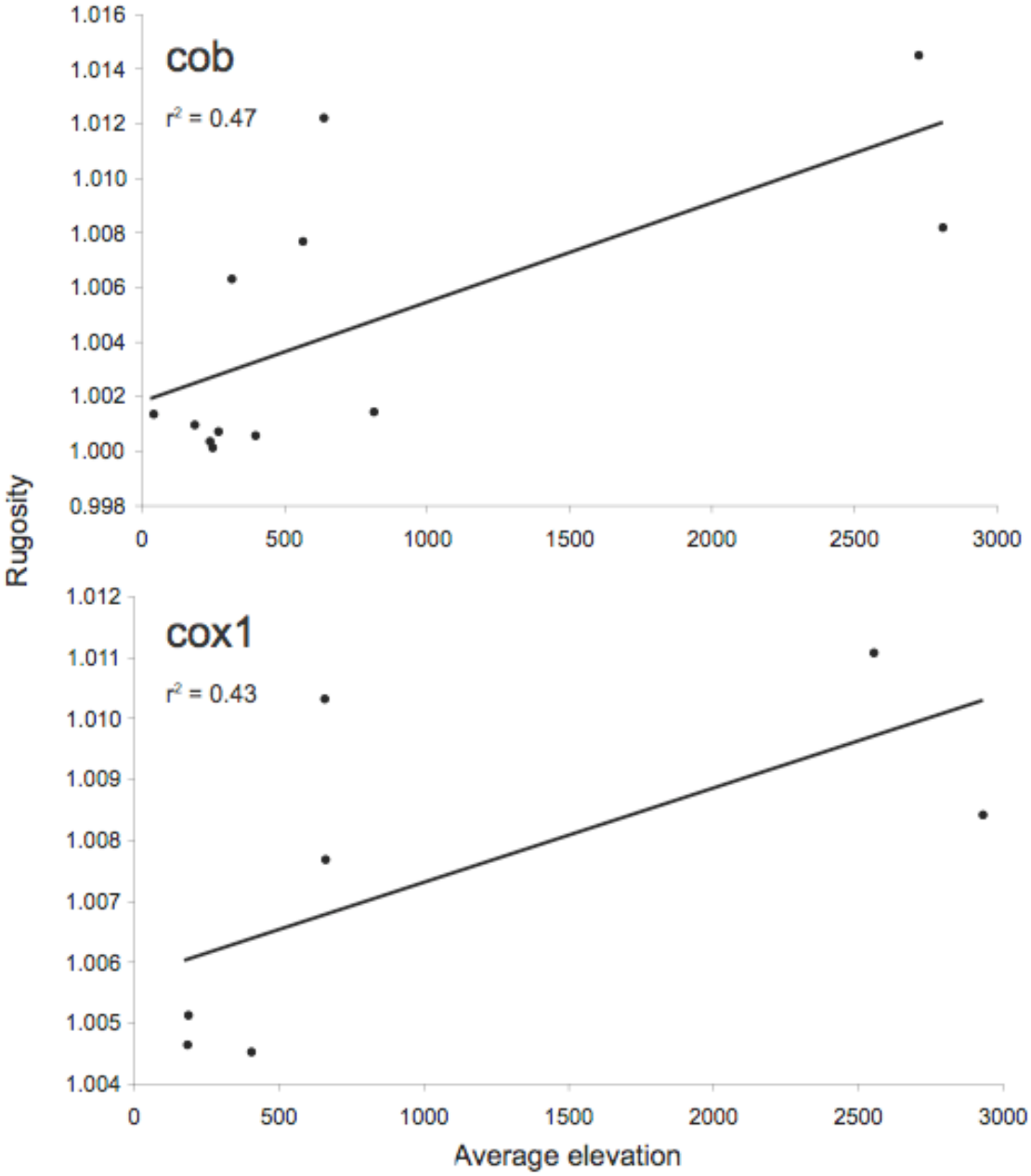


Figure 2.7. Maximum likelihood gene genealogies used to estimate rate heterogeneity across taxa. Left: *cob*, right: *cox1*. Nodes A, B, C, D, are associated to Table 2. Numbers on nodes correspond to bootstrap values. Outgroups are described in the text.

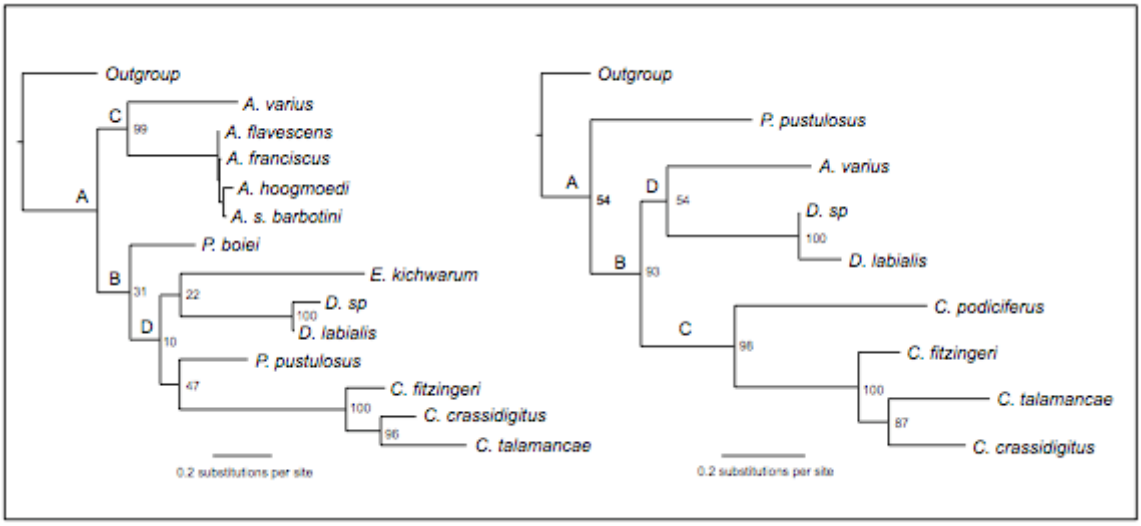


Figure 3.1. Geographic distribution of the localities included in this study. The dashed line delineates the contact zone between both *Dendropsophus labialis* clades. Depicted is the Táchira Depression, which is a low elevation point (<900 m) that connects the Andes of Colombia and Venezuela. Names and geographic coordinates of each locality described in Table 1. Only elevations above 500 m are shown.

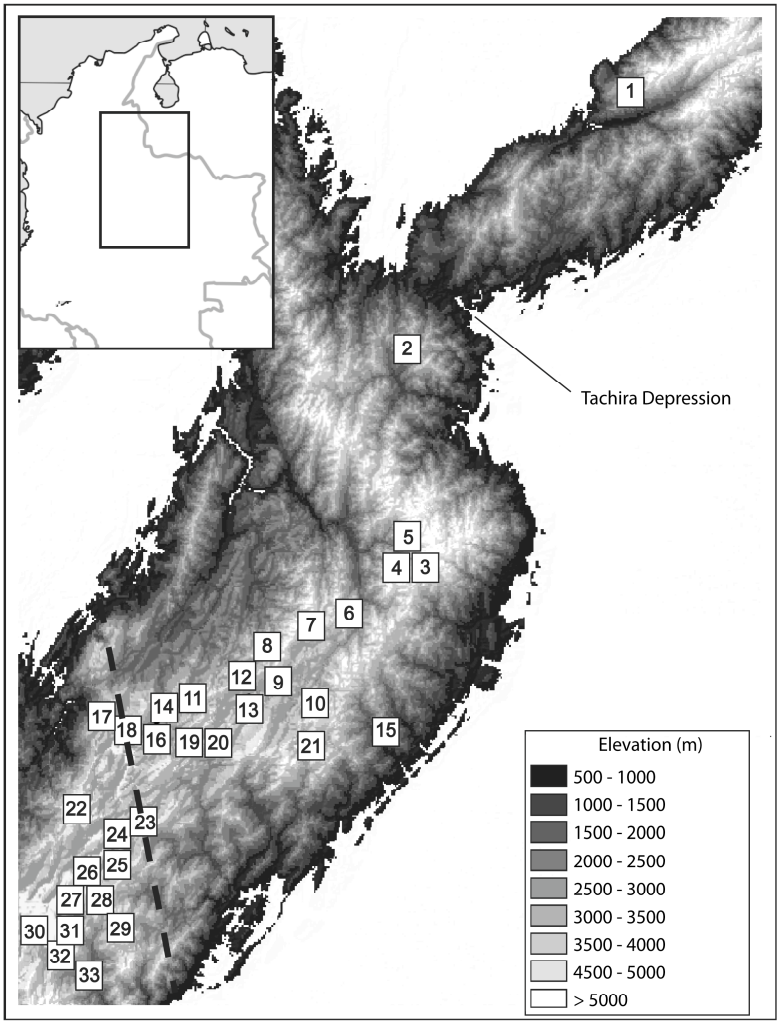


Figure 3.2. Maximum likelihood gene trees using *left*: the complete mitochondrial 12s-16s rRNA and *right*: the nuclear POMC gene. Numbers on top of the branches correspond to Bayesian posterior probabilities and below to bootstrap support values. Numbers in parenthesis correspond to the map codes displayed in table 1. Transparent grey bars delineate the *D. meridensis* clade (top), *D. labialis* northern clade (middle), and *D. labialis* southern clade (bottom).

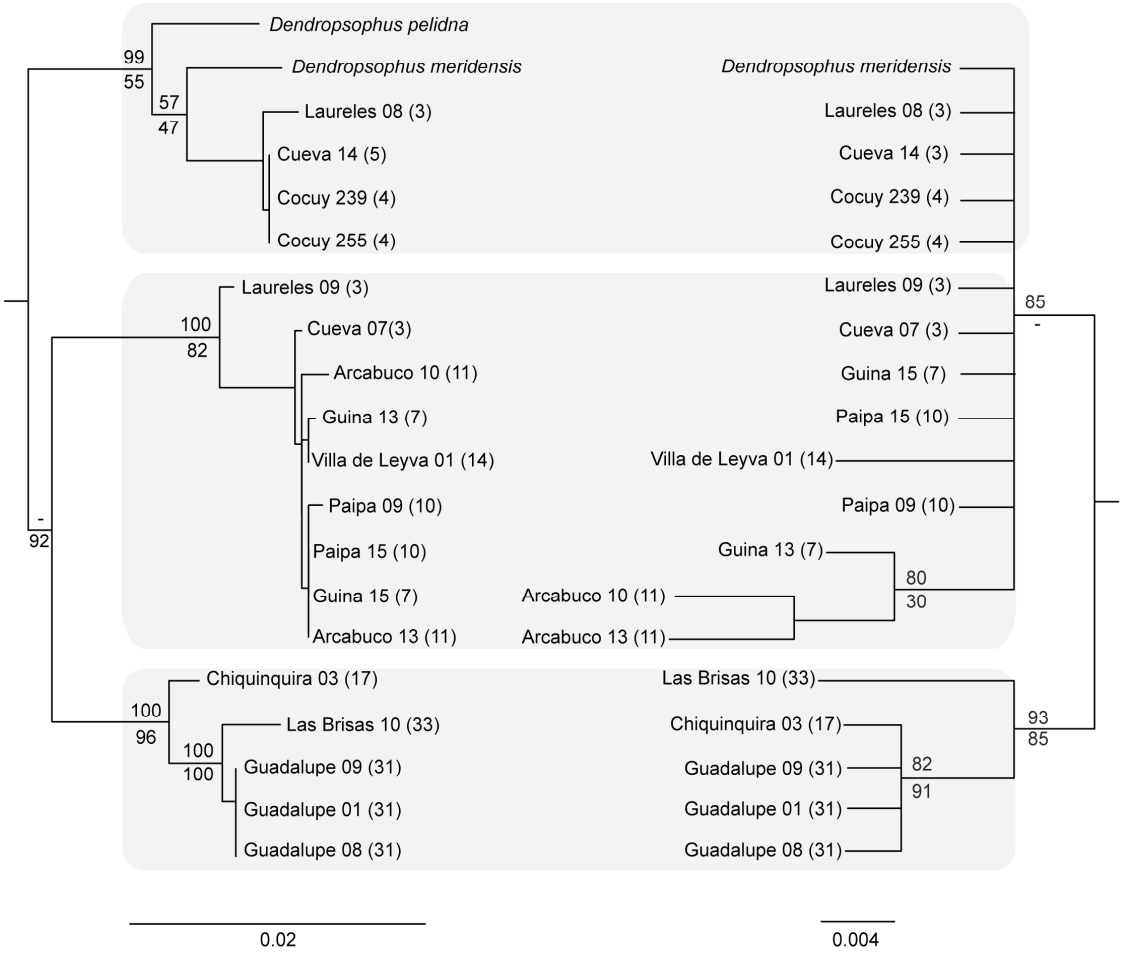


Figure 3.3. Principal component analysis plot that summarizes *top*: temperature-independent variation in call parameters *bottom*: size-independent variation in external morphology among frogs grouped in two phylogenetic clades. White dots: northern clade, black dots: southern clade. Grey triangles: sympatric locality.

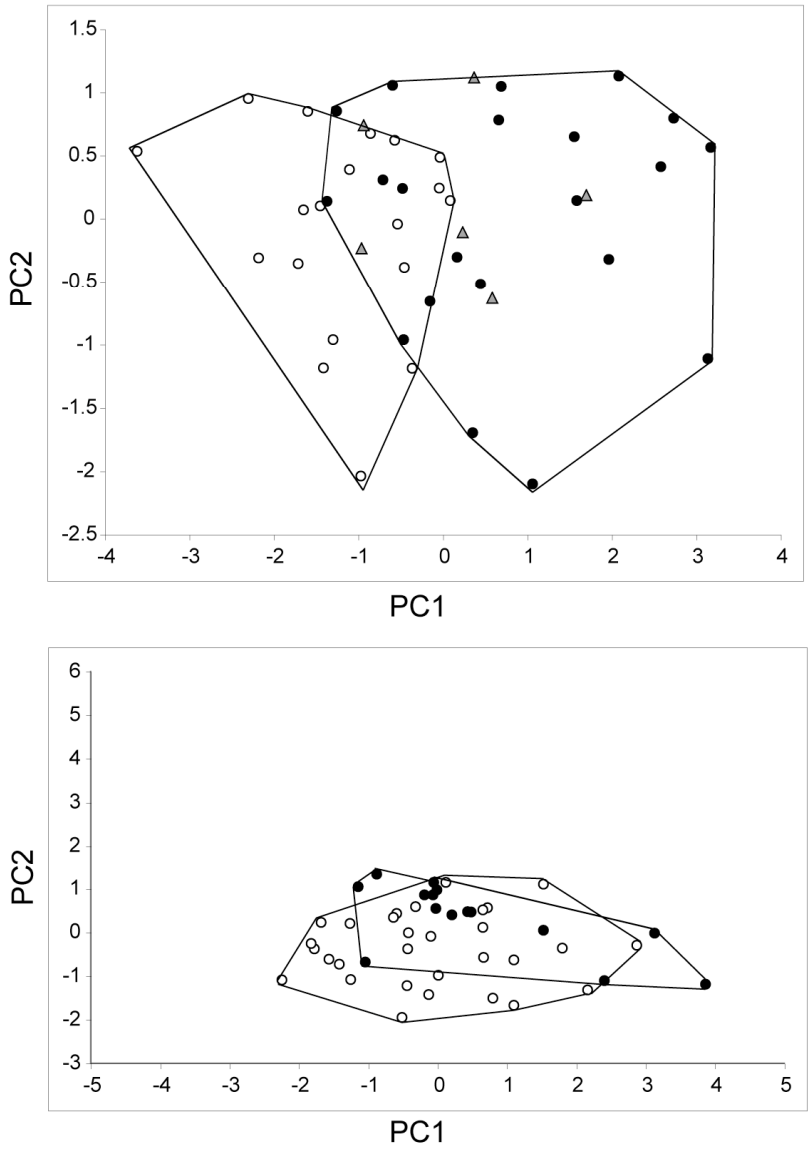


Figure 3.4. Plot depicting geographic vs. genetic distances in intra-clade (black dots) and inter-clade (white dots) pairwise comparisons. *Top*: Hypothetical scenario where inter-clade comparisons only occur at large geographic distances (> 100 km) having no overlap with intra-clade comparisons across the x-axis. This scenario is expected when large genetic distances are the result of an intermediate spatial sampling gap. *Bottom*: *Dendropsophus labialis* 12s-16s data. Notice that intra and inter-clade comparisons completely overlap across the x-axis. See text for discussion.

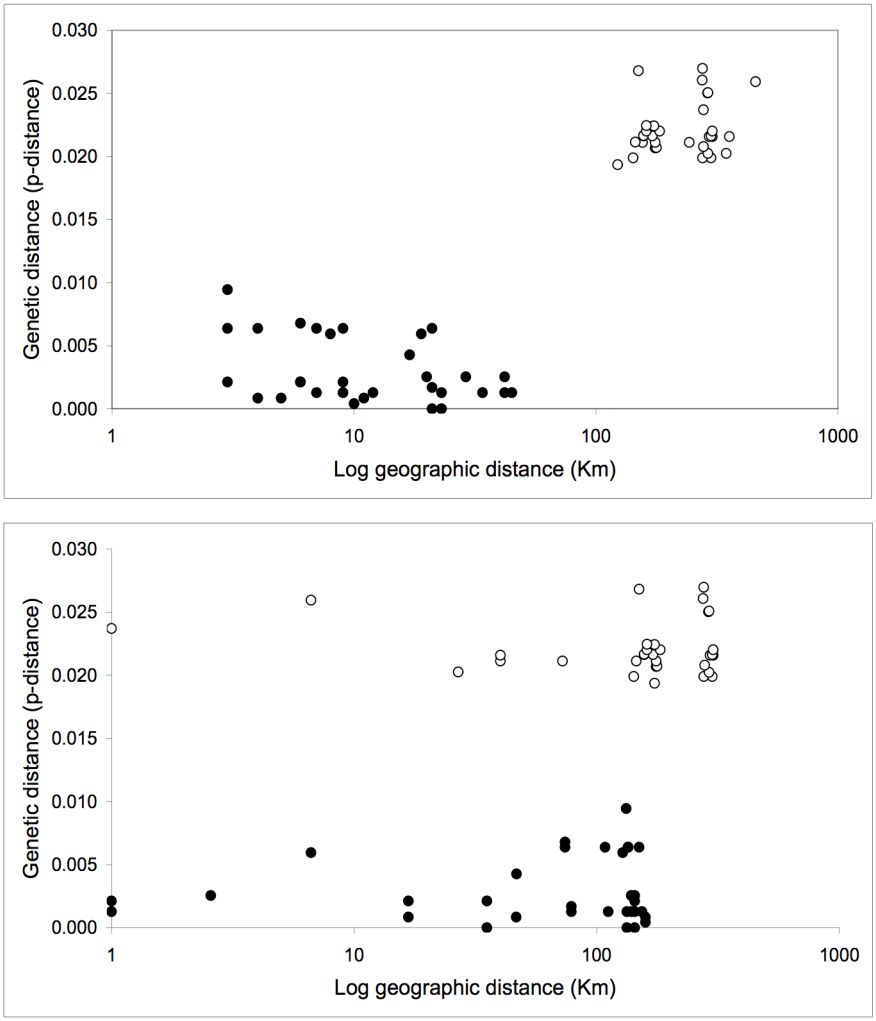


Figure 3.5. Geographic variation of *D. labialis* and *D. luddeckei* across the contact zone for *top*: pulse rate, *middle*: pulses per call, and *bottom*: haplotype frequencies of the Southern clade (Based on the populations sampled in Guarnizo et al 2009). The dotted line corresponds to the contact zone that passes through the sympatric locality San Carlos (map code 18). The negative values in the x-axis correspond to the region west of the contact zone (*D. labialis*) and the positive values to the region east of the contact zone (*D. luddeckei)*.

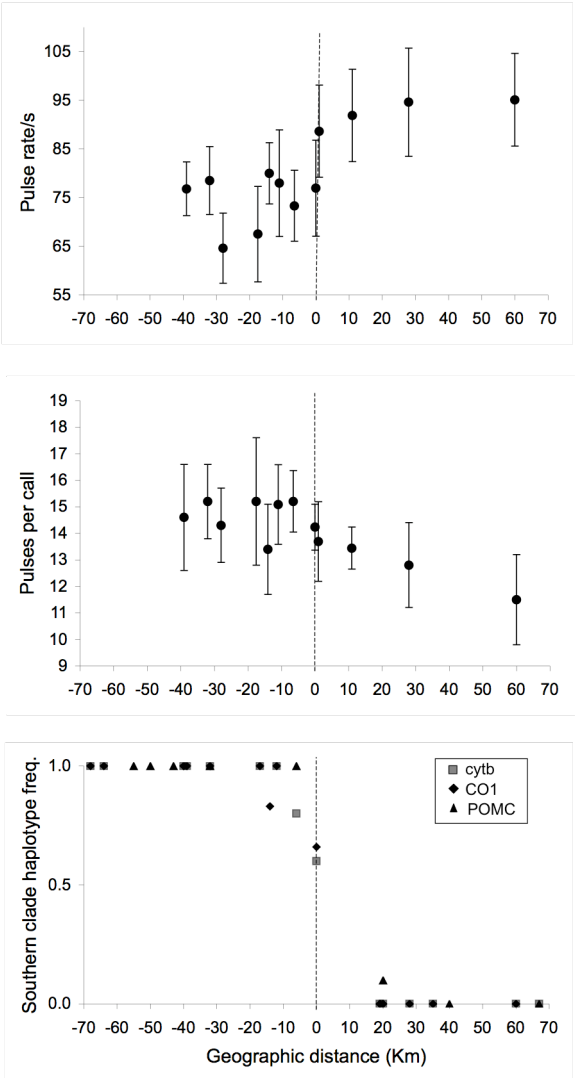


Figure 3.6. Elevation profiles and the level of sequence divergence (for the complete 12s-16s rRNA) within the species pairs *D. labialis – D. luddeckei* and *D. pelidna – D. meridensis*. Gray areas depict the presumed elevation range of each species based on the IUCN red list webpage (see text). The arrow points to the Táchira Depression, which reaches elevations below the minimum elevation registered for any of the four species.

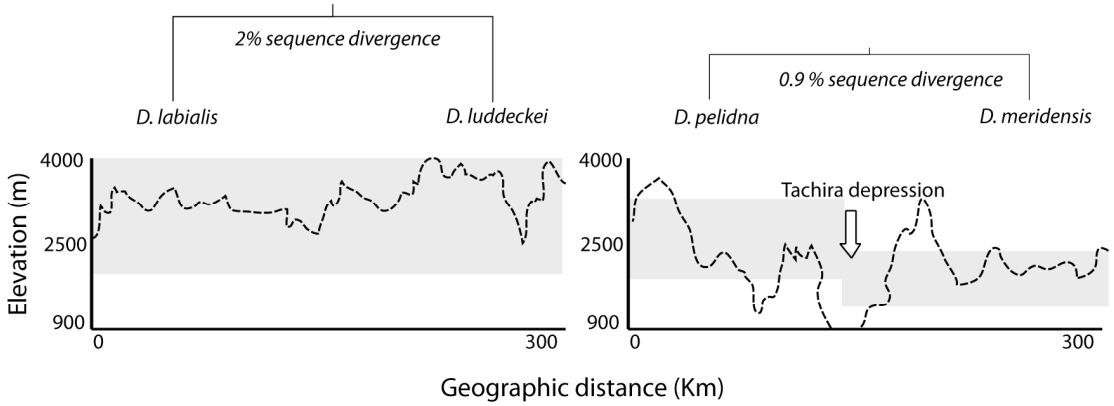
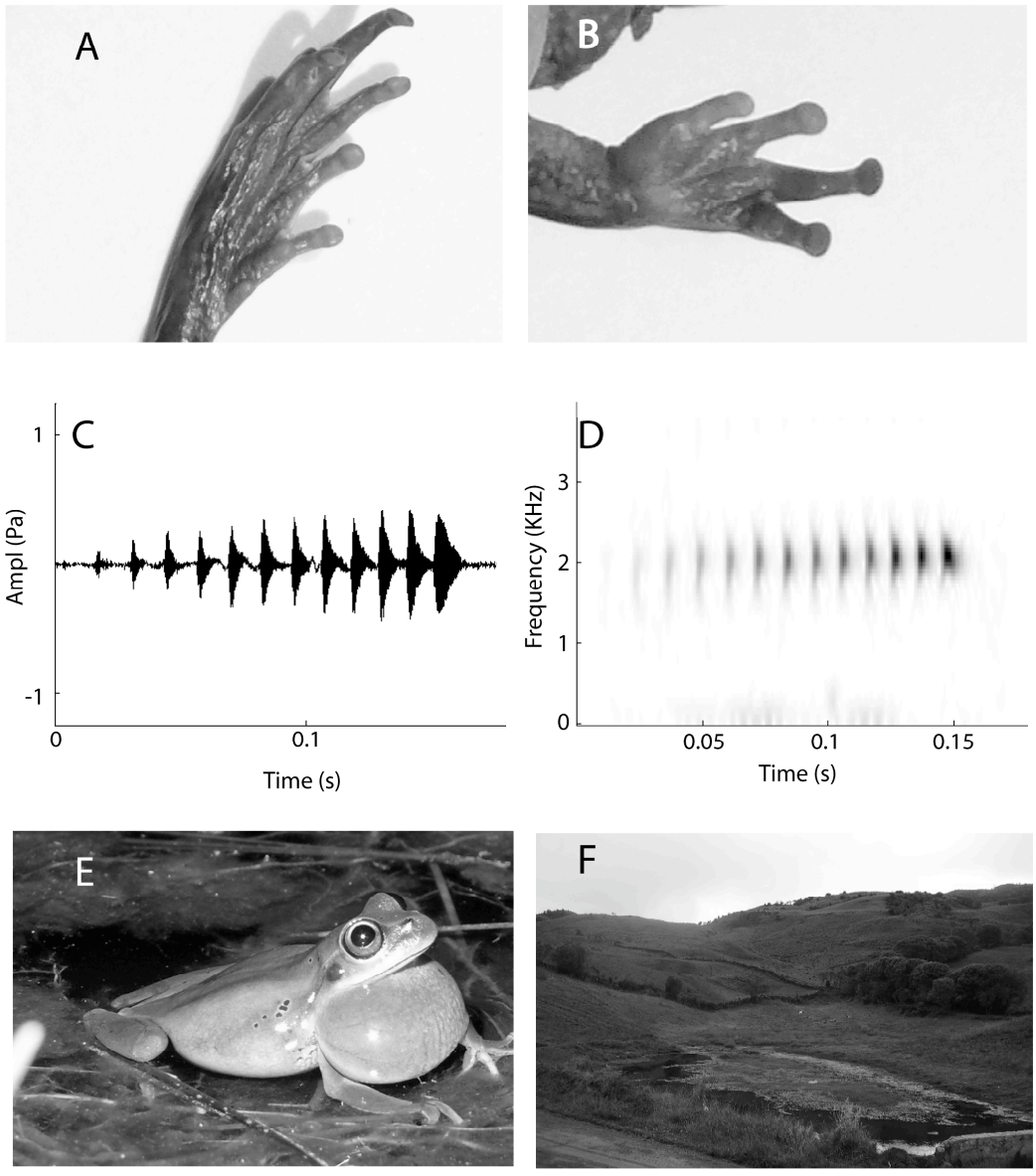


Figure 3.7. Holotype right foot (A), holotype left hand (B), oscillogram (C), sonogram (D), holotype advertisement call (E), and typical habitat (F), of *Dendropsophus luddeckei*. Photos of the holotype by C. Escallon and habitat by C. Guarnizo.



TABLES

Table 1.1. Sampling sites, map codes, geographic coordinates, elevation (above sea level), and the number of individuals analyzed for each locus. Populations within each species are ordered from north to south.

Species	Population	Code	Coordinates (lat N, Long W)	Elevation (m)	n	# of individuals sequenced
	La Cueva	A	6°24'15.20", 72°22'55.90"	3729	14	14 (control region), 11 (POMC), 04 (c-myc)
	Laureles	B	6°24'54.84", 72°26'21.76"	2930	12	12 (control region), 12 (POMC), 04 (c-myc)
	Cocuy	C	6°24'19.30", 72°26'47.60"	2691	17	17 (control region), 09 (POMC), 05 (c-myc)
D. sp	Guina	D	6°05'51.90", 72°52'45.84"	3288	18	18 (control region), 18 (POMC), 06 (c-myc)
	Paipa	E	5°44'50.60", 73°08'01.60"	2642	20	20 (control region), 19 (POMC), 01 (c-myc)
	Arcabuco	F	5°45'14.22", 73°26'40.30"	2575	20	20 (control region), 19 (POMC), 13 (c-myc)
	Villa de Leyva	G	5°38'55.20", 73°31'56.10"	2125	13	13 (control region), 12 (POMC), 04 (c-myc)
	Chiquinquira	H	5°36'27.70", 73°46'10.92"	2542	17	17 (control region), 16 (POMC), 09 (c-myc)
	Las Pilas	I	5°07'0.130", 74°07'37.10"	2313	6	06 (control region), 06 (POMC), 01 (c-myc)
D. labialis	Pantano	J	5°00'14.40", 74°10'32.20"	3192	15	15 (control region), 12 (POMC), 01 (c-myc)
	Guadalupe	K	4°35'44.20", 74°01'49.76"	3276	18	18 (control region), 18 (POMC), 04 (c-myc)
	Las Brisas	L	4°25'58.62", 73°55'13.19"	1970	18	18 (control region), 17 (POMC), 05 (c-myc)
	Aguacate	M	0°44'17.10", 79°55'25.00"	49	19	17 (control region), 19 (POMC), -- (c-myc)
P. achatinus	Chihuilpe	N	0°19'49.70", 79°12'59.20"	197	18	18 (control region), 18 (POMC), -- (c-myc)
	Otongachi	O	0°19'32.80", 78°56'56.40"	639	14	14 (control region), 14 (POMC), 02 (c-myc)
	Omontana	P	0°25'25.80", 78°47'10.90"	2241	10	09 (control region), 10 (POMC), 01 (c-myc)

Table 1.2. Causal model scheme of the expected relationship between geographic features and migration rate or FST (see models explanation in text). L = Least Cost Path distances; T = topographic complexity ratio; A = altitudinal difference; m = migration rate. The "x" indicates a correlation between two matrices. The "." Indicates the co-variable that is controlled in the partial Mantel test. Sig = significant correlation, NS = non-significant correlation.

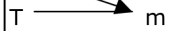
Model A		L x m.T	Sig.
		L x m.A	Sig.
		T x m.L	N.S.
		A x m.L	N.S.
Model B		T x m.L	Sig.
		T x m.A	Sig.
		L x m.T	N.S.
		A x m.T	N.S.
Model C		A x m.L	Sig.
		A x m.T	Sig.
		T x m.A	N.S.
		L x m.A	N.S.
Model D		L x m.A	Sig.
		T x m.A	Sig.
		L x m.T	Sig.
		T x m.L	Sig.
		A x m.L	N.S.
		A x m.T	N.S.
Model E		T x m.L	Sig.
		A x m.L	Sig.
		T x m.A	Sig.
		A x m.T	Sig.
		L x m.T	N.S.
		L x m.A	N.S.
Model F		L x m.T	Sig.
		A x m.T	Sig.
		L x m.A	Sig.
		A x m.L	Sig.
		T x m.L	N.S.
		T x m.A	N.S.
Model G		L x m.T	Sig.
		L x m.A	Sig.
		T x m.L	Sig.
		T x m.A	Sig.
		A x m.L	Sig.
		A x m.T	Sig.

Table 1.3. Demographic parameters (peak probability distributions after smoothing) calculated for each species. θ1 and θ2 are the effective sizes of the populations 1 and 2 respectively. *m*1 and *m*2 are the maximum migration rates from population 1 to population 2 and vice-versa. *m* corresponds to a single migration rate parameter. The posterior probability upwards is estimated as 1 – posterior probability downwards. In parentheses are the 90% high posterior density intervals. Asterisks indicate not-contiguous high posterior density intervals (when there were multiple peaks or rough surfaces along the interval). The last column corresponds to pairwise F_{ST} values.

Species	Population 1	Population 2	θ_1	θ_2	m_1	m_2	m	Posterior probability downwards $2N_1m_1>2N_2m_2$	Fst
	A	B	0.1980 (0.0849–1.2729)	5.4028 (1.9518–20.000)*	2.2900 (0.7100–19.370)*	0.1700 (0.0100–9.2500)	3.8300 (0.2900-10.0000)*	0.9723	0.4693
	C	A	3.1637 (1.1493–7.1927)	1.7691 (0.4520–5.0749)	0.0550 (0.0150–2.3850)	0.6050 (0.0150–3.8950)	0.5300 (0.0500-4.3100)	0.6132	0.6091
	C	B	1.1613 (0.3372–4.1584)	4.4581 (1.5360–20.000)*	0.0625 (0.0125–12.287)*	4.3125 (0.0125–16.587)*	7.2900 (2.0700-17.200)*	0.0321	0.0307
	D	A	1.7435 (0.6946–3.8130)	1.8569 (0.6662–4.4934)	0.0050 (0.0050–0.4550)	0.2350 (0.0250–0.8350)	0.1900 (0.0500-0.6700)	0.5081	0.3189
	D	B	0.9943 (0.3097–2.4612)	1.8744 (0.5705–4.5475)	0.0050 (0.0050–0.7250)	0.2450 (0.0250–0.9750)	0.2900 (0.0700-0.7900)	0.8840	0.8510
	C	D	2.9210 (1.3544–5.7277)	2.5946 (1.1912–4.8139)	0.0650 (0.0050–0.3450)	0.0050 (0.0050–0.2550)	0.0700 (0.0300-0.3500)	0.5489	0.9509
	G	A	2.7900 (1.0605–5.4985)	2.7248 (0.9953–5.6943)	0.0050 (0.0050–0.8150)	0.1950 (0.0050–0.9450)	0.2900 (0.0500-0.9300)	0.6496	0.4306
	B	G	3.2252 (1.0991–7.7297)	2.3604 (0.6306–5.4234)	0.1250 (0.0050–0.9750)	0.0150 (0.0150–1.9900)	0.2500 (0.0500-0.8900)	0.7115	0.8940
	C	G	2.9391 (1.3782–5.8949)	3.0388 (1.3450–6.0609)	0.0350 (0.0050–0.5150)	0.0050 (0.0050–0.6450)	0.1900 (0.0500-0.7300)	0.7399	0.9853
	G	D	1.6476 (0.3444–4.2167)	1.7220 (0.2513–3.9561)	0.4950 (0.0050–2.9750)*	0.0050 (0.0050–2.4050)*	0.4500 (0.1100-1.3100)	0.8365	0.6056
D. sp	F	A	4.9049 (2.5584–8.6529)	1.6784 (0.5703–3.6991)	0.0450 (0.0050–0.6550)	0.2350 (0.0150–1.2550)	0.1700 (0.0005-0.5100)	0.5867	0.3173
	F	B	3.4298 (1.7949–6.4865)	2.6835 (1.1196–5.6690)	0.1250 (0.0050–0.5950)	0.1650 (0.0150–0.6950)	0.1500 (0.0500-0.4500)	0.6086	0.8544
	F	C	5.0886 (2.8389–8.9095)	2.6247 (1.1963–5.2672)	0.0650 (0.0050–0.4250)	0.0550 (0.0050–0.4250)	0.0900 (0.0300-0.3300)	0.8358	0.9527
	F	D	2.1820 (0.7521–5.5989)	0.5664 (0.1393–2.2006)	0.0050 (0.0050–5.8750)*	2.2950 (0.0050–8.3750)*	0.7700 (0.1900-2.1900)	0.7244	0.1915
	F	G	6.1052 (3.0647–12.668)	0.1689 (0.0724–1.2307)	0.0100 (0.0100–2.4900)	1.7700 (0.2900–16.150)*	0.6100 (0.1900-1.5700)	0.4266	0.6157
	E	A	4.6448 (2.4877–8.3448)	1.6847 (0.7243–4.1882)	0.0750 (0.0050–0.4450)	0.1350 (0.0150–0.7550)	0.1100 (0.0300-0.4100)	0.8917	0.3770
	B	E	1.6744 (0.4661–4.2638)	1.6744 (0.5697–3.7459)	0.1950 (0.0150–1.2350)	0.1350 (0.0050–1.0350)	0.1500 (0.0500-0.4900)	0.4814	0.8626
	C	E	2.6824 (1.1496–5.4148)*	4.1819 (2.0493–7.3142)*	0.1250 (0.0050–0.5850)	0.0550 (0.0050–0.3850)	0.1100 (0.0300-0.3700)	–	0.9577
	D	E	0.6269 (0.0950–2.2606)	2.2986 (0.7409–4.9962)	0.0500 (0.0100–11.710)*	0.0100 (0.0100–4.7500)*	0.8500 (0.2500-2.2500)	0.5839	0.3754
	E	G	1.7879 (0.3694–4.4476)	4.8613 (1.8765–12.866)	0.0050 (0.0050–4.3350)*	0.0250 (0.0050–1.9050)	0.1700 (0.0300-0.5900)	0.1215	0.6973
	F	E	4.1665 (1.3483–15.366)*	1.2269 (0.4009–3.5591)	0.0550 (0.0050–7.8150)*	1.7850 (0.0050–8.4250)*	1.3300 (0.4700-3.9700)	–	0.2867
	K	L	0.6050 (0.1460–1.7316)	1.8151 (0.7302–3.8597)	0.0100 (0.0100–1.5900)	0.1500 (0.0100–1.0300)	0.2300 (0.0100-0.7100)	0.7275	0.8251
	K	J	0.4531 (0.1256–1.8070)	1.0536 (0.2784–3.3902)	0.0450 (0.0050–9.0750)*	3.6850 (0.0350–8.5550)*	2.2500 (0.5300-7.5700)*	0.9095	0.1948
	L	J	2.4305 (1.0844–4.6990)	1.9320 (0.7852–4.2004)	0.0850 (0.0050–0.7750)	0.0050 (0.0050–0.7550)	0.0100 (0.0100-0.3700)	0.6364	0.7620
	H	K	0.7246 (0.2143–2.0309)	1.6635 (0.5817–4.1536)	0.0150 (0.0150–2.0550)	0.0150 (0.0150–2.3850)	0.1500 (0.0100-0.6900)	0.8989	0.8971
	H	L	2.4163 (1.1094–4.7870)	2.1428 (0.7750–4.4831)	0.1850 (0.0050–0.7450)	0.0050 (0.0050–0.5550)	0.0100 (0.0100-0.2500)	0.6686	0.8804
D. labialis	H	J	2.1603 (0.9346–4.4891)	2.1296 (0.8733–4.5197)	0.0050 (0.0050–0.4850)	0.2550 (0.0050–0.9550)	0.1300 (0.0300-0.4900)	0.1470	0.8614
	K	I	0.2192 (0.0296–1.0250)	0.6932 (0.0652–4.1768)*	0.0050 (0.0050–7.6950)*	2.2650 (0.0050–8.0150)*	1.1500 (0.0100-10.000)*	0.8291	0.6546
	L	I	2.1450 (0.9308–4.4923)	1.1737 (0.2833–3.4400)	0.0550 (0.0050–0.8050)	0.0050 (0.0050–1.1650)	0.1100 (0.0100-0.5100)	0.6453	0.8300
	J	I	2.8961 (0.5448–6.9105)	0.2581 (0.0287–5.6488)	0.0100 (0.0100–12.530)*	1.7300 (0.0100–16.730)*	0.5700 (0.0900-4.0700)*	0.3157	0.7131
	H	I	1.3367 (0.3947–3.4501)	1.2604 (0.0382–4.6722)	0.0050 (0.0150–1.2150)	0.5450 (0.0150–2.9250)	0.2300 (0.0100-0.8300)	0.5001	0.7748
	N	M	3.0690 (1.3950–6.0184)	7.7721 (4.7430–12.873)	0.4700 (0.1100–1.2900)	0.0100 (0.0100–0.3500)	0.1900 (0.0700–0.5900)	0.1858	0.8878
	P	N	1.3000 (0.3634–2.9355)	4.4592 (2.0409–7.5764)	0.0050 (0.0050–0.5050)	0.1450 (0.0150–0.5150)	0.0900 (0.0300–0.3900)	0.8472	0.9201
P. achatinus	M	P	0.7813 (0.0397–5.7072)	6.3428 (1.9730–17.280)*	0.0050 (0.0050–5.3150)*	0.0050 (0.0050–1.8750)	1.5100 (0.4500–3.4300)	0.6925	0.4998
	N	O	4.8478 (2.5187–9.6144)	0.7312 (0.1354–2.3020)	0.0500 (0.0100–1.2700)	1.0700 (0.0900–8.0100)	0.4300 (0.1100–1.3700)	0.4179	0.6817
	M	O	1.7630 (0.0745–9.2123)	7.4244 (1.7630–20.000)*	0.1450 (0.0050–3.8550)*	0.0050 (0.0050–1.2450)	0.9700 (0.1700–2.9300)	0.4681	0.2783
	P	O	0.6714 (0.0172–19.400)*	1.3255 (0.0516–12.342)	1.1250 (0.0050–8.8450)*	0.0750 (0.0050–7.6050)*	1.5300 (0.3500–16.130)*	0.3696	0.1989

Table 1.4. Mantel correlation coefficients (Mantel's r) and P-values from partial Mantel tests. The "x" indicates a correlation between two matrices, and the "." Indicates a third co-variable that was controlled. m = migration rate. A = altitudinal difference. E = Euclidean distance. L = Least Cost Path distances (log). T = topographic complexity. The top half of the table indicates correlation between geographic features and migration rate. The bottom half displays correlations between geographic features and F_{ST}. Euclidean distance correlations are not displayed as they were highly correlated with LCP distances.

	D. sp		***D. labialis***		***P. achatinus***	
Correlation	***r***	***P***	***r***	***P***	***r***	***P***
A x m.L	-0.043556	0.477183	-0.515270	0.058333	0.4341	0.3334
A x m.T	-0.223947	0.181746	-0.540726	0.050000	0.1790	0.4202
L x m.A	-0.818281	0.007341	-0.443544	0.133333	0.2254	0.6253
L x m.T	-0.597388	0.002778	0.274946	0.191667	-0.4551	0.2887
T x m.A	-0.714909	0.011706	0.607630	0.050000	0.4495	0.2541
T x m.L	0.005519	0.502183	0.496500	0.083333	0.6642	0.1197
A x F_{ST}.L	-0.387965	0.052778	0.541502	0.066667	0.0661	0.4215
A x F_{ST}.T	-0.307936	0.144246	0.614070	0.041667	0.3143	0.3385
L x F_{ST}.A	0.540621	0.015873	0.463935	0.133300	-0.1458	0.4979
L x F_{ST}.T	0.049156	0.394246	-0.401944	0.150000	0.2174	0.4194
T x F_{ST}.A	0.543056	0.011508	-0.702316	0.025000	-0.4548	0.2588
T x F_{ST}.L	0.259394	0.138690	-0.621048	0.033330	-0.3834	0.2472

Table 2.1. Species analyzed and the number of haplotypes downloaded form Genbank for each species.

Family	Species	*cox1*	*cob*
Bufonidae	*Atelopus flavescens*	–	19
Bufonidae	*Atelopus franciscus*	–	14
Bufonidae	*Atelopus hoogmoedi*	–	29
Bufonidae	*Atelopus spumarius barbotini*	–	9
Bufonidae	*Atelopus varius*	25	25
Craugastoridae	*Craugastor crassidigitus*	27	27
Craugastoridae	*Craugastor fitzingeri*	30	20
Craugastoridae	*Craugastor talamancae*	5	7
Hylidae	*Dendropsophus labialis*	21	41
Hylidae	*Dendropsophus sp.*	13	37
Strabomantidae	*Pristimantis ockendeni*	–	68
Cycloramphidae	*Proceratophrys boiei*	–	54
Leiuperidae	*Physalaemus pustulosus*	24	–

Table 2.2. Relative rate test for each gene estimated with the program r8s. The species assigned to each node (A, B, C, D) are depicted in Supplemental figure 2. Asterisks indicate significant departures from clocklike evolution (P < 0.001). Hyphens indicate cases where the evolution occurring in a clocklike manner cannot be rejected. LR = likelihood ratio test statistic; d.f. = degrees of freedom.

	Node	LR statistic	d.f.	P
cob	A	37.43	1	***
	B	128.65	1	***
	C	1.26	1	–
	D	0.48	1	–
cox1	A	58.97	1	***
	B	67.79	1	***
	C	1.54	1	–
	D	0.35	1	–

Table 2.3. Results of Mantel tests. Significant correlations in bold between geographic and genetic (K2P) distances.

Species	*cox1*		*cob*	
	Mantel r	**P**	**Mantel r**	**P**
A. flavescens	–	–	**0.8192**	**0.0001**
A. franciscus	–	–	0.1585	0.1807
A. hoogmoedi	–	–	**0.2221**	**0.0212**
A. barbotini	–	–	-0.0382	0.4733
A. varius	0.1357	0.1519	0.0190	0.3905
C. crassidigitus	**0.5292**	**0.0001**	**0.6495**	**0.0001**
C. fitzingeri	**0.4464**	**0.0001**	**0.5733**	**0.0001**
C. talamancae	0.8043	0.1000	**0.8027**	**0.0024**
D. labialis	**0.4750**	**0.0001**	**0.5736**	**0.0001**
D. sp.	0.0259	0.4154	**0.1966**	**0.0300**
P. ockendeni	–	–	**0.6623**	**0.0001**
P. boiei	–	–	**0.6049**	**0.0001**
P. pustulosus	**0.5177**	**0.0015**	–	–

Table 2.4. Genetic and geographic parameters for each species/gene included in the analysis. All estimations are based on the sampled localities only.

Gene	Species	max gen dist/km	Aver. gen dist/km	S.D. gen dist/km	Rugosity	Aver. elevation (m)	Elevation range (m)
cob	*A. S. barbotini*	0.006192	0.000866	0.001552	1.00013	248.0	101
cob	*A. flavecens*	0.005970	0.001261	0.001372	1.00135	41.3	101
cob	*A. varius*	0.033346	0.001811	0.005035	1.01221	638.9	929
cob	*C. crassidigitus*	0.030504	0.000658	0.001688	1.00768	564.4	1376
cob	*C. fitzingeri*	0.002976	0.000091	0.000307	1.00631	314.7	1075
cob	*A. franciscus*	0.001309	0.001309	0.002896	1.00035	238.8	336
cob	*A. hoogmoedi*	0.008982	0.000540	0.001511	1.00071	268.7	233
cob	*D. labialis*	0.005970	0.000272	0.000718	1.00819	2809.8	1716
cob	*D. sp*	0.011983	0.000368	0.001266	1.01451	2725.9	1239
cob	*P. ockendeni*	0.002976	0.000083	0.000439	1.00058	397.7	393
cob	*C. talamancae*	0.000960	0.000398	0.000262	1.00097	184.8	295
cob	*P. boiei*	0.008982	0.000737	0.001728	1.00144	814.3	462
cox1	*C. crassidigitus*	0.002781	0.000592	0.000538	1.00769	660.6	1150
cox1	*C. fitzingeri*	0.000975	0.000102	0.000117	1.00453	403.9	1289
cox1	*D. labialis*	0.008150	0.000651	0.001254	1.00842	2930.1	1123
cox1	*D. sp*	0.004065	0.000447	0.000758	1.01109	2554.5	838
cox1	*P. pustulosus*	0.002384	0.000147	0.000201	1.00465	183.2	802
cox1	*C. talamancae*	0.000527	0.000268	0.000185	1.00513	186.0	528
cox1	*A. varius*	0.010225	0.000948	0.001933	1.01033	657.6	1086

Table 3.1. Sampling sites, map codes, assigned clade, geographic coordinates, and the number of individuals analyzed for acoustics, morphology, and the phylogenetic analysis. Localities are ordered from north to south. Asterisks indicate samples sequenced earlier for cytb and CO1 in Guarnizo et al. (2009).

Locality	Map code	Clade	Lat N	Long W	Acoustics (# individuals)	Morphology (# individuals)	Phylogeny (# individuals)
Los Suarez, Merida	1	*D. meridensis*	8°38'34.7"	71°22'55.9"	-	-	1
Betania, Tachira	2	*D. pelidna*	7°24'38.2"	72°25'32.2"	-	-	1
Laureles	3	Sympatric	6°24'54.8"	72°26'21.7"	-	-	1
Cocuy	4	Sympatric	6°24'19.3"	72°26'47.6"	-	-	2
Cueva	5	Sympatric	6°24'15.2"	72°22'55.9"	-	-	2
Sativa Norte	6	North	6°08'0.23"	72°42'31.3"	-	2	-
Guina	7	North	6°05'51.9"	72°52'45.8"	-	-	2, 8*
Paramo de la Rusia	8	North	5°59'20.3"	73°05'10.7"	-	3	-
Duitama	9	North	5°49'35.9"	73°02'17.0"	-	5	-
Paipa	10	North	5°46'54.5"	73°08'1.60"	-	3	1, 5*
Arcabuco	11	North	5°45'14.2"	73°26'40.3"	-	-	2, 5*
Manzano	12	North	5°45'0.50"	73°10'40.0"	22	-	2*
Santa Sofia	13	North	5°42'25.5"	73°36'22.4"	-	5	-
Villa de Leyva	14	North	5°38'55.2"	73°31'56.1"	-	-	1, 9*
Combita	15	North	5°38'56.8"	72°26'57.9"	-	5	-
Sutamarchan	16	North	5°37'44.2"	73°36'41.6"	9	-	-
Chiquinquira	17	South	5°36'27.7"	73°46'10.9"	7	-	3, 5*
San Carlos	18	Sympatric	5°35'53.9"	73°43'0.12"	7	-	6*
Cucaita	19	North	5°32'44.9"	73°27'0.36"	25	-	2*
Tunja	20	North	5°32'03.8"	73°22'13.9"	-	5	-
Aquitania	21	North	5°31'3.84"	72°52'50.1"	-	5	-
Paramo de Guerrero	22	South	5°13'1.48"	73°59'34.7"	-	5	-
Choconta	23	North	5°10'16.2"	73°40'5.52"	9	-	1*
Teneria	24	South	5°05'9.80"	73°46'27.1"	8	-	-
Cucunuba	25	South	5°0'36.68"	73°47'44.8"	11	-	3*
La Caro	26	South	4°51'48.9"	74°01'43.6"	-	3	-
Cota	27	South	4°48'34.9"	74°00'36.6"	12	-	2*
Encenillo	28	South	4°47'43.2"	73°54'38.9"	21	-	-
Chingaza	29	South	4°41'25.0"	73°48'23.0"	11	-	2*
La Granja	30	South	4°36'52.2"	74°10'38.1"	-	5	-
Guadalupe	31	South	4°35'44.2"	74° 1'49.7"	-	-	3, 4*
Paramo de Cruz Verde	32	South	4°33'26.0"	74°03'32.7"	-	2	-
Las Brisas	33	South	4°25'58.6"	73°55'13.1"	34	-	1, 8*

Table 3.2. Summary of the principal component analysis on the covariance matrix between acoustic and morphological characters in *Dendropsophus labialis.* All variables are temperature -independent. Only the variables with greater discriminating power (more heavily loaded) are shown.

Acoustics			
	PC1	PC2	
Eigenvalue	2.3251	0.6584	
% of variation	77.500	21.950	
	Factor loads		
Pulses per call	0.6512	-	
pulse rate	-	-0.7926	
Morphology			
	PC1	PC2	PC3
Eigenvalue	2.5124	1.4382	1.1035
% of variation	27.915	15.980	12.261
	Factor loads		
Head width	0.4816	-	-
Eye to nostril dist.	0.4470	-	-
Head length	-	0.5123	-
Foot length	-	-0.5923	-
Eye diameter	-	0.4088	0.5400
Tibia length	-	-	0.4526

Table 3.3. Acoustic and morphological measurements of *D. labialis* and *D. luddeckei*. The sample size for acoustic traits in *D. labialis* was 105, and 66 in *D. luddeckei* . The sample size for morphological traits in *D. labialis* was 15 and 33 in *D. luddeckei*. All morphological traits are in centimeters.

	Dendropsophus labialis			*Dendropsophus luddeckei*		
	Average	SD	Range	Average	SD	Range
Call duration (s)	203.8143	18.4146	178.3 - 231	148.1000	21.1079	122.3 - 178.3
Pulse rate/s	73.6571	5.5193	64.3 - 78.5	89.7400	7.6265	76.9 - 95.1
Pulses per call	14.7429	0.6161	13.6 - 15.2	13.0000	0.9083	11.5 - 13.7
Peak frequency (KHz)	1.7500	0.2193	1.12 - 2.34	2.0600	0.2298	1.65 - 2.44
SVL (cm)	4.6266	0.9631	3.263 - 6.106	3.8693	0.6990	2.64 - 5.00
Tibia length	2.4612	0.9274	1.641 - 5.54	1.9617	0.3360	1.37 - 2.544
Foot length	2.1838	0.5172	1.472 - 3.046	1.8080	0.3802	1.12 - 2.35
Head length	1.3890	0.2554	0.994 - 1.799	1.1969	0.2123	0.85 - 1.80
Head width	1.3709	0.2437	1.026 - 1.79	1.1506	0.1929	0.84 - 1.48
Interorbital distance	1.1807	0.2268	0.884 - 1.58	1.0308	0.1661	0.755 - 1.35
Eyes to nostril distance	0.3628	0.0631	0.289 - 0.47	0.3049	0.0509	0.21 - 0.412
Eye diameter	0.4643	0.0583	0.376 - 0.582	0.3883	0.0466	0.306 - 0.49
Tympanum diameter	0.2830	0.0655	0.215 - 0.413	0.2330	0.0538	0.16 - 0.434
Eye to timpanum distance	0.2838	0.0616	0.198 - 0.401	0.2030	0.0565	0.111 - 0.303

APPENDIX A

Table A1. Species used, Genbank accession numbers and their associated geographic coordinates.

Locus	Species	Accesion number	Lat	Long
cox1	*Atelopus varius*	EF494967	*	*
cox1	*Atelopus varius*	EF494968	*	*
cox1	*Atelopus varius*	EF494969	*	*
cox1	*Atelopus varius*	EF494970	*	*
cox1	*Atelopus varius*	EF494971	*	*
cox1	*Atelopus varius*	EF494972	*	*
cox1	*Atelopus varius*	EF494973	*	*
cox1	*Atelopus varius*	EF494974	*	*
cox1	*Atelopus varius*	EF494975	*	*
cox1	*Atelopus varius*	EF494976	*	*
cox1	*Atelopus varius*	EF494977	*	*
cox1	*Atelopus varius*	EF494978	*	*
cox1	*Atelopus varius*	EF494979	*	*
cox1	*Atelopus varius*	EF494980	*	*
cox1	*Atelopus varius*	EF494981	*	*
cox1	*Atelopus varius*	EF494982	*	*
cox1	*Atelopus varius*	EF494983	*	*
cox1	*Atelopus varius*	EF494984	*	*
cox1	*Atelopus varius*	EF494985	*	*
cox1	*Atelopus varius*	EF494986	*	*
cox1	*Atelopus varius*	EF494987	*	*
cox1	*Atelopus varius*	EF494988	*	*
cox1	*Atelopus varius*	EF494989	*	*
cox1	*Atelopus varius*	EF494990	*	*
cox1	*Atelopus varius*	EF494991	*	*
cox1	*Atelopus varius*	EF494992	*	*
cox1	*Atelopus varius*	EF494993	*	*
cox1	*Atelopus varius*	EF494994	*	*
cox1	*Atelopus varius*	EF494995	*	*
cox1	*Craugastor crassidigitus*	DQ350166	10.04328	–83.54863
cox1	*Craugastor crassidigitus*	DQ350167	9.44005	–83.68966
cox1	*Craugastor crassidigitus*	DQ350168	8.64850	–83.62476
cox1	*Craugastor crassidigitus*	DQ350169	8.70552	–83.52403
cox1	*Craugastor crassidigitus*	DQ350170	8.78333	–82.97582
cox1	*Craugastor crassidigitus*	DQ350171	8.61700	–81.05000
cox1	*Craugastor crassidigitus*	DQ350172	8.66667	–80.59167
cox1	*Craugastor crassidigitus*	DQ350173	8.70000	–79.95000
cox1	*Craugastor crassidigitus*	DQ350174	9.22175	–79.40327
cox1	*Craugastor crassidigitus*	DQ350175	9.31670	–78.98330
cox1	*Craugastor crassidigitus*	DQ350176	10.93167	–85.45972
cox1	*Craugastor crassidigitus*	EF629400	10.04328	–83.54863
cox1	*Craugastor crassidigitus*	EF629401	10.04328	–83.54863
cox1	*Craugastor crassidigitus*	EF629402	10.04328	–83.54863

cox1	*Craugastor crassidigitus*	EF629403	10.00523	–83.53555
cox1	*Craugastor crassidigitus*	EF629404	10.04328	–83.54863
cox1	*Craugastor crassidigitus*	EF629405	10.04328	–83.54863
cox1	*Craugastor crassidigitus*	EF629406	10.04328	–83.54863
cox1	*Craugastor crassidigitus*	EF629407	10.04328	–83.54863
cox1	*Craugastor crassidigitus*	EF629408	10.04328	–83.54863
cox1	*Craugastor crassidigitus*	EF629409	10.21889	–84.44028
cox1	*Craugastor crassidigitus*	EF629410	9.00343	–78.74873
cox1	*Craugastor crassidigitus*	EF629411	8.70000	–79.95000
cox1	*Craugastor crassidigitus*	EF629412	9.29505	–83.76663
cox1	*Craugastor crassidigitus*	EF629413	9.29505	–83.76663
cox1	*Craugastor crassidigitus*	EF629414	8.63337	–80.07830
cox1	*Craugastor crassidigitus*	EF629415	10.30000	–84.70000
cox1	*Craugastor fitzingeri*	DQ350178	14.73333	–85.16667
cox1	*Craugastor fitzingeri*	DQ350179	14.73333	–85.16667
cox1	*Craugastor fitzingeri*	DQ350180	10.23570	–83.56737
cox1	*Craugastor fitzingeri*	DQ350181	10.04328	–83.54863
cox1	*Craugastor fitzingeri*	DQ350182	10.18223	–84.55855
cox1	*Craugastor fitzingeri*	DQ350183	9.88333	–84.28333
cox1	*Craugastor fitzingeri*	DQ350184	9.65000	–85.09167
cox1	*Craugastor fitzingeri*	DQ350185	9.31763	–83.85650
cox1	*Craugastor fitzingeri*	DQ350186	9.31228	–83.77203
cox1	*Craugastor fitzingeri*	DQ350187	8.70600	–83.88717
cox1	*Craugastor fitzingeri*	DQ350188	8.67833	–83.66333
cox1	*Craugastor fitzingeri*	DQ350189	8.64850	–83.62475
cox1	*Craugastor fitzingeri*	DQ350190	8.70552	–83.52403
cox1	*Craugastor fitzingeri*	DQ350191	8.78333	–82.97500
cox1	*Craugastor fitzingeri*	DQ350192	9.00000	–81.75000
cox1	*Craugastor fitzingeri*	DQ350193	8.66667	–80.59167
cox1	*Craugastor fitzingeri*	DQ350194	9.11700	–79.70000
cox1	*Craugastor fitzingeri*	DQ350195	9.11670	–79.70000
cox1	*Craugastor fitzingeri*	DQ350196	9.22175	–79.40327
cox1	*Craugastor fitzingeri*	DQ350197	9.31670	–78.98330
cox1	*Craugastor fitzingeri*	DQ350198	7.63333	–78.18583
cox1	*Craugastor fitzingeri*	EF629416	9.70000	–84.65000
cox1	*Craugastor fitzingeri*	EF629417	10.00523	–83.53555
cox1	*Craugastor fitzingeri*	EF629418	10.21400	–83.71840
cox1	*Craugastor fitzingeri*	EF629419	7.75607	–77.68406
cox1	*Craugastor fitzingeri*	EF629420	7.63333	–78.18583
cox1	*Craugastor fitzingeri*	EF629421	9.07933	–78.79568
cox1	*Craugastor fitzingeri*	EF629422	9.00343	–78.74873
cox1	*Craugastor fitzingeri*	EF629423	9.28022	–79.97542
cox1	*Craugastor fitzingeri*	EF635371	8.60183	–80.14375
cox1	*Craugastor talamancae*	EF629429	10.43030	–84.00700
cox1	*Craugastor talamancae*	EF629430	9.22344	–82.11008
cox1	*Craugastor talamancae*	EF629431	9.22344	–82.11008
cox1	*Craugastor talamancae*	EF629432	9.67323	–83.02412
cox1	*Craugastor talamancae*	EF629435	10.23570	–83.56737
cox1	*Dendropsophus labialis*	EF653827	5.59833	-73.71670
cox1	*Dendropsophus labialis*	EF653827	5.59833	-73.71670

cox1	*Dendropsophus labialis*	EF653827	5.01019	-73.79580
cox1	*Dendropsophus labialis*	EF653827	5.01019	-73.79580
cox1	*Dendropsophus labialis*	EF653823	4.69028	-73.80639
cox1	*Dendropsophus labialis*	EF653824	4.69028	-73.80639
cox1	*Dendropsophus labialis*	EF653822	4.63833	-74.01111
cox1	*Dendropsophus labialis*	EF653825	4.80972	-74.01017
cox1	*Dendropsophus labialis*	EF653823	4.80972	-74.01017
cox1	*Dendropsophus labialis*	EF653828	5.01019	-73.79580
cox1	*Dendropsophus labialis*	EF653823	4.64083	-74.22111
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cox1	*Dendropsophus labialis*	EF653833	4.68344	-74.26361
cox1	*Dendropsophus labialis*	EF653833	4.68344	-74.26361
cox1	*Dendropsophus labialis*	EF653836	5.00850	-73.77720
cox1	*Dendropsophus labialis*	EF653837	5.00850	-73.77720
cox1	*Dendropsophus labialis*	EF653838	5.00850	-73.77720
cox1	*Dendropsophus labialis*	EF653838	5.00850	-73.77720
cox1	*Dendropsophus labialis*	EF653837	5.00850	-73.77720
cox1	*Dendropsophus labialis*	EF653831	4.43295	-73.92033
cox1	*Dendropsophus labialis*	EF653831	4.43295	-73.92033
cox1	*Dendropsophus sp.*	EF653826	5.54583	-73.45010
cox1	*Dendropsophus sp.*	EF653826	5.54583	-73.45010
cox1	*Dendropsophus sp.*	EF653829	5.54444	-73.38353
cox1	*Dendropsophus sp.*	EF653830	5.54444	-73.38353
cox1	*Dendropsophus sp.*	EF653829	5.75014	-73.17780
cox1	*Dendropsophus sp.*	EF653832	5.75014	-73.17780
cox1	*Dendropsophus sp.*	EF653834	5.76194	-73.54278
cox1	*Dendropsophus sp.*	EF653834	5.76194	-73.54278
cox1	*Dendropsophus sp.*	EF653839	5.64867	-73.53225
cox1	*Dendropsophus sp.*	EF653835	5.64867	-73.53225
cox1	*Dendropsophus sp.*	EF653835	5.64867	-73.53225
cox1	*Dendropsophus sp.*	EF653835	5.59833	-73.71670
cox1	*Dendropsophus sp.*	EF653835	5.59833	-73.71670
cox1	*Physalaemus pustulosus*	DQ120018	10.61000	-85.45000
cox1	*Physalaemus pustulosus*	DQ120019	8.27000	-82.86000
cox1	*Physalaemus pustulosus*	DQ120020	8.53000	-82.29000
cox1	*Physalaemus pustulosus*	DQ120021	8.13000	-82.29000
cox1	*Physalaemus pustulosus*	DQ120022	8.40000	-80.24000
cox1	*Physalaemus pustulosus*	DQ120023	9.25000	-79.95000
cox1	*Physalaemus pustulosus*	DQ120024	9.28000	-79.92000
cox1	*Physalaemus pustulosus*	DQ120025	9.17000	-79.85000
cox1	*Physalaemus pustulosus*	DQ120026	9.16000	-79.73000
cox1	*Physalaemus pustulosus*	DQ120027	9.12000	-79.70000
cox1	*Physalaemus pustulosus*	DQ120028	9.11000	-79.69000
cox1	*Physalaemus pustulosus*	DQ120029	9.07000	-79.65000
cox1	*Physalaemus pustulosus*	DQ120030	9.02000	-79.59000
cox1	*Physalaemus pustulosus*	DQ120031	8.97000	-79.59000
cox1	*Physalaemus pustulosus*	DQ120032	8.90000	-79.59000
cox1	*Physalaemus pustulosus*	DQ120033	8.80000	-79.55000
cox1	*Physalaemus pustulosus*	DQ120034	8.45000	-78.85000
cox1	*Physalaemus pustulosus*	DQ120035	8.50000	-77.97000

cox1	*Physalaemus pustulosus*	DQ120036	8.13000	-77.73000
cox1	*Physalaemus pustulosus*	DQ120037	5.18000	-74.90000
cox1	*Physalaemus pustulosus*	DQ120038	8.56000	-71.63000
cox1	*Physalaemus pustulosus*	DQ120039	8.98000	-67.35000
cox1	*Physalaemus pustulosus*	DQ120040	10.64000	-63.22000
cox1	*Physalaemus pustulosus*	DQ120041	10.63000	-61.28000
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cob	*Atelopus flavescens*	AY995959	4.50000	-52.03333
cob	*Atelopus flavescens*	AY995960	4.50000	-52.03333
cob	*Atelopus flavescens*	AY995964	4.48333	-52.03333
cob	*Atelopus flavescens*	AY995965	4.48333	-52.03333
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cob	*Atelopus flavescens*	AY995967	4.48333	-52.03333
cob	*Atelopus flavescens*	AY995968	4.48333	-52.03333
cob	*Atelopus flavescens*	AY995969	4.48333	-52.03333
cob	*Atelopus flavescens*	AY995970	4.48333	-52.03333
cob	*Atelopus flavescens*	AY995971	4.48333	-52.03333
cob	*Atelopus flavescens*	AY995972	4.48333	-52.03333
cob	*Atelopus flavescens*	AY995973	4.48333	-52.03333
cob	*Atelopus flavescens*	AY995974	4.48333	-52.03333
cob	*Atelopus flavescens*	AY995978	4.50000	-52.03333
cob	*Atelopus flavescens*	AY995984	4.21667	-51.85000
cob	*Atelopus flavescens*	AY995985	4.21667	-51.85000
cob	*Atelopus flavescens*	AY995987	4.86667	-52.33333
cob	*Atelopus flavescens*	AY995988	4.86667	-52.33333
cob	*Atelopus franciscus*	AY995956	4.11667	-52.66667
cob	*Atelopus franciscus*	AY995963	4.60000	-53.36667
cob	*Atelopus franciscus*	AY995975	4.11667	-52.66667
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cob	*Atelopus franciscus*	AY995977	4.11667	-52.66667
cob	*Atelopus franciscus*	AY995979	3.76667	-52.33333
cob	*Atelopus franciscus*	AY995980	3.76667	-52.33333
cob	*Atelopus franciscus*	AY995981	4.60000	-53.36667
cob	*Atelopus franciscus*	AY995982	3.75000	-53.03333
cob	*Atelopus franciscus*	AY995983	3.81667	-52.75000
cob	*Atelopus franciscus*	AY995986	3.81667	-52.75000
cob	*Atelopus franciscus*	AY995989	3.81667	-52.75000
cob	*Atelopus franciscus*	AY995990	3.81667	-52.75000
cob	*Atelopus franciscus*	AY995991	3.81667	-52.75000
cob	*Atelopus hoogmoedi*	AY996008	2.26667	-54.53333
cob	*Atelopus hoogmoedi*	AY996009	2.26667	-54.53333
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cob	*Atelopus hoogmoedi*	AY996011	4.86667	-55.21667
cob	*Atelopus hoogmoedi*	AY996012	4.86667	-55.21667
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cob	*Atelopus hoogmoedi*	AY996016	4.86667	-55.21667
cob	*Atelopus hoogmoedi*	AY996017	4.38333	-58.76667
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cob	*Atelopus hoogmoedi*	AY996019	4.38333	-58.76667
cob	*Atelopus hoogmoedi*	AY996020	3.03333	-52.70000
cob	*Atelopus hoogmoedi*	AY996021	3.03333	-52.70000
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cob	*Atelopus hoogmoedi*	AY996029	3.03333	-52.70000
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cob	*Atelopus hoogmoedi*	AY996033	4.38333	-58.76667
cob	*Atelopus hoogmoedi*	AY996034	1.30000	-58.75000
cob	*Atelopus hoogmoedi*	AY996035	2.26667	-54.53333
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cob	*Atelopus barbotini*	AY995996	3.25000	-53.06667
cob	*Atelopus barbotini*	AY995997	3.25000	-53.06667
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cob	*Atelopus varius*	EF494922	*	*
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cob	*Atelopus varius*	EF494947	*	*
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cob	*Craugastor crassidigitus*	DQ350209	–83.54863	10.04328
cob	*Craugastor crassidigitus*	DQ350210	–83.68966	9.44005
cob	*Craugastor crassidigitus*	DQ350211	–83.62476	8.64850
cob	*Craugastor crassidigitus*	DQ350212	–83.52403	8.70552
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cob	*Craugastor crassidigitus*	DQ350215	–80.59167	8.66667
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cob	*Craugastor fitzingeri*	DQ350226	–84.28333	9.88333
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cob	*Craugastor fitzingeri*	DQ350228	–83.85650	9.31763
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cob	*Craugastor fitzingeri*	DQ350230	–83.88717	8.70600
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cob	*Craugastor fitzingeri*	DQ350234	–82.97500	8.78333
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cob	*Craugastor fitzingeri*	DQ350239	–79.40327	9.22175
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cob	*Craugastor fitzingeri*	DQ350241	–78.18583	7.63333
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cob	*Craugastor fitzingeri*	EF629455	–78.18583	7.63333
cob	*Craugastor fitzingeri*	EF629458	–78.79568	9.07933
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cob	*Craugastor fitzingeri*	EF629462	–79.97542	9.28022
cob	*Craugastor talamancae*	DQ350250	–80.59167	8.66667

cob	*Craugastor talamancae*	DQ350251	–79.40327	9.22175
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