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The relative roles of vicariance versus elevational gradients in the genetic differentiation of the high Andean tree frog, *Dendropsophus labialis*

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ABSTRACT

There are two main competing hypotheses (vicariance and vertical ecotones) that attempt to explain the tremendous diversity of the tropical Andes. We test these hypotheses at the intraspecific level by analyzing mitochondrial and nuclear DNA sequences from 24 populations of the high Andean frog, *Dendropsophus labialis* (Anura: Hylidae). This species displays geographic variation in a number of phenotypic traits. Most of these traits covary with elevation, while few vary along the horizontal (latitudinal) axis. We found that, both, vicariance and elevation had important effects on the genetic differentiation in this species. We detected two highly divergent clades along the south–north axis using independent information from mitochondrial and nuclear genes, suggesting that this differentiation was the result of long-term barriers to gene flow rather than stochastic processes. We hypothesize mechanisms for *D. labialis* strong differentiation in light of geological and paleoenvironmental models of evolution in the northern Andean highlands.

1. Introduction

Tropical Andes have the highest level of vertebrate and plant endemism in the world (Duellman, 1999; Myers et al., 2000; Orme et al., 2005). The complex geological and environmental history of the northern Andes is thought to have played a key role in promoting speciation in the region (Lynch, 1999; Willmott et al., 2001; Doan, 2003). Several mechanisms have been invoked to explain the role of Andean orogenesis in stimulating speciation, including vertical speciation across elevation gradients and horizontal speciation between mountains or valleys, (Chapman, 1917; Willmott et al., 2001; Hall, 2005). The relative contribution of processes promoting vertical (altitudinal) versus horizontal (latitudinal or longitudinal) divergence within Andean species is not well understood (Hall, 2005).

Most tropical Andes models of diversification invoke vicariance as the primary mode of differentiation because many sister-species in the Andes are distributed between geographically adjacent areas of similar altitude rather than among different elevations (Patton and Smith, 1992; Lynch et al., 1997; Rull, 2005; Roberts et al., 2006; Brumfield and Edwards, 2007). A different perspective (Endler 1977; Moritz et al., 2000; Smith et al., 2001; Graham et al., 2004; Rull, 2005; Hughes and Eastwood, 2006; Brumfield and Edwards, 2007) proposes that on recently formed mountains (like the northern Andes cordillera), rapid diversification results from ecological gradients and opportunities like the availability of new habitats and the absence of competition. The latter argument assumes that organisms such amphibians would be more heavily differentiated by ecotones because of their restricted dispersal abilities (Rowe et al., 2000; Beebee, 2005) high sensitivity to temperature in early developmental stages (Berven, 1982), adaptations to particular elevations (Lüddecke and Sánchez, 2002; Navas, 2006; Anguilletta et al., 2006; Bonin et al., 2006), and niche conservatism (Wiens et al., 2005).

Under the model of diversification through ecological gradients (the ecotone model), differential adaptation associated with the ecological gradient should lead to parapatric speciation along the Andes vertical axis. The ecotone model of Andean speciation is considered to promote rapid speciation in comparison to allopatric divergence (Orr and Smith, 1998), thus providing a viable explanation for the existence of a high diversity of exclusive Andean species that might have evolved more recently than major vicariance effects (Fjeldså, 1994).

This study is among the first that directly attempts to test the vicariance versus ecotone hypoteses at intraspecific level using molecular data. We used as a model the high Andean frog, *Dendropsophus labialis.* This species has a wide vertical distribution (between 1900 and 4100 m.a.s.l., Ruiz-Carranza et al., 1996; Guarnizo and Escallón, unpublished data) and latitudinal range (from 04°26′N to 08°50′N; IUCN et al., 2004), in the Eastern Andes of Colombia. Although frog species from temperate regions usually have much wider geographical ranges (Kozak and Wiens, 2007),

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D. labialis has an exceptionally high horizontal and vertical distribution. Previous research on *D. labialis* has demonstrated significant phenotypic differentiation among populations along both vertical (body size, reproductive effort, and metabolic rate; Lüddecke, 1997; Amézquita, 1999) and horizontal (call temporal traits; Amézquita, 2001) transects. In fact, based on the substantial differences in body size (highland individuals can be three times bigger than lowland ones), highland populations were previously considered as different sub-species (*Hyla labialis krausi*; Cochran and Goin, 1970). No previous studies have tested whether horizontal and vertical phenotypic differentiation among populations is paralleled by genetic differences.

Here, we use mtDNA and nuclear phylogeographic approaches to assess genetic differences among populations of *D. labialis* representing the altitudinal and latitudinal breadth of the species' distribution. We specifically, investigate how haplotype variation is distributed along elevation and horizontal axes for two mitochondrial (mtDNA) and one nuclear gene. Our prediction under the ecotone model of variation is that the intraspecific tree topology will be correlated with elevation, in other words, sister clades will be found mostly at non-overlapping elevational ranges (Patton and Smith, 1992; Hall, 2005). In contrast, under the vicariance model we expect independence between the tree topology and elevation, or, most sister clades found at overlapping elevational ranges (Fig. 1). Finally, we characterize the phylogeographic pattern and history of diversification in *D. labialis,* and interpret this species' history in light of geological and paleoenvironmental models of evolution in the northern Andean highlands.

2. Materials and methods

2.1. Sampling

Dendropsophus labialis tissues were obtained by clipping the toe of adult frogs or the tail tips of larvae. We sampled 80 individuals from 24 localities representing most of the geographic distribution of this species (Table 1; Fig. 2). Samples were collected taking care of covering different elevations that range from 1970 to 3550 m.a.s.l. Tissues were preserved in 97% ethanol and one individual from each of four different localities was collected as a voucher specimen. The vouchers were deposited in the Universidad de Los Andes biological collection (Bogotá, Colombia) with Accession Nos.: UA001, UA006, UA009, UA010. Geographic coordinates for each site were recorded using GPS (Table 1). The mitochondrial Cytochrome *b* gene (cyt *b*) was sequenced for all 24 localities (79 individuals), whereas the Cytochrome Oxidase 1 gene (COI) was sequenced for 13 localities (43 individuals). In order to obtain an independent source of information we also sequenced the nuclear gene proopiomelanocortin A (POMC) for a subset of seven localities (48 individuals) (Table 1).

Table 1
Sampling sites, geographic coordinates, elevation, and number of individuals/loci sequenced for *Dendropsophus labialis* in this study. CO1: Cytochrome oxidase 1, cyt *b*: Cytochrome b, POMC: nuclear proopiomelanocortin A gene. Voucher specimens were collected from the populations indicated with asterisks and deposited at Universidad de los Andes (Bogotá, Colombia). Localities are ordered from north to south.

Locality	Code	Coordinates (Lat N, Long W)	Elevation (m)	# of individuals sequenced
Guina	A	06.05.51, 72.52.45	3311	8(cyt *b*), 6(POMC)
Paipa	B	05.44.51, 73.08.01	2563	5(cyt *b*), 4(POMC)
Manzano	C	05.45.05, 73.10.40	2577	2(cyt *b*), 2(CO1)
Periquera	D	05.45.43, 73.32.34	2450	2(cyt *b*), 2(CO1)
Arcabuco	E	05.36.31, 73.20.53	3108	5(cyt *b*), 5(POMC)
Villa de Leyva*	F	05.38.55, 73.31.56	2170	7(cyt *b*), 3(CO1). 9(POMC)
Chiquinquira	G	05.36.28, 73.46.11	2610	5(cyt *b*), 5(POMC)
Caldas	H	05.32.98, 73.49.58	2650	1(cyt *b*)
San Carlos	I	05.35.54, 73.43.02	2590	6(cyt *b*), 4(CO1)
Cucaita	J	05.32.45, 73.27.03	2688	2(cyt *b*), 2(CO1)
El Carmen	K	05.32.40, 73.23.07	3000	2(cyt *b*), 2(CO1)
Chorroblanco	L	05.30.15, 73.23.20	3090	1(cyt *b*)
El Roble	M	05.25.37, 73.45.34	2585	2(cyt *b*), 1(CO1)
Ventaquemada	N	05.21.03, 73.33.23	3085	2(cyt *b*)
Choconta	0	05.10.16, 73.40.05	2670	1(cyt *b*)
Suesca	P	05.05.06, 73.46.38	2678	5(cyt *b*), 5(CO1)
Cucunuba	Q	05.06.07, 73.47.45	2592	3(cyt *b*), 3(CO1)
Cota*	R	04.48.35, 74.06.06	2600	2(cyt *b*), 2(CO1)
Chingaza*	S	04.41.25, 73.48.23	3550	2(cyt *b*), 2(CO1)
Mondonedo	T	04.41.04, 74.15.49	2600	2(cyt *b*), 1(CO1)
La Calera	U	04.38.18, 74.00.40	2833	1(cyt *b*), 1(CO1)
Las Juntas	V	04.38.27, 74.13.16	2650	2(cyt *b*), 2(CO1)
Guadalupe	W	04.35.44, 74.01.50	3298	4(cyt *b*), 6(POMC)
Las Brisas	X	04.26.12, 73.55.10	1970	7(cyt *b*), 8(POMC)

* Localities with voucher specimens.

2.2. PCR and sequencing methods

Total genomic DNA was extracted using the standard phenol-chloroform method (Sambrook et al., 1989). The three DNA fragments were amplified using published primers for COI (COIa: 5′-AGTATAAGCGTCTGGGTAGTC-3′, COIf: 5′-CCTGCAGGAGGAGGAGAYCC-3′; Palumbi et al., 1991), cyt *b* (Cb1: 5′-CCATCCAACATCTCAGCATGATGAA-3′, Cb3: 5′-GGCAAATAGGAARTATCATTC-3′; Palumbi et al., 1991), and POMC (PomcR1: 5′- GGCRTTYTTGAAWAGAGTCATTAGWGG-3′, PomcF1: 5′:ATATGTCATGASCCAYTTYCGCTGGAA3′; Vieites et al., 2007). Amplifications were performed with a reaction mix containing: 2.5 μl of 10× PCR buffer (500 mM KCl, 100 mM Tris–HCl, pH 8.5), 2.5 μl of 8 μM dNTP's, 13.875 μl of dH_2O (Sigma), 1.0 μl of $MgCl_2$, 0.125 μl of Qiagen DNA polymerase, 1.25 μl of each primer (10 μM), and 2.5 μL of the DNA extract for a final volume of

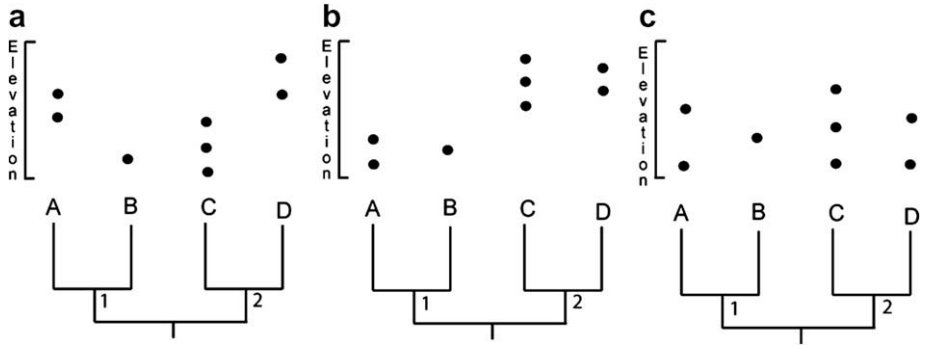

Fig. 1. Hypothetical representation of the effect of elevation on intraspecific phylogenies. (a) Elevation stimulates divergence *within* 1 and 2. (b) Elevation stimulates divergence *between* 1 and 2. (c) Scenario where the ecotone model of differentiation is not supported (absence of correlation between elevation and tree topology). Given that this is an intraspecific phylogeny, some tips might be associated to more that one elevation (in the cases where individuals from different elevations share the same haplotype).

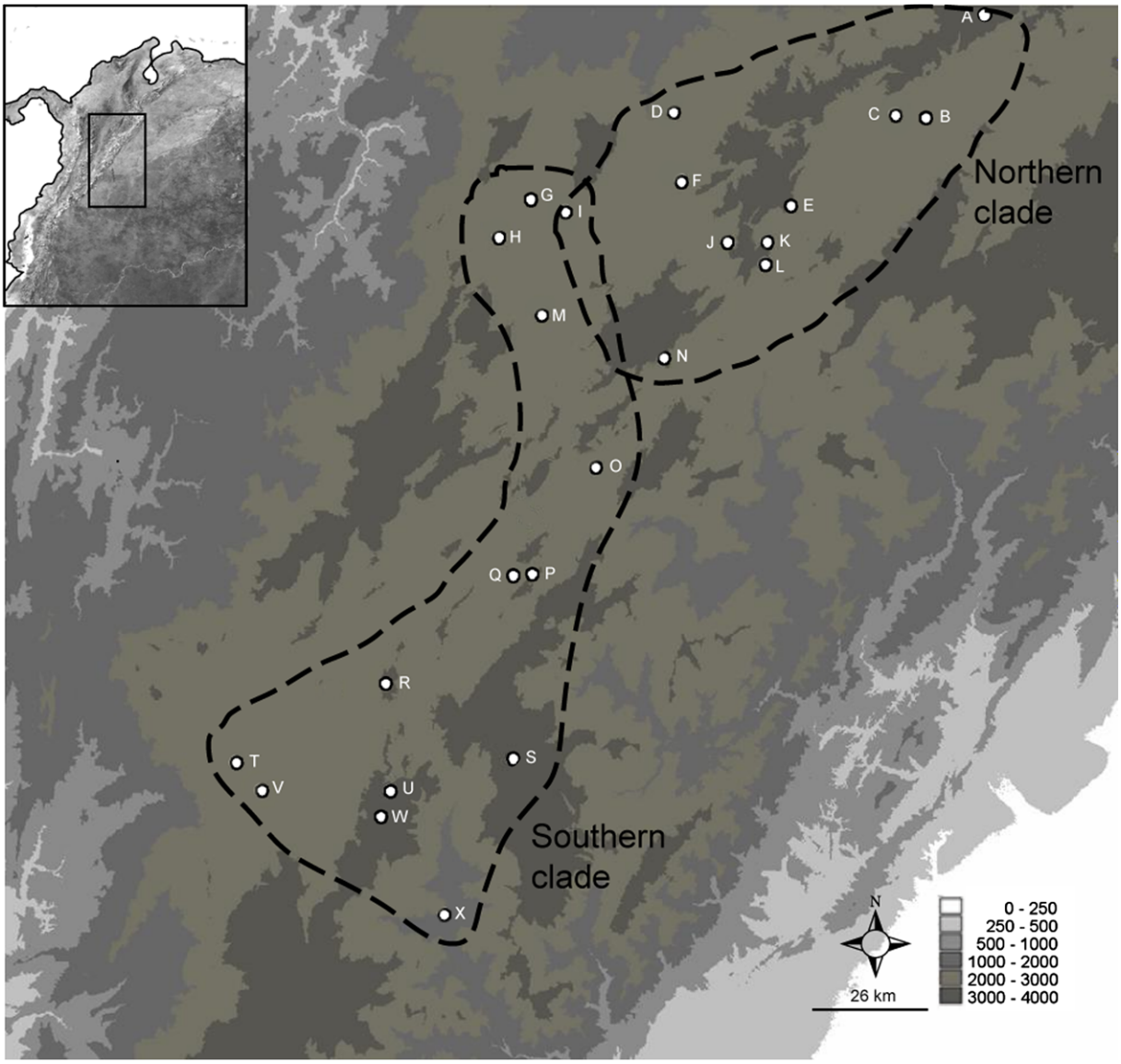

Fig. 2. Geographic distribution of *Dendropsophus labialis* samples along the eastern Cordillera of Colombia. The elevation range of the sampling sites goes from 1970 to 3550 m.a.s.l. The dotted lines delineate the sampling sites that belong to the northern and southern clades shown in Fig. 3.

25 μL. PCR cycles for COI, cyt *b*, and POMC involved a touchdown procedure and included an initial denaturing step at 96 °C for 6 min, then five amplification cycles (45 s at 96 °C, 45 s at 50 °C, 1:30 s at 72 °C), then 29 cycles (45 s at 96 °C, 45 s at 56 °C, 1:30 s at 72 °C), and a final extension of 72 °C for 6 min. PCR products were purified by cutting bands from low melting point agarose gels and treating them with Gelase.

Cycle sequencing was performed using PCR primers. Sequencing products were analyzed on a MJ Research Genescan or ABI 3100 automated sequencer following manufacturer's protocols. Each fragment was sequenced in both directions to confirm base calls. Nucleotide sequences were aligned using Sequencher 3.1 (Gene Codes Corporation Inc., 1998), and edited by eye. Protein-coding sequences were translated to amino acid sequences using MacClade 4.0 (Maddison and Maddison, 1992) to check for premature stop codons or mis-sense mutations.

2.3. Molecular analysis

Phylogenetic trees were constructed using Maximum Parsimony (MP), Maximum Likelihood (ML), and Bayesian analyses. MP trees were obtained by stepwise addition with the tree bisection–reconnection (TBR) branch-swapping algorithm. Equal character weights were used in heuristic searches using 10,000 random addition sequence replicates. Support was assessed using the nonparametric bootstrap with 1000 pseudo-replicates. The Akaike information criterion was used in MODELTEST v. 3.6 (Posada and Crandall, 1998) to identify the optimal model for ML and Bayesian analysis (we made an independent analysis for each gene partition). ML trees were constructed with TBR branch swapping in PAUP*4.0b2. (Swofford, 1999). For the Bayesian phylogenetic analysis we used MrBayes v. 3.1.2 (Huelsenbeck and Ronquist 2001). We made two independent runs of 1000,000 generations implementing Metropolis-coupled Markov chain Monte Carlo (MCMC), each with four incrementally heated Markov chains, sampling every 100 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.

We rooted the trees using *D. labialis* more closely related species that have already been sequenced for cyt *b* and POMC. For the cyt *b* tree we used *Dendropsophus carnifex* (GenBank Accession No.

AY843842) and for the POMC tree we used *Dendropsophus ebraccata* (Accession No. AY819117).

In order to determine if the mitochondrial genes CO1 and cyt *b* provided significantly different tree topologies, we evaluated first their potential phylogenetic heterogeneity using the partition homogeneity test (Farris et al., 1995) in PAUP*. A parsimony network analysis (Posada and Crandall, 1998) was implemented to visually inspect if there was an association between genetic variation and geography. Networks were calculated with the software TCS 1.13 (Clement et al., 2000), using the method of Templeton (1992). Parsimony connections were statistically justified ($P \geqslant 0.95$) for haplotype divergences of less than 14 mutational differences. Because of the diploid nature of nuclear DNA, to calculate the POMC parsimony network we previously determined the two alleles in heterozygous individuals using the PHASE algorithm (Stephens et al., 2001), under the default settings of the program DNAsp 4.5 (Rozas and Rozas, 1999).

We conducted a non-parametric partial Mantel test to determine if there was a correlation between geographic and genetic distances. Mantel tests were performed with the software The R Package (Legendre and Vaudor, 1991) using 10,000 permutations on each analysis. Geographic distances between GPS points were calculated with the software The R Package (Legendre and Vaudor, 1991).

To determine if the phylogeny correctly predicted the patterns of covariance among individuals on a given elevation, we calculated the phylogenetic scaling parameter lambda (λ), defined by Pagel (1999). A value of $\lambda = 0$ indicates that the evolution of the trait (in this case elevation) is independent of phylogeny, while a value of $\lambda = 1$ indicates that the trait (elevation) is evolving according to Brownian motion on the given phylogeny. λ parameters were fitted by maximum likelihood using the program Continuous (Pagel 2000). A likelihood ratio test (LRT) was used to compare between a model where λ was constrained to 1 and a model where λ was allowed to take its ML value. If the LRT test is significant it indicates that lambda <1.0 (the elevation is not correlated with the phylogeny). Because Continuous only receives bifurcating trees, some individuals were excluded of the analysis when polytomies were present (trying to maximize the number of elevations compared, see Fig. 4). Whenever, a single tip of the phylogeny contained individuals from different elevations, these were averaged.

To calculate molecular diversity we used (π) per site, which measures heterozygosity based on the average number of differences between all pairs of *n* sequences (Nei, 1987). To measure the level of population genetic structure we calculated the fixation index, Fst, corrected for DNA sequences (Hudson et al., 1992) among the groups obtained in the phylogenetic analysis. All parameters were calculated with DNAsp 4.5 (Rozas and Rozas, 1999). We used the haplotype determination algorithm of Stephens et al. (2001), to avoid heterozygous ambiguity codes in the calculation of POMC nucleotide diversity and Fst values.

To determine intraspecific divergence times, we first made a LRT to evaluate if the cyt *b* dataset evolved under a molecular clock assumption (Felsenstein, 1981). Divergence times using genetic distances were calculated using a fast frog mitochondrial rate of evolution (0.0191 total divergence per My; Crawford, 2003), and a slow rate (0.0069 total divergence per My; Macey et al., 1998). The uncorrected (*p*-distance) genetic distances and twice their standard error (calculated with the program MEGA 4; Tamura et al., 2007) were divided by the fast and slow rates to obtain a maximum–minimum divergence time estimate.

3. Results

We recovered 43 unique haplotypes among 80 individuals for the cyt *b* gene (620 bp), 18 unique haplotypes among 34 individuals for the COI gene fragment (505 bp), and 17 unique haplotypes among 44 individuals for POMC (413 bp) (see Appendix for information on individuals identity and GenBank accession numbers).

3.1. Phylogeographic pattern

All inference methods using the mitochondrial data (either cyt *b*, CO1 or the combined dataset) produced consistent results (the partition homogeneity test indicated that cyt *b* and CO1 could be combined; $P = 0.240$). The model of substitution recommended by MODELTEST for each gene partition was HKY85 + G (Hasegawa et al., 1985; Yang, 1993). Two main groups were formed with the mitochondrial and nuclear data (Fig. 3a): the "northern" and "southern" clades. The northern clade is composed of haplotypes from the Northeast part of the distributional range of *D. labialis*, while the Southern clade corresponds to the Southwest populations, near Bogotá D.C. Within the southern clade the mitochondrial data formed a well-supported sub-clade we call the central clade. The Northern and Southern clades are highly differentiated, with corresponding Fst values of 0.93 (northern vs. southern), 0.94 (central vs. northern), and 0.84 (southern vs. central). The differentiation between the northern and southern clades with the nuclear gene (POMC) was lower, with an Fst of 0.53.

Three non-connected haplotype networks within the northern clade and three within the southern clade were recovered by TCS using the cyt *b* information (Fig. 3b). POMC reflected its low nucleotide variation with a single network that separates the northern and southern clades by a single nucleotide step. Within each clade, the mitochondrial haplotype diversity is similar, being slightly higher in the southern division (northern $\pi = 0.0033$, central $\pi = 0.00315$, southern $\pi = 0.0088$). POMC also reflected similar nucleotide diversities within each clade (northern $\pi = 0.0038$, southern $\pi = 0.0040$). At the intraclade level, it seems that the geographic distribution of haplotypes is not concordant with the network topology. In other words, the geographically closest individuals are not always most genetically similar. For example, Fig. 3b shows how individuals can share identical DNA sequences in spite of being from localities far away from each other, or conversely, display very differentiated haplotypes within a single locality. At the interclade level, however, for the mitochondrial and the nuclear gene the geographic distribution of haplotypes was concordant with the north–south classification, with the exception of the individuals collected from San Carlos (locality I), where haplotypes from the northern and southern clades co-occur.

The Mantel correlation test using the mitochondrial dataset detected a positive correlation between geographic and genetic distances ($r = 0.5818$, $P < 0.001$). Within each clade, however, we did not detect a significant correlation between geographic and genetic distances (northern clade: $r = -0.0994$, $P = 0.469$; southern clade: $r = -0.047$, $P = 0.502$). In other words, isolation by distance was supported between but not within clades. We did not use POMC for the Mantel tests because there was to little nucleotide variation present.

The phylogenetic scaling parameter (λ) was not significantly different from 1.0 in the northern clade ($\lambda = 1$, $P = 1.0$), and the southern clade ($\lambda = 0$, $P = 0.090$), indicating that elevation and the cyt *b* tree topology are correlated. This suggests that elevation might have an effect on the intraspecific differentiation within the northern and the southern clades.

The LRT did not reject the molecular clock hypothesis ($\chi^2 = 2(2268.10–2251.76) = 32.66$; d.f. = 33, $P > 0.05$). The divergence time between the northern and southern clades using the slow rate of mitochondrial evolution (0.69% total divergence per My) ranged from 5.5 to 10.14 My (late Miocene). In contrast, the divergence time using the fast rate (1.9% total divergence per

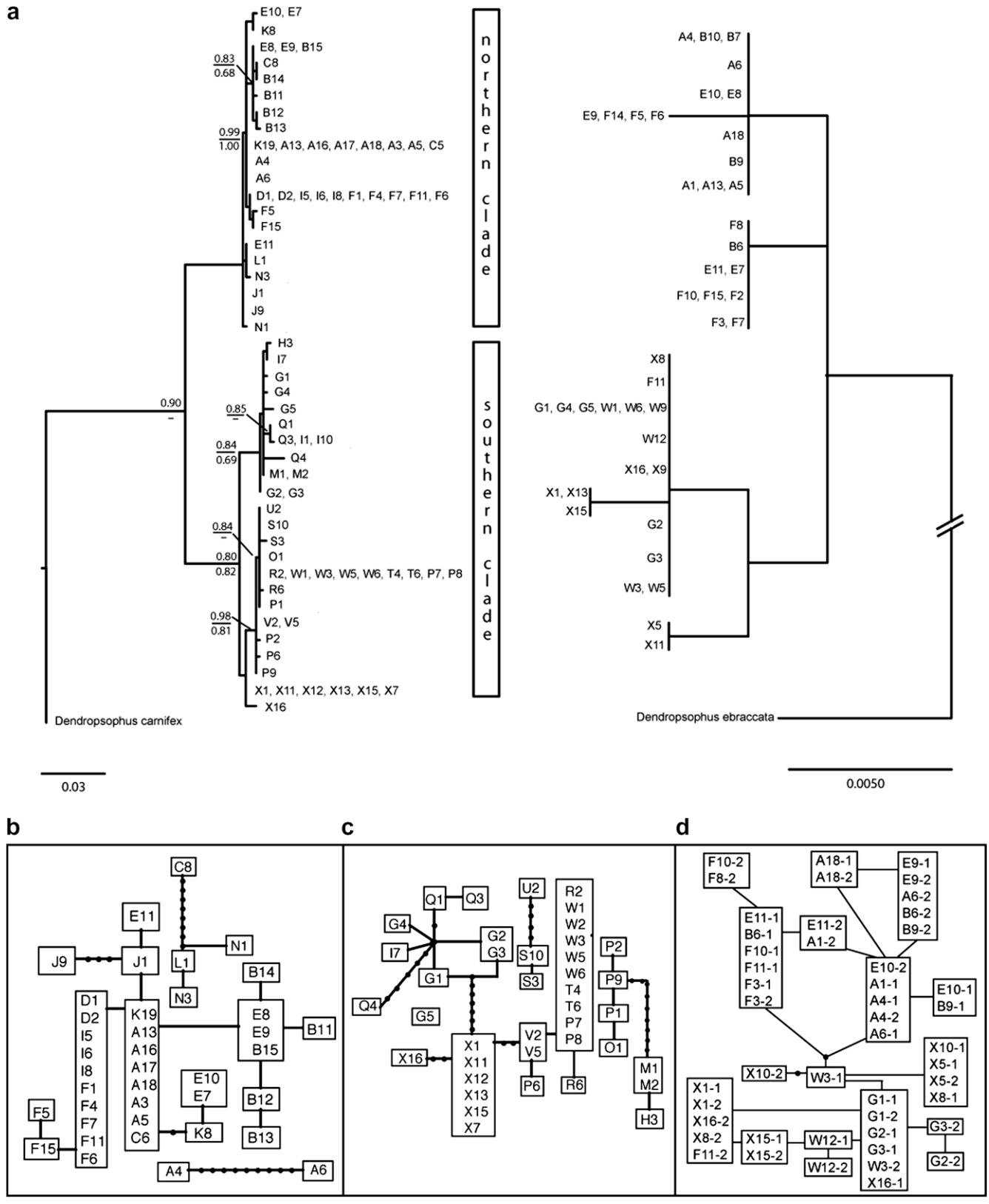

Fig. 3. (a) Maximum likelihood *Dendropsophus labialis* gene trees based on data from *left:* cyt *b*, and *right*: POMC genes. Numbers on branches correspond to Bayesian posterior probabilities/Maximum likelihood bootstrap values (only values above 65% are displayed). The sampling sites codes are in Table 1. *Below*: parsimony networks from (b) northern clade (cyt *b*), (c) southern clade (cyt *b*), (d) POMC. Open rectangles indicate haplotypes observed. Black dots correspond to hypothetical intermediate haplotypes not observed. Each connecting line corresponds to a single nucleotide change. POMC network was reconstructed using statistical haplotype determination (see text).

My; Crawford 2003) ranged from 1.9 to 3.6 My (Pliocene). In order to be conservative (acknowledging all the potential problems derived from the absence of calibration points for this species, Arbogast et al. 2002), we will assume that the northern vs. southern

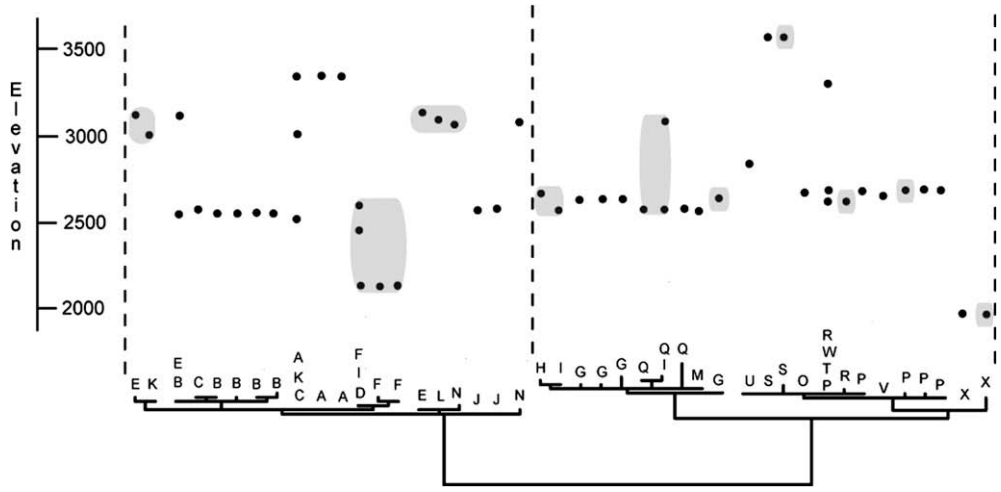

Fig. 4. Association between *Dendropsophus labialis* elevation and the cyt *b* tree topology. Dotted line delineates the northern and southern clades. Shaded regions indicate the sampling sites used for the phylogeny-elevation correlation test (see text for explanation).

clade divergence time ranged from the oldest age obtained under the slow rate, to the youngest age under the fast rate: 1.9–10.4 My.

4. Discussion

4.1. The role of vicariance

We found two highly divergent clades distributed along the north–south axis of the eastern Cordillera of Colombia. This supports the predominant effect of vicariance on the differentiation of *D. labialis*. The high sequence divergence and Fst values between northern and southern populations (compared to other frogs with greater distributional ranges; Funk et al., 2007; Austin and Zamudio, 2008) suggest that they have been under reproductive isolation. This pattern agrees with Avise's (2000) phylogeographic category I, which states that major genetic discontinuities that display a geographic component result from long-term extrinsic barriers to gene flow. Irwin (2002) concluded that deep phylogeographic breaks could be formed within a continuously distributed species even in the absence of barriers to gene flow. Nevertheless, the fact that we consistently obtain the two major clades with data from two mitochondrial fragments and an independent nuclear gene, suggests that both clades are the result of reproductive isolation and not a byproduct of stochastic processes.

Our data suggests that the divergence time between the northern and southern clades range from the late Pliocene (1.9 My) to the late Miocene (10.4 My). The abundant paleobotanical data from the Eastern Cordillera of Colombia indicate that in the middle Miocene through early Pliocene, elevations were fairly low, no more than 40% of their modern values (Gregory-Wodzicki, 2000; Garzione et al., 2008, Fig. 5a). Elevations then increased rapidly between 2 and 5 Ma, at rates on the order of 0.5–3 mm/yr, reaching modern elevations by around 2.7 My (Gregory-Wodzicki 2000). We hypothesize (based on our divergence time estimates) that the strong differentiation between *D. labialis* clades occurred as a result of long-term isolation between populations located in the top of the mountains during the Late Miocene and early Pliocene.

We suggest the mechanism of differentiation was as follows: During the late Miocene the Northern Andes elevation remained relatively stable, it was approximately 40% shorter, and on average 10 °C warmer (assuming a rate of 0.66 °C per 100 m; van der Hammen and Gonzalez, 1963). Therefore, only the peak elevations of that time might get cold enough to reach the warmer temperatures in which *D. labialis* currently occurs. If *D. labialis* was limited to high mountains during the Miocene, then these mountains might have resembled islands, isolating populations for millions years (Fig. 5a). Afterwards (during the early Pliocene), the Northern Andes started to rise at a very fast rate. As the mountains uplifted they became colder (Garzione et al. 2008), deleting the thermal geographic barrier previously present, and stimulating population range expansion and secondary contact (Fig. 5b).

Ancient long-term isolation between habitats at mountain peaks would explain why we observe deep divergences in *D. labialis* in the absence of geographic barriers to gene flow today. Our observations support the hypothesis that divergence among most extant taxa in tropical mountains predated the recent Pleistocene alternation of glacial and interglacial climates (Moritz et al., 2000). It still under debate, however, exactly when most of the tremendous diversification of the Northern Andes occurred (Roy et al., 1997; García-Moreno et al., 1999; Chesser, 2000; Koscinski et al., 2008; Rull, 2006).

If our data agrees with this hypothesis, then we would find *D. labialis* major clades in close geographic association to current peak Andean elevations. The examination of the topography of the Eastern Andes of Colombia confirms our hypothesis, finding a close correspondence between the geographic distribution of *D. labialis* clades and the major regions above 3800 m today (Fig. 5c). Furthermore, the sister species of *D. labialis* (*D. pelidna* and *D. meridensis*), have geographic distributions very close the other two major regions above 3800 m in the eastern Andes: Paramo del Cocuy in Colombia (6°24′N) and the paramos of Mérida in Venezuela (8°51′N; IUCN et al., 2004).

Our biogeographic hypothesis makes a fundamental prediction regarding the geographic distribution of major intraspecific clades in high elevation species. We expect that major clades within high Andean species will be geographically close (but not restricted to), peak elevation mountains that behaved as isolated cold habitats before the fast Andes uplift. The correlation of the geographic distribution of the major clades and maximum peaks in elevation does not provide causality, so it is fundamental to sample more cold-adapted populations of high-Andean species in order to obtain comparative phylogeographic data that support/reject our hypothesis.

4.2. Intraclade level variation

Within each clade we detected an effect of geography on the genetic structure. For example, a continuous ridge at least 2500 m

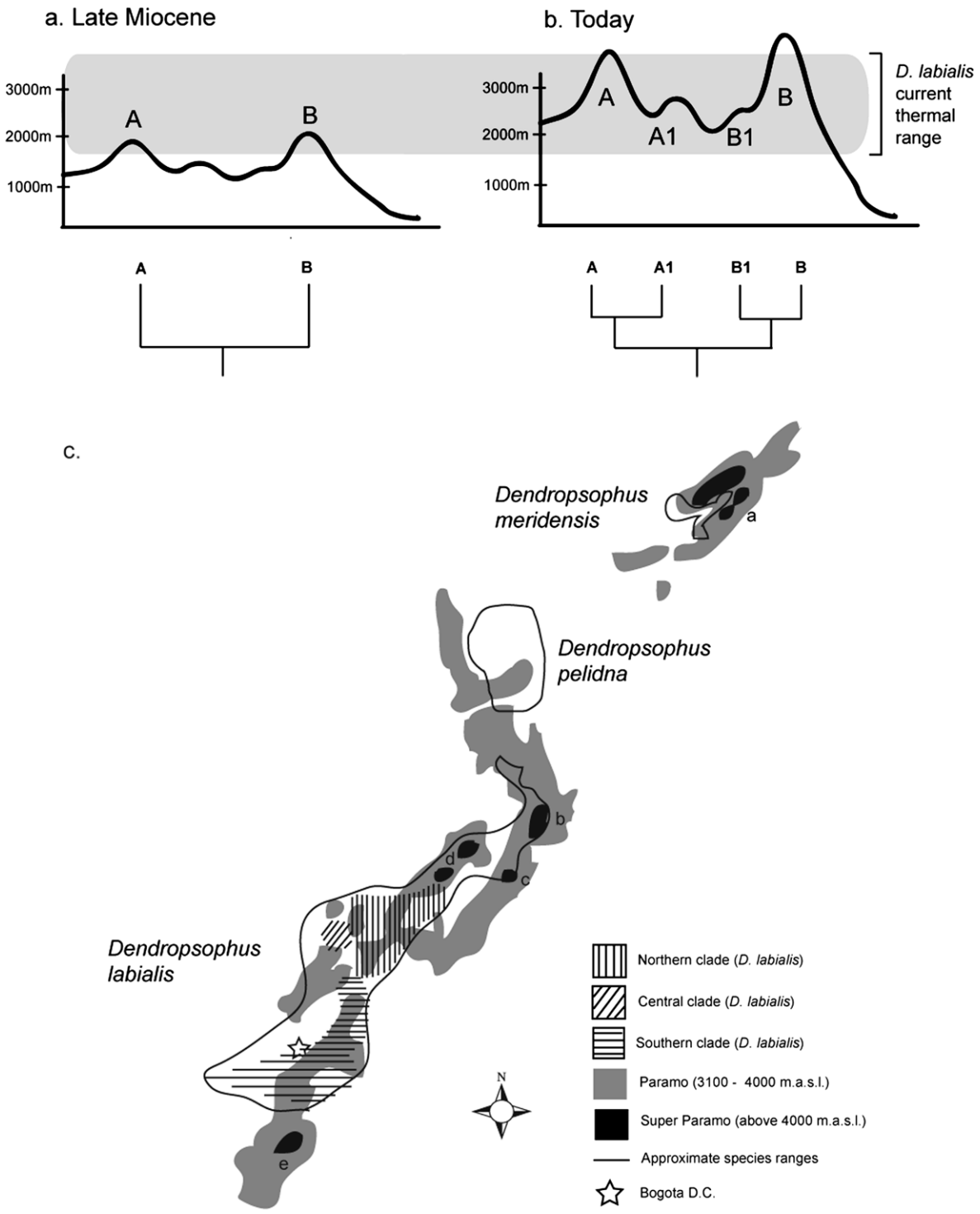

Fig. 5. (a) Hypothetical profile of the eastern Cordillera of Colombia during the Late Miocene when average elevation was no more than 40% of the modern values (Gregory-Wodzicki 2000). *D. labialis* cold-adapted populations might have become isolated on the top of the highest mountains. (b) Hypothetical profile of the eastern Cordillera of Colombia during the Pliocene (between 2 and 5 My). The fast Andean uplift might have stimulated rage expansion and secondary contact. (c) Profile of the high elevation regions in the Eastern Colombian Andes and Venezuelan Andes. The three sister species and the three clades within *D. labialis* are geographically close (but not restricted) to the peak elevations (gray and black areas). (a) Paramo of Merida, (b) Paramo of Cocuy, (c) Paramo of Pisba, (d) Paramo of Guativa. (e) Paramo of Sumapaz.

high that separates populations from opposite slopes of the eastern cordillera seems to be affecting the southern clade genetic variation. The two mitochondrial and the nuclear gene support the strong differentiation of the single locality from the eastern slope (locality las Brisas, code X), against all the other samples from the western slope. This seems to indicate that the ridge restricts dispersal from one slope to the other (a similar effect was found in the frog *Colostheus palmatus*, which is sympatric to *D. labialis* in many localities, including Las Brisas; Bernal et al., 2005).

Our data do not support isolation by distance at the intraclade level in spite of the high genetic variability observed. We were expecting strong isolation by distance because (a) multiple studies suggest that in general, frog adults rarely move distances more than a few km (Berven and Grudzien, 1990; Gulve, 1994; Monsen

and Blouin, 2004). And (b) montane habitats, in particular, appear to promote very strong genetic isolation among frog populations (Monsen and Blouin, 2003; Funk et al., 2005). The absence of isolation by distance might be explained if *D. labialis* had high dispersal abilities that restricted continuous differentiation along geographic distance. The positive correlation observed between geographic and genetic distances using the complete dataset we assume is an artifact of analyzing two very divergent clades that happen to be in a linear formation.

Another interesting observation at the intraclade level is that only one population (San Carlos; code D) contained haplotypes from the two main clades. This population might correspond to a secondary contact zone between the two main clades. Future research should be focused on the study of this and other intermediate geographic regions in order to analyze allele frequencies, and discontinuous trends in vocalizations and mate choice.

4.3. The role of ecotones

Within each clade we found a correlation between elevation and the cyt *b* phylogeny (phylogenetic scaling parameter $\lambda = 1$) and also the presence of some sister clades at non-overlapping elevations (Fig. 4). Although this result could be interpreted as an evidence for ecotonal (adaptive) variation along the elevation gradient, we suggest that it reflects more a vicariant mechanism among regions that became elevationally differentiated after the very dynamic uplifting process of the Northern Andes (Garzione et al., 2008). Ecotonal differentiation might have been supported in this species with a phylogeny displaying deeper divergences between elevationally parapatric sister clades (such as Fig. 1a and b).

Ecotonal divergence might appear in the morphological and life history characters before its seen in neutral genetic markers (Smith et al. 2001). Therefore, the strong (and presumably adaptive) elevation-dependent phenotypic variation found in *D. labialis* might not be detected at the molecular level, unless candidate genes under direct elevation-dependent selection are used. Bonin et al. (2006) performed a genome-wide survey to reveal selection signatures along an elevation gradient in frogs. They found few loci that were presumably adapted to elevation. Future research should be focused on the mechanism and rates of diversification between phenotypic traits and candidate genes under selection. Additionally, independent datasets would be needed to test for isolating mechanisms between elevationally parapatric populations, such as call variation, female phonotaxis experiments, and population crosses. That approach would give us a fine grain perspective on the effect of ecotones and vicariant processes on the intraspecific diversification of mountain regions.

The analysis of the elevational distribution of the genus Dendropsophus indicates that *D. labialis* is the species that reaches highest elevations, followed by *D. pelidna, D. meridensis*, and *D. carnifex* (IUCN et al., 2004). The other species that belong to this genus have elevational ranges under 1500 m (based on the phylogeny of Wiens et al. 2005). This observation indicates elevation might have played an important role in the diversification of the montane representatives of the Dendropsophus genus.

This is just one of the initial attempts to understand the effect of the Andes on the mitochondrial and nuclear genetic structure of anuran populations; Many other Andean species with wide altitudinal ranges most be analyzed, integrating the information from other disciplines such as geology and palynology to get a general perspective on the complex orogenic history of the region. The upward expansion of anurans and other organisms to newly available habitats as a consequence recent climate change (Seimon et al., 2007), the upward range extension of the chytridiomycosis disease that is now infecting frogs in most of their elevational range (Seimon et al., 2007), and the fact that amphibian extinctions (and all the organisms that share the same montane ecosystems) are increasing especially at high elevations (Corn and Fogleman, 1984; Lips, 1998; Stuart et al., 2004; Pounds et al., 2006; Bosch et al., 2007; Seimon et al., 2007), makes phylogeographic, population genetic, and natural history studies on montane species a high priority.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2008.10.005.

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