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CONGRUENCE BETWEEN ACOUSTIC TRAITS AND GENEALOGICAL HISTORY REVEALS A NEW SPECIES OF *DENDROPSOPHUS* (ANURA: HYLIDAE) IN THE HIGH ANDES OF COLOMBIA

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ABSTRACT: The High-Andean Frog (*Dendropsophus labialis*) is distributed along the Eastern Andes of Colombia between 1900 and 3600 m. We analyzed multiple traits to determine if acoustics and morphology covary with genealogical history or if they evolved following independent trajectories. We generated a phylogeny of *D. labialis* populations with the use of mitochondrial ribosomal gene (12S, val-RNA, 16S) and nuclear gene (proopiomelanocortin A [POMC]) sequences. Two well-supported clades that correspond to geographic distribution were recovered. Acoustic variation diverges according to genealogical history, but external morphology does not follow this pattern. The strong and congruent divergence observed in acoustic and genetic characters indicates that these two clades correspond to morphologically cryptic, parapatric species. We describe the Northern Clade as a new species (*Dendropsophus luddeckei*; the Southern Clade retains the name *D. labialis*). The low level of molecular divergence between two outgroup species, *Dendropsophus pelidna* and *Dendropsophus meridensis*, suggests *D. pelidna* is a junior subjective synonym of *D. meridensis*.

Key words: Acoustic divergence; *Dendropsophus*; *labialis* group; Morphological variation; New species; Tropical Andes

THE NEOTROPICS are characterized by high endemism (Myers et al., 2000; Orme et al., 2005). Historically, most tropical species have been described as morphospecies. Inferring species boundaries by morphology alone (or any phenotypic trait) is problematic when genealogical history and that trait do not evolve in a coupled fashion, as may happen when selection breaks correlation patterns between phenotypic traits (Lougheed et al., 2006). Even if phenotypes evolve under neutral processes, problems with species delimitation still may occur if the phenotypic trait has not fully diverged after a speciation event (de Queiroz, 2007). As a result, the number of morphospecies will tend to underestimate species diversity based on molecular data.

We used the High-Andean Frog *Dendropsophus labialis* (Peters, 1863) to contrast patterns of evolution of multiple characters (mitochondrial DNA, nuclear DNA, acoustics, and morphology) following recent divergence, and to test for the possible existence of cryptic species.

The nominal species *D. labialis* is distributed along the Eastern Andes of Colombia between 3.5 and 6.3°N and 1900 and 3600 m in elevation (Ruiz-Carranza et al., 1996). Mitochondrial and nuclear DNA sequences indicate two strongly differentiated clades with parapatric distribution within *D. labialis* (Guarnizo et al., 2009). However, taxonomic analyses have treated these populations 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 Southern Clade (Amézquita, 1999; Lüddecke, 2002; Lüddecke and Sánchez, 2002), no studies have compared the phenotypic variation between the Northern and Southern Clades.

We conducted a multitrait analysis to determine whether acoustics and morphology codiverged according to genealogical history. We also tested if the climatic characteristics associated with each clade were statistically different. These analyses allowed us to deter-

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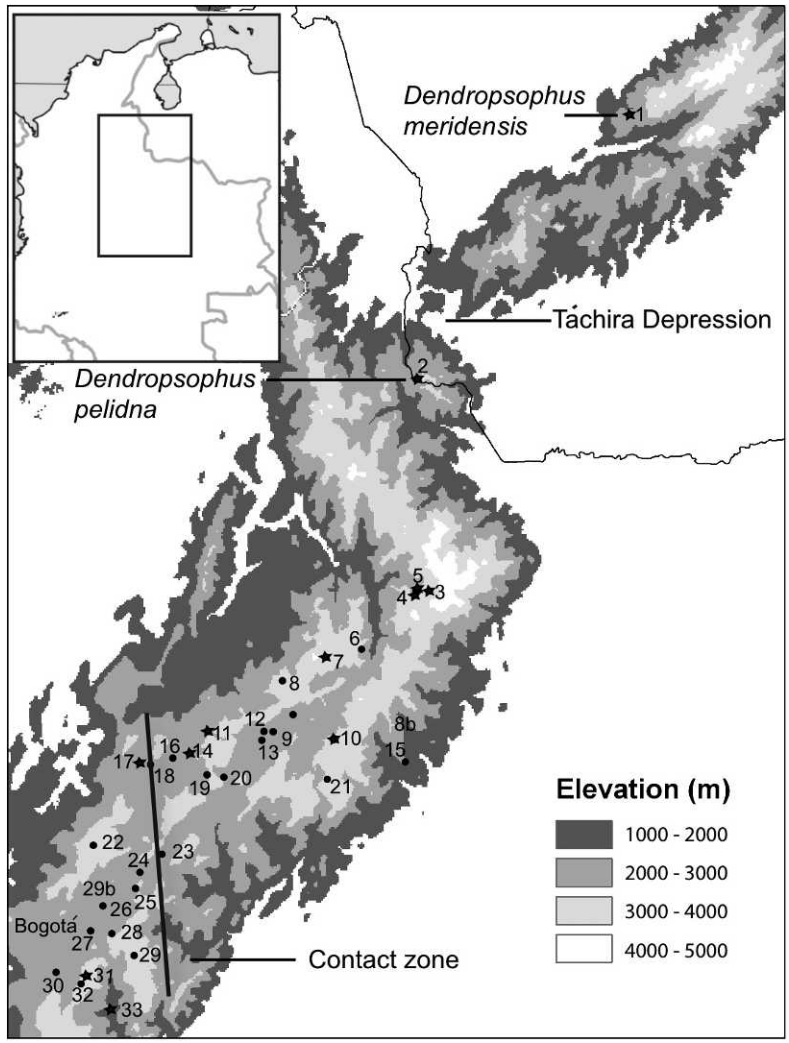

FIG 1.—Map of the localities included in this study. The vertical line delineates the contact zone between the Northern and Southern Clades. The Táchira Depression is a low-elevation region (< 900 m) that separates the Andes of Colombia and Venezuela. Names and geographic coordinates of each locality are given in Table 1. Stars indicate the localities used to build the 12S–16S and proopiomelanocortin A phylogenies. Only elevations above 1000 m are shown.

mine the degree to which acoustic and morphological traits differentiate because of divergence events that occurred in the very young northern Andes (Fjeldsaå and Lovett, 1997; Young et al., 2002).

MATERIALS AND METHODS

DNA Sequencing

Tissue samples from the nominal species *D. labialis* were obtained from 10 populations (Fig. 1). Three of these populations geographically correspond to the Southern Clade (Guarnizo et al., 2009), and seven to the Northern Clade (Table 1). Tissues were obtained by toe-clipping adult frogs (IACUC protocol 07101901). Total genomic DNA was extracted with the use of a Viogene Blood and Tissue Genomic DNA Extraction Miniprep System (Viogene, Inc., Taipei, Taiwan). Four primer pairs (Symula et al., 2008) were used to amplify the almost-complete 12S–16S

TABLE 1.—Sampling sites, map codes, assigned clade, geographic coordinates, and the number of individuals analyzed for acoustics, morphology, and the phylogenetic analysis (12S-16S and proopiomelanocortin A [POMC]). Localities are ordered from north to south. Asterisks indicate additional localities sequenced earlier for cytb and CO1 in Guarnizo et al. (2009). The North–South Clade corresponds to a locality that contained haplotypes from both clades (sympatric locality).

Locality	Map code	Clade	Latitude N	Longitude W	Acoustics (*n* individuals)	Morphology (*n* individuals)	*n* individuals sequenced for 12S–16S	*n* individuals sequenced for POMC
Los Suarez, Mérida	1	*Dendropsophus meridensis*	8°38′34.7″	71°22′55.9″	–	–	1	–
Betania, Táchira	2	*Dendropsophus pelidna*	7°24′38.2″	72°25′32.2″	–	–	1	–
Laureles	3	North	6°24′54.8″	72°26′21.7″	–	–	2	2
Cocuy	4	North	6°24′19.3″	72°26′47.6″	–	–	2	2
Cueva	5	North	6°24′15.2″	72°22′55.9″	–	–	2	2
Sativa Norte	6	North	6°08′00.2″	72°42′31.3″	–	2	–	–
Guina	7	North	6°05′51.9″	72°52′45.8″	–	–	2	2
Páramo de la Rusia	8	North	5°59′20.3″	73°05′10.7″	–	3	–	–
Vado Hondo	8b	North	5°29′52.6″	72°44′52.2″		24	–	–
Duitama	9	North	5°49′35.9″	73°02′17.0″	–	5	–	–
Paipa	10	North	5°46′54.5″	73°08′01.6″	–	3	2	2
Arcabuco	11	North	5°45′14.2″	73°26′40.3″	–	–	2	2
Manzano	12	North	5°45′00.5″	73°10′40.0″	22	–	2*	–
Santa Sofía	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	1
Cómbita	15	North	5°38′56.8″	72°26′57.9″	–	5	–	–
Sutamarchán	16	North	5°37′44.2″	73°36′41.6″	9	–	–	–
Chiquinquirá	17	South	5°36′27.7″	73°46′10.9″	7	–	1	1
San Carlos	18	North–South	5°35′53.9″	73°43′00.1″	7	–	6*	–
Cucaita	19	North	5°32′44.9″	73°27′00.4″	25	–	2*	–
Tunja	20	North	5°32′03.8″	73°22′13.9″	–	5	–	–
Aquitania	21	North	5°31′03.8″	72°52′50.1″	–	5	–	–
Páramo de Guerrero	22	South	5°13′1.48″	73°59′34.7″	–	5	–	–
Chocontá	23	North	5°10′16.2″	73°40′05.5″	9	–	1*	–
Tenería	24	South	5°05′9.80″	73°46′27.1″	8	–	–	–
Cucunubá	25	South	5°00′36.7″	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*	–
Bogotá	29b	South	4°47′09.8″	74°02′30.3″	–	16	–	–
La Granja	30	South	4°36′52.2″	74°10′38.1″	–	5	–	–
Guadalupe	31	South	4°35′44.2″	74°01′49.7″	–	–	3	3
Páramo 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	1

rRNA region. One primer pair (Guarnizo et al., 2009) was used to amplify the nuclear gene proopiomelanocortin A (POMC); we sequenced additional populations that were not included in Guarnizo et al. (2009).

The polymerase chain reaction (PCR) cycle for the 12S–16S region included an initial denaturing step of 2 min at 94°C, followed by 35 amplification cycles (30 s at 94°C, 30 s at 48°C, 60 s at 72°C), and a final extension of 7 min at 72°C. For POMC we used the PCR cycle protocol in Guarnizo et al. (2009). PCR products were purified with the use of a Viogene Gel Purification Kit and were sequenced at the University of Texas ICMB Core Research Facility with ABI 3730 DNA sequencers. Each fragment was sequenced in both directions to confirm base calls. Nucleotide sequences were aligned with the use of MUSCLE (Edgar, 2004).

Phylogenetic Analyses

Nucleotide diversity (a measure of genetic differences within a group of sequences; Nei and Li, 1979), was calculated with the use of DNAsp (Rozas and Rozas, 1999). Genetic distances between clades were estimated with the program MEGA 5.0 (Tamura et al., 2011). To reconstruct the haplotypes for the nuclear POMC locus, we used PHASE 2.1 (Stephens and Donnelly, 2003) as implemented in DNASP v. 5 (Librado and Rozas, 2009). Phylogenetic trees were constructed with the use of 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. For each independent gene we used MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) to generate two 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), sampled every 1000 generations, and preliminary analyses indicated a burn-in of 25% to be enough for discarding the first proportion of the Markov chain. Convergence of the two runs was indicated when the average standard deviation of the split frequencies was less than 0.01. ML trees were generated with RAxML-VI-HPC (Stamatakis, 2006) with the use of a GTRGAMMA model of evolution (GTR + Γ, following the recommendation of Stamatakis, 2006) and implementing a standard bootstrap search with 1000 replicates.

We included in the phylogenetic analysis two other members of the *labialis* group (Duellman, 1989): *Dendropsophus meridensis* from Mérida (2200 m) Venezuela (12S–16S and POMC sequenced by CEG), and *Dendropsophus pelidna* from Táchira (2800 m) Venezuela (GenBank accession AY819434); no GenBank POMC sequences were available for *D. pelidna*. The most distant outgroup was *D. carnifex* (12S and 16S only; GenBank AY843616; following Faivovich et al., 2005, and Wiens et al., 2010). GenBank numbers for new sequences are JF422585–JF422622.

Acoustic data were obtained from fieldwork and morphometric data were collected from museum specimens. Unfortunately, no morphometric data were collected from individuals used for acoustic analysis because the purpose of the companion study (Guarnizo et al., 2009) was to assess the relation of ecological variation to phylogeny. Therefore, each individual was analyzed either for acoustic or morphologic variation, but not both. Table 1 includes sample sizes, localities, and analyses performed.

Acoustic Analyses

We analyzed the calls of 176 individuals from 12 localities across most of the geographic range of the species (Fig. 1; Table 1). We included four localities in the Northern Clade, seven in the Southern Clade, and one from a locality with haplotypes from both clades (San Carlos, Locality 18; see also Guarnizo et al., 2009). The call data from seven localities were obtained from Amézquita (2002) as averages for each population. Between 9 and 14 consecutive advertisement calls from each of 8–12 males per locality were recorded by placing a Sennheiser ME67 directional microphone in the frontal plane of a calling male. The distance between the microphone and the male was $>$ 50 cm. The calls were recorded on TDK MA90 metal bias tapes with a Sony WM D6C Professional Walkman. Each male was recorded calling from shallow water at the edges of ponds. Air temperature (used to correct certain call variables) was measured with a digital thermometer placed within 1 m of the male.

Terminology for call parameters follows Cocroft and Ryan (1995). Male calls were digitized at 44.5 KHz and 16 bits, and were analyzed with Cool Edit (Syntrillium Software Corporation, USA). Oscillograms were used to measure call duration and number of pulses per call; these call traits display the strongest levels of geographic variation in *D. labialis* (Amézquita, 2002). Pulse rate was calculated as the number of pulses/call duration. To remove the effect of ambient temperature in among-locality comparisons of call traits (range of 8.2–20.0°C), we used the residuals of pulse rate on temperature regressions as temperature-independent call descriptors.

For each of the three call variables (call duration, number of pulses/call, and pulse rate), we used a two-tailed *t*-test to test for

differences in means between the Northern and Southern Clades. The Central Clade was included within the Southern Clade.

To reduce the number of variables needed to describe overall variation in acoustic parameters, we performed a principal-component analysis (PCA) with the use of the correlation matrix. Analysis of variance (ANOVA) was used to test for differences between the PC factor scores using clade (Northern or Southern) as the grouping variable.

It is important to control for the confounding effect of geographic distance on acoustic differentiation, because phenotypic analyses that include a spatial component may suffer from spatial autocorrelation (Legendre and Fortin, 1989). We performed a partial Mantel test to determine if acoustic distance (estimated as pairwise Euclidean distances using scores of the first principal component) 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. The test was performed with the program zt (Bonnet and Van de Peer, 2002) using 10,000 permutations. Although some uses of partial mantel tests have been criticized, the use of these tests for analysis of pairwise distance data is appropriate (Legendere and Fortin, 2010).

Morphometric Analyses

We performed morphometric analyses of 102 individuals from 16 localities (Fig. 1, Table 1), 7 in the Southern Clade and 9 in the Northern Clade. Measurements were taken with a digital caliper (precision of 0.01 mm) from preserved frogs in the collections of the Instituto de Ciencias Naturales at the Universidad Nacional de Colombia (ICN) and the University of Kansas Biodiversity Institute (KU). We collected data from eight external variables following Ron et al. (2005): snout–vent length, tibia length, foot length, head length, head width, eye diameter, tympanum diameter, and eye–tympanum distance. All measurements were taken from the right side of the specimen and $\log_{10}$ transformed. Given the small sample size of females, only males were analyzed. We used individual scores on PC1 (assuming the presence of uniform loadings of the same sign) to reflect overall size (Bookstein, 1989). Although residuals from variables regressed on SVL are often used as size-free variables, we did not use this approach because these residuals often do not completely remove the effect of size (Bookstein et al., 1985).

To determine if morphometric variation differs significantly between clades, excluding the North–South locality, we performed a PCA with the use of the correlation matrix. A two-tailed *t*-test was used to test for differences between scores from the first two PC factors grouped by clade membership. Morphological distance between individuals was estimated as the pairwise Euclidean distance between factor scores. Partial Mantel tests were performed to detect correlation between morphological distance and clade membership, controlling for the effect of geographic distance between populations, as described above.

Climatic Analysis

An association between genetic and climatic variation might indicate ecological mechanisms in the process of diversification (Ogden and Thorpe, 2002). To determine whether localities grouped by clade can be differentiated by climatic properties of the sampled localities, we used the program DIVA–GIS (Hijmans et al., 2001) to extract 30-arcsec resolution layers of annual temperature and precipitation averages (Hijmans et al., 2005) from the localities. ANOVA was used to determine if there were significant differences in mean precipitation and mean temperature variables between the two clades. Each locality was used as a data point; $n = 19$ for the Northern Clade and $n = 12$ for the Southern Clade.

Results

Phylogenetic Analysis

We sequenced 2369 base pairs (bp) of the 12S–16S region of mitochondrial DNA and 490 bp of the nuclear gene POMC. All sites in both genes were included because no sites were ambiguously aligned. The Bayesian (best-fit model $GTR + \Gamma + I$) and ML analysis of the 12S–16S region recovered the same clades (Southern and Northern) found by

TABLE 2.—Summary of the principal-component analysis on the correlation matrix of acoustic and morphological characters of *Dendropsophus labialis*. All acoustic variables are corrected for temperature when appropriate.

	Factor loadings	
	PC1	PC2
Acoustics		
Eigenvalue	2.325	0.658
Percent of variation	77.500	21.950
Pulses per call	0.651	–
Pulse rate	–	−0.793
Morphology		
Eigenvalue	4.758	0.919
Percen of variation	59.471	11.488
Log snout–vent length	0.916	0.146
Log foot length	0.904	0.020
Log tibia length	0.887	−0.079
Log head width	0.814	−0.031
Log head length	0.800	−0.118
Log eye–tympanum	0.596	−0.506
Log eye diameter	0.479	0.785
Log tympanum diameter	0.654	−0.056

Guarnizo et al. (2009), who used the faster-evolving mitochondrial genes cytochrome b and cytochrome oxidase 1 (Fig. 2). The species *D. meridensis*, *D. pelidna*, and individuals from the Serranía del Cocuy, which lies north of the Northern Clade (Localities 3, 4, and 5; Fig. 1). in Colombia formed a third clade (here termed the *meridensis* Clade). These three clades are individually well supported (> 80% bootstrap), however, the branch that groups the Northern and Southern Clade has a bootstrap value of 55% The maximum p distance (uncorrected) between the Northern and Southern Clades (12S–16S) was 2.7%, and that between the *meridensis*-Northern and *meridensis*-Southern Clades were 2.5% and 2.2%, respectively. The Southern Clade contains haplotypes from localities southwest of Chiquinquira (Locality 17, Fig. 1), and the Northern Clade includes localities northeast of the city. The *meridensis* Clade, as mentioned earlier, included individuals from the Venezuelan Andes and the Serranía del Cocuy in Colombia. POMC also recovered the Southern Clade (Fig. 2), but was unable to resolve the Northern and *meridensis* Clades.

Guarnizo et al. (2009) recovered a central clade. In this analysis, which includes additional data and localities, the distinctiveness of this clade is less apparent, and we have subsumed it into the Southern Clade.

Acoustic Analyses

Call duration was highly correlated with number of pulses per call (Pearson's $r = 0.78$, $P < 0.0001$, $n = 176$ individuals). Therefore, to avoid redundancies in later analyses we used only pulse rate and pulses per call. Frogs calling at higher temperatures produced calls with slightly fewer pulses per call (linear regression, $r^2 = 0.06$, $F = 11.3$, $P = 0.009$, $n = 186$ individuals) produced at a higher pulse rate ($r^2 = 0.21$, $F = 50.0$, $P < 0.0001$). To remove the effect of ambient temperature in among-locality comparisons of call features (range of 8.2–20.0°C), we used the residuals of these regressions as temperature-independent call descriptors for subsequent analyses.

Basic statistics for the three call variables are summarized in Table 3. The analysis of covariance (ANCOVA) between the Northern and Southern Clades (using temperature as a covariate) showed that call duration was significantly different ($F = 41.12$, $P < 0.001$, $n = 21$ for Northern, $n = 21$ for Southern); number of pulses/call was significantly different ($F = 17.30$, $P < 0.001$), and pulse rate was significantly different ($F = 33.66$, $P < 0.001$).

Call data for the two clades were pooled for the PCA. Two principal components summarized more than 99% of the acoustic variance (Table 2). The highest loading for PC1 was pulses per call, and the highest loading for PC2 was pulse rate. After the effects of the temperature were statistically controlled, we found significant differences between the two clades when using the PC1 as a descriptor of the acoustic variation (ANOVA, $F = 26.67$, $P < 0.001$). Also, we found a significant correlation between acoustic (Euclidean) distance and clade assignment when controlling for geographic distance (partial Mantel test, $r = 0.19$, $P < 0.001$), which indicates that the variation in calls is dependent on clade, independent of geographic distance.

Morphometric Analyses

Two components with eigenvalues higher than 0.9 were extracted from the PCA and account for 71% of the variance. The highest loadings for PC1 were SVL and foot length,

TABLE 3.—Acoustic and morphological measurements of *Dendropsophus labialis* and *Dendropsophus luddeckei*. Sample sizes (*n*, below in table) are given in number of individuals distributed in seven localities. All morphometric data are in millimeters. SD = standard deviation.

	Dendropsophus labialis (Southern Clade)			*Dendropsophus luddeckei* (Northern Clade)		
	Mean (n = 105)	SD	Range	Mean (n = 66)	SD	Range
Call duration (s)	197.4	23.94	152–252.1	140.5	14.65	108.2–212.7
Pulse rate/s	75.2	6.38	61.8–88.2	92.9	2.96	71.2–106.3
Pulses per call	14.5	0.74	13.4–17.2	12.8	0.98	11.1–15.6
	Mean (n = 31)	SD	Range	Mean (n = 57)	SD	Range
Snout–vent length	46.2	0.96	32.6–61.0	38.6	0.69	26.4–50.0
Tibia length	24.6	0.92	16.4–55.4	19.6	0.33	13.7–25.4
Foot length	21.8	0.51	14.7–30.4	18.0	0.38	11.2–23.5
Head length	13.8	0.25	9.9–17.9	11.9	0.21	8.5–18.0
Head width	13.7	0.24	10.2–17.9	11.5	0.19	8.4–14.8
Interorbital distance	11.8	0.22	8.8–15.8	10.3	0.16	7.5–13.5
Eyes–nostril distance	3.6	0.06	2.8–4.7	3.0	0.05	2.1–4.1
Eye diameter	4.6	0.05	3.7–5.8	3.8	0.04	3.0–4.9
Tympanum diameter	2.8	0.06	2.1–4.1	2.3	0.05	1.6–4.3
Eye–tympanum distance	2.8	0.06	1.9–4.0	2.0	0.05	1.1–3.0

whereas the highest loadings for PC2 were eye diameter, eye–tympanum distance, and tympanum diameter (Table 2). We found broad overlap in morphological space (Fig. 3) and no statistical differences among males between the clades (PC1: $t = -0.93$, $P = 0.36$; PC2: $t = 0.31$, $P = 0.76$). We did not find a significant correlation between morphological (Euclidean) distance and clade assignment when controlling for geographic distance (partial Mantel test, $r = 0.07$, $P = 0.20$), which indicates that morphological variation is not dependent on clade origin even after controlling for geographic distance.

Climatic Analyses

We did not find statistical differences in annual averages of temperature and precipitation across the sampled localities between the two clades (ANOVA; temperature: $F = 1.07$, $P = 0.32$; precipitation: $F = 2.22$, $P = 0.17$).

DISCUSSION

Genealogical History of D. labialis

The tree topology obtained with 12S–16S indicates *D. labialis* is composed of two highly divergent clades distributed across the Eastern Cordillera in Colombia, confirming the results of Guarnizo et al. (2009), who used faster-evolving mitochondrial genes. We also found a third clade including *D. meridensis*, *D. pelidna* (Venezuela), and individuals from Serranía del Cocuy in Colombia. POMC is unable to resolve the polytomy that includes the Northern and *meridensis* clades. This is similar to patterns expected in recently diverged sister species (Guayasamín et al., 2008; Gvozdik et al., 2010; Rodríguez et al., 2010) where there is not enough time or sufficiently small effective population size of the nuclear genes to counteract incomplete lineage sorting (Moore, 1995).

Given the geographic distribution of the sampled individuals, it might first appear that the Southern and Northern Clades result from the sampling gap between them (Fig. 1). However, the relationship between geographic and genetic distances (Fig. 4) indicates that this gap does not explain the divergence between the two clades, because large interclade genetic distances (> 2%) completely overlap low intraclade genetic distances (< 1%) along the geographic distance axis. A meaningful effect of a spatial sampling gap on genetic divergence would be indicated if large interclade genetic distances did not overlap with low intraclade genetic distances along this axis.

Phenotypic Divergence Between the Northern and Southern Clades

We found that acoustic (temporal call characteristics) but not morphological traits

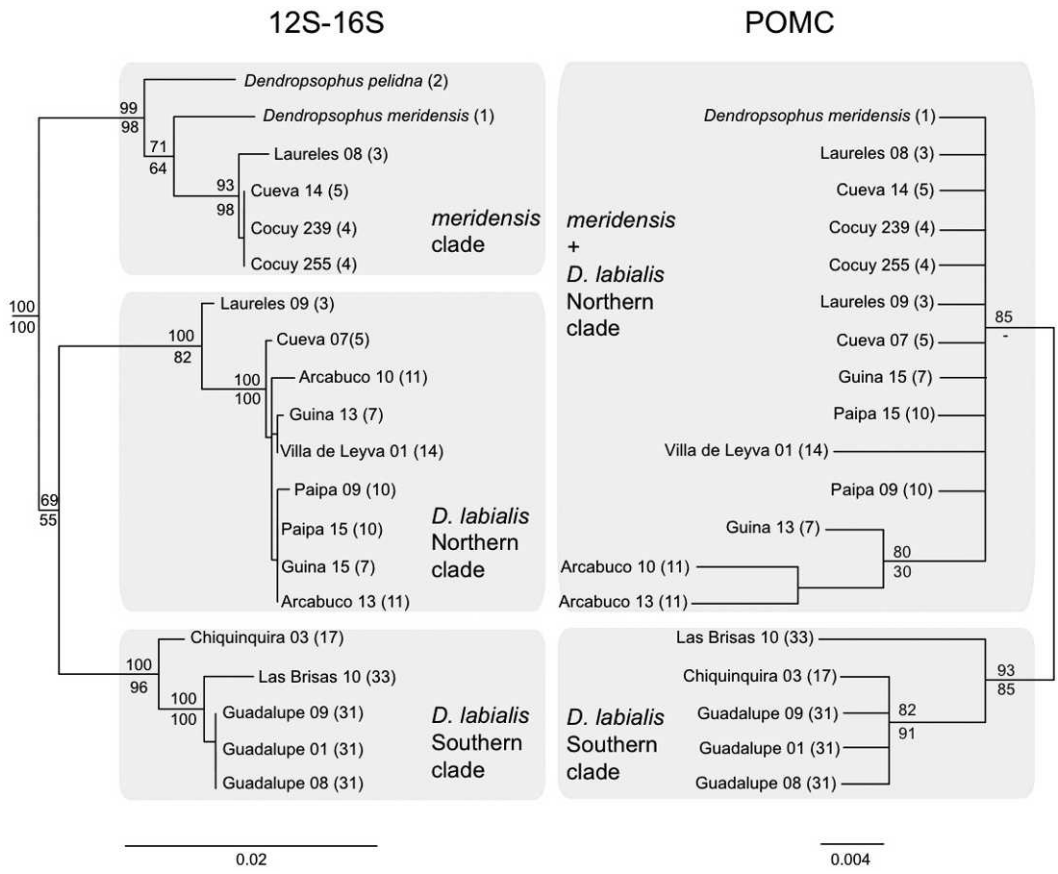

FIG. 2.—Maximum-likelihood gene trees with the mitochondrial 12S–16S (left) and the nuclear proopiomelanocortin A genes (right). Numbers above the branches are Bayesian posterior probabilities and below are bootstrap support values. Numbers after locality names indicate the specimen identifier number. Numbers in parentheses correspond to the map codes in Table 1. Grey boxes delineate the *Dendropsophus meridensis* Clade (top), *Dendropsophus labialis* Northern Clade (middle), and *D. labialis* Southern Clade (bottom).

differ statistically when individuals were grouped according to their clade membership. We found a significant difference in acoustic traits between individuals grouped by clade. However, we did not find a difference between clades in morphometric traits. Assuming that the mtDNA tree reflects population divergence, two alternative hypotheses are: Either acoustic traits diverge according to mtDNA history and morphological traits do not exhibit divergence (e.g., they are constrained by selection), or both acoustic and morphological traits track mtDNA history, but the rate of acoustic divergence is faster. Thus, the two clades are distinguishable based on acoustic traits but not by morphology. Both character complexes suggest that the likely scenario for *D. labialis* is that morphology and acoustic variation covary with mtDNA population history, but morphology is diverging at a slower rate than acoustic traits.

For frogs, it is well established in some species that morphological traits underestimate the amount of divergence and/or reproductive isolation between closely related populations or cryptic species, whether there is character displacement (Blair, 1955; Littlejohn and Martin, 1964) or not (Padial et al., 2009). Thus, morphological traits seem to provide a conservative delineation of species boundaries. However, it can also be argued that morphological characters appear to be

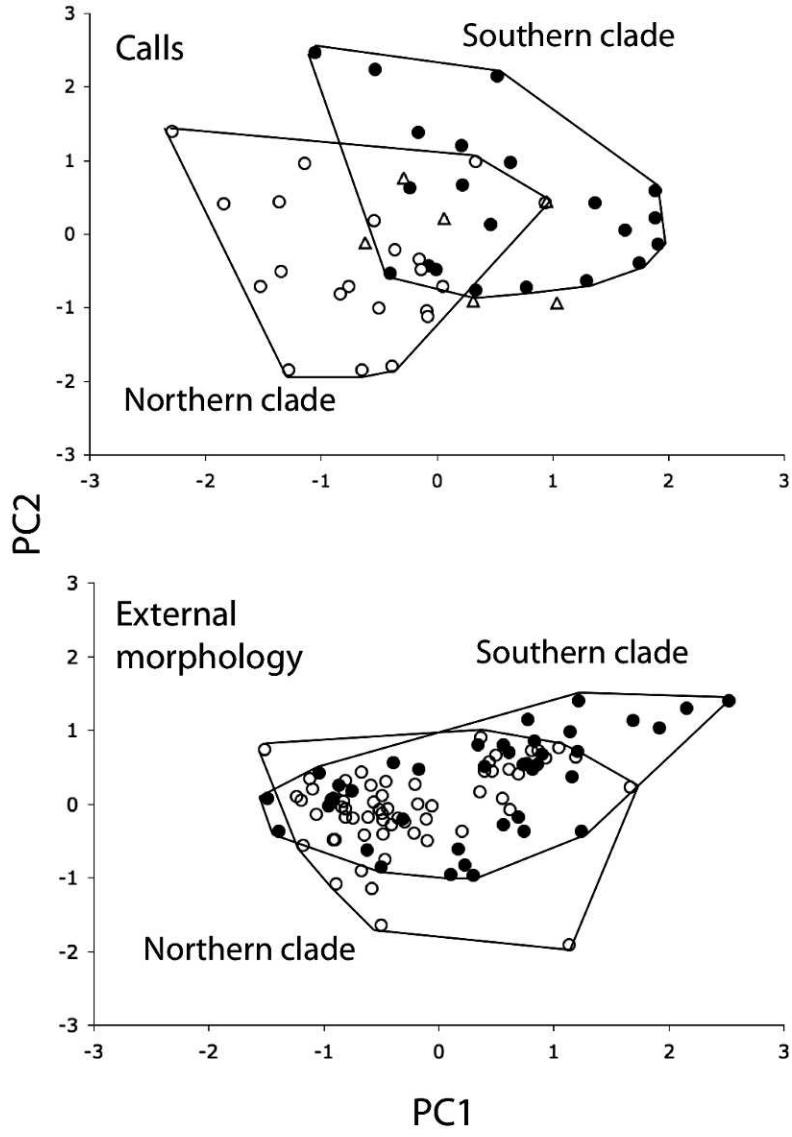

FIG. 3.—Principal-component analysis plot that summarizes variation in call parameters (top) and variation in external morphology (bottom) among frogs grouped in two clades. White dots: Northern Clade (*Dendropsophus luddeckei*), black dots: Southern Clade (*Dendropsophus labialis*), white triangles: sympatric occurrence.

conservative simply because they are not thoroughly studied. For example, with the use of mtDNA, Elmer and Cannatella (2008) delineated three new species formerly included under *Pristimantis* "*ockendeni*." Given the phylogeny, they were able a posteriori to identify phenotypic traits that distinguished the three species.

Multitrait divergence as evidence of speciation.—Dendropsophus labialis exhibits an abrupt (~30 km) change in haplotype frequencies and acoustic traits (Fig. 5), which suggests a secondary contact zone (Barton and Hewitt, 1985). The species also displays significant differences in temporal call characters (particularly pulse rate) between the Southern Clade and the Northern Clade. This

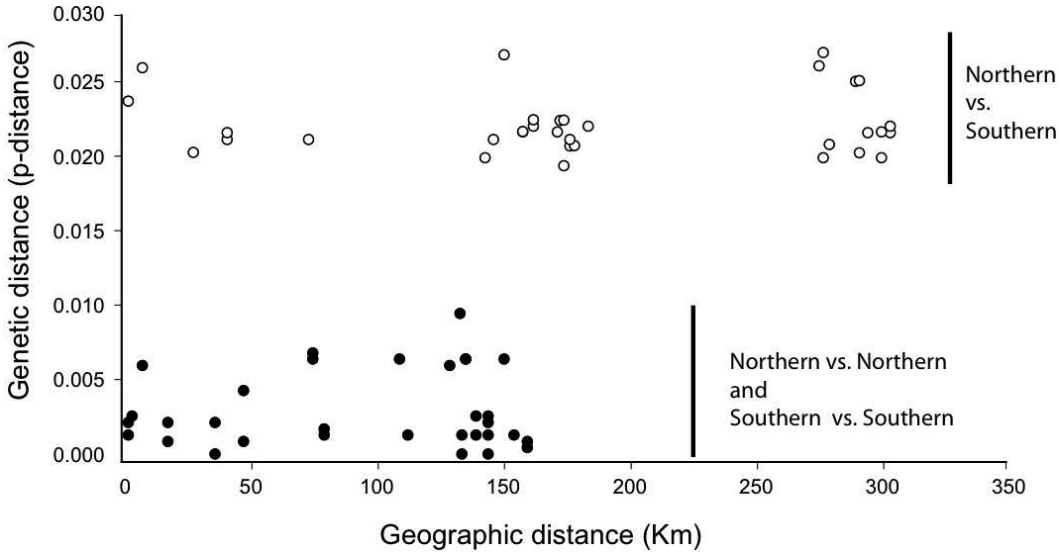

FIG. 4.—Scatter plot depicting geographic vs. genetic distances in *Dendropsophus labialis* using 12S–16S. Black dots indicate intraclade (Southern vs. Southern and Northern vs. Northern) and white dots indicate interclade (Southern vs. Northern) pairwise comparisons of individuals in Fig. 2. None of the individuals of the *meridensis* clade was included.

agreement between two independent character systems, temporal acoustic signals—crucial cues in species recognition—and mitochondrial genes, indicates that the two clades are morphologically cryptic species with parapatric distributions. For this reason we designate the Northern Clade as a new species (see species description below). Given that the holotype of *D. labialis* (ZMB 4913) geographically corresponds to the Southern Clade ("vicinity of Bogotá" in Cundinamarca), the name *D. labialis* remains associated with the Southern Clade.

Are the Northern and Southern Clades Exchanging Alleles?

Our evidence indicates that there is no movement of mitochondrial alleles either west or east of the locality of Chiquinquira (Locality 17; Fig. 1). In other words, there are no mitochondrial haplotypes from the Southern Clade within the geographic range of the Northern Clade and vice versa.

Fifty-nine percent of the individuals were heterozygous for POMC. After phasing this locus, we found nine alleles restricted to the Northern Clade and five alleles restricted to the Southern Clade. However, two heterozygous individuals contained alleles from both clades, suggesting limited hybridization. These two individuals are from Villa de Leyva (Locality 14) and Arcabuco (Locality 11; Fig. 1), which are within 30 km of the region where the two clades meet (the contact zone; Fig. 1). Interestingly, we found that the two hybrids had nuclear alleles from both clades, but only mitochondrial haplotypes from the Northern Clade. In other words, these two hybrids seem to have Northern Clade mothers and Southern Clade fathers.

Based on these very limited data, we offer some speculative remarks; these are meant to point out areas of needed research rather than to suggest definitive answers to the questions that are posed.

Are hybrids under a selective disadvantage? We found only 2 hybrid individuals out of 18 sequenced for both 16S and POMC, suggesting that they are uncommon. Barton and Hewitt (1985) indicate that it is hard to distinguish whether parapatric forms remain distinct because they are adapted to different environments or because hybrids are selected against. We did not find annual temperature or precipitation differences between the two species at a macrogeographical scale (although our data are admittedly coarse). If this truly indicates a lack of local ecological differences

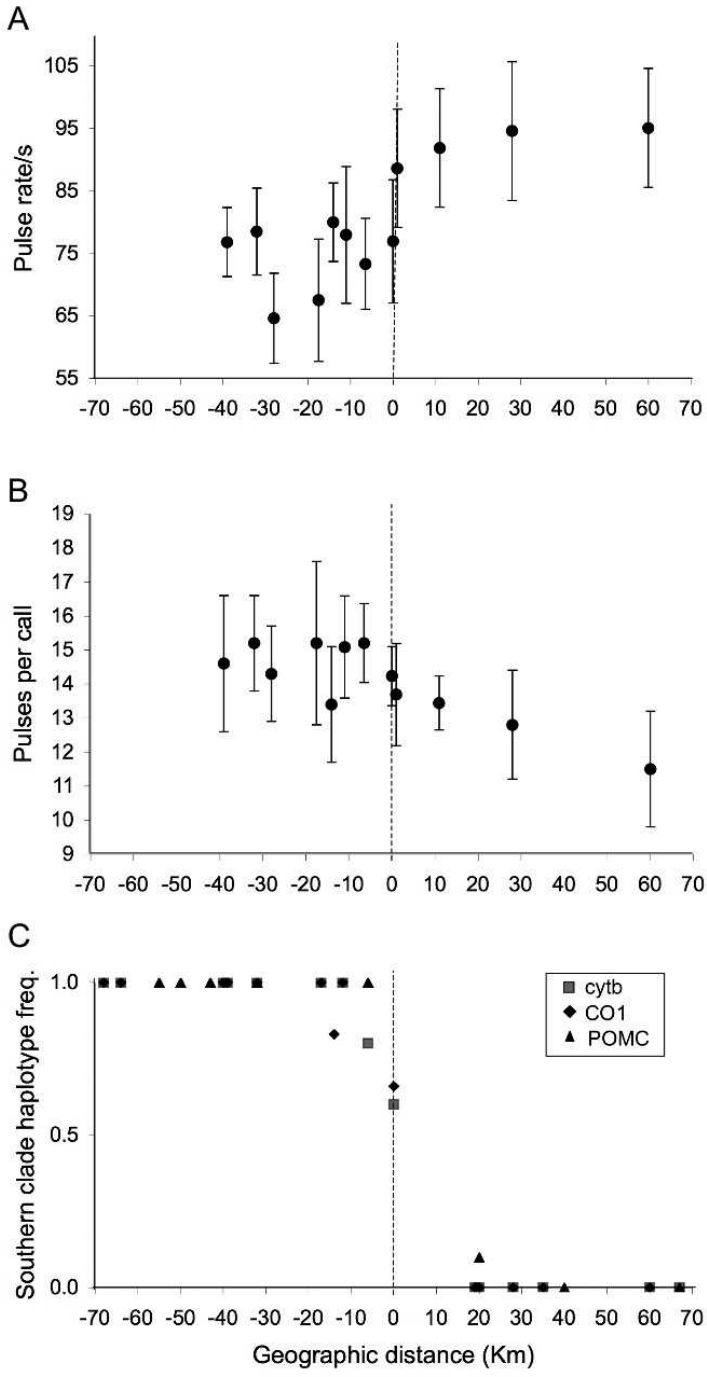

Fig. 5.—Variation of the Southern and Northern Clades across the contact zone for (A) pulse rate, (B) pulses per call, and (C) haplotype frequencies, based on the populations sampled by Guarnizo et al. (2009). The dotted line corresponds to the contact zone that passes through the sympatric locality San Carlos (map code 18). The negative values in the x-axis correspond to the region southwest of the contact zone (Southern Clade) and the positive values to the region northeast of the contact zone (Northern Clade). From left to right, the map codes in A and B are 27, 33, 28 29, 25, 24, 17 18, 23, 16, 19, and 12.

between the two regions, then perhaps hybrids are uncommon as a result of a selective disadvantage.

Hybrids may be subject to postzygotic barriers that reduce fitness. Ulloa (2003) found that Southern Clade individuals mate with Northern Clade individuals under laboratory conditions. To test for postzygotic barriers, she measured larval survival (% of living larvae per day) of crosses between and within the two clades. Although she did not find a significant difference (Mann–Whitney: $Z = -1.46$, $P = 0.14$, $n = 11$ clutches; Appendix), she found that the median hybrid larval survival between the two clades was lower than the median larval survival in the intraclade crosses.

If hybrid individuals are being selected against, we would expect to find acoustic character displacement preventing maladaptive hybridization. Our very limited data indicate that one locality (San Carlos, Locality 18; Guarnizo et al., 2009) contains mtDNA haplotypes of both clades in sympatry. However, individuals from this locality show no evidence of character displacement in call parameters; they are located clearly within the principal-component space delimited by the Northern and Southern Clades (white triangles in Fig. 3, top graph). Hybrid inviability between sister species calling sympatrically does not mean, however, that acoustic character displacement is occurring. Niche partitioning (spatial or temporal) might prevent females of one species from syntopic contact with the males of the other species. Our data do not contain fine-grained spatial or temporal acoustic information; therefore we cannot make firm conclusions.

As an alternative, behavioral prezygotic mechanisms such as assortative mating might explain rarity of hybrids, but we do not have data needed to make firm conclusions. A more complete description of the genetic variation across the contact zone, together with experiments that may reveal an effect of prezygotic barriers (such as phonotaxis experiments), or the effect of postzygotic barriers (proportion of fertilized eggs in interspecific crosses and fitness assessment of the F1 and F2 generations), are needed to characterize the mechanisms that prevent exchange of alleles.

Comments on phylogenetic relationships and biogeography of the meridensis clade.— We found surprisingly low 12S–16S sequence divergence (maximum uncorrected pairwise difference of 0.9%) between *D. meridensis* and *D. pelidna*, compared to 2.7% between *D. luddeckei* and *D. labialis* (Fig. 6). Given that the tissue samples of both species are from localities relatively close to their type localities, it seems likely that the sequences are correctly associated with their nominal species. This low level of divergence suggests that *D. pelidna* and *D. meridensis* are conspecific. If so, *D. pelidna* is a junior subjective synonym of *D. meridensis*. However, we do not formally propose this taxonomic reassignment because our sample size is low and other data such as advertisement calls and morphometric analysis are highly desirable in making these assessments.

If *D. meridensis* and *D. pelidna* are conspecific, then *D. meridensis* sensu lato occurs on both sides of the point where the Eastern Andes reaches its lowest elevation (Táchira Depression: 900 m), which is below the current altitudinal range of the species (1200–2400 m; La Marca, 2004). A possible explanation for such a distribution is that originally *D. meridensis* was found only west of the Táchira depression. Because of Pleistocene glaciations, populations of *D. meridensis* might have been pushed downwards to warmer elevations, allowing them to cross the altitudinal barrier. Following this, interglacial warmer periods might have, again, displaced populations upward and isolated a small relict of *D. meridensis* in the Mérida Andes in Venezuela. This would explain why *D. meridensis* has a restricted distribution and *D. pelidna* is very abundant (La Marca, 2004). However, this scenario is speculative.

Equally interesting is the fact that some specimens (Laureles 08 at Locality 3, Cueva 14 at Locality 5, and Cocuy 239 and 255 at Locality 4; Figs. 1 and 2) were syntopic with the Northern Clade (Laureles 09 and Cueva 07, both from Locality 3; Figs. 1 and 2), but clustered within the *D. meridensis* clade. However, we have not referred these specimens to *D. meridensis*. Comparing

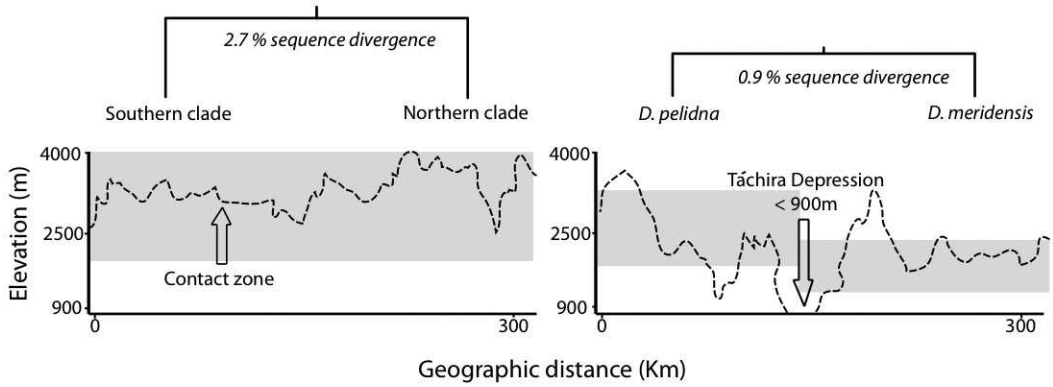

FIG. 6.—Elevation profiles and the level of sequence divergence (for the 12S–16S genes) within the species pairs *Dendropsophus labialis* (Southern Clade)–*Dendropsophus luddeckei* (Northern Clade) and *Dendropsophus pelidna*–*Dendropsophus meridensis*. Gray areas depict the presumed elevation range of each species based on their geographic distribution. The arrow points to the Táchira Depression, which reaches elevations (900 m) below the minimum elevation registered for any of the four species.

the morphology between the individuals of the *D. meridensis* and *D. luddeckei* clades from the syntopic locality would be interesting and informative; unfortunately, we do not have access to the specimens of this locality. If further studies determine that all samples in the *meridensis* clade prove to be a single species, this would indicate that the distribution of *D. meridensis* extends from the Mérida Andes in Venezuela to the Serranía del Cocuy in Colombia, crossing the Táchira Depression.

This study provides an additional example of a widespread frog species, abundant in urban areas, that was delimited by the lack of morphometric divergence among populations (presumably because morphological traits evolve slowly), but is found to have other aspects of the phenotype (acoustic traits) and genetic diversity that reveal cryptic species.

SPECIES ACCOUNT

Dendropsophus **luddeckei** *sp. nov.*

Holotype.—Deposited at Museo de Historia Natural Andes, Universidad de los Andes, Bogotá, Colombia. Catalogue number ANDES-A 0603 (Fig. 7). An adult male from Sotaquira, (05°45′02.8″N; 73°16′39.7″W), 2765 m elevation, Departamento de Boyacá, Colombia, collected 16 June 2010 by C. Escallón.

Paratypes.—57 (Male adult: ANDES-A0604, collected at the type locality (05°45′02.8″N; 73°16′39.7″W) with the holotype by C. Escallón, 33 adults from the collection of Instituto de Ciencias Naturales, Universidad Nacional de Colombia: ICN 5588, 5658, 33263, 5661, 5686: Aquitania, Departamento de Boyacá. ICN 33615, 33626, 33633, 33618, 33629: Combita, Boyacá. ICN 12585, 12563, 12584, 12568, 12585: Duitama, Boyacá. ICN 52895, 52897, 52896: Paipa, Boyacá. ICN 4423, 959, 4424: Páramo de la Rusia, Boyacá. ICN 52941, 52944, 52945, 52942, 52943: Santa Sofia, Boyacá. ICN 5856, 5810: Sativa Norte, Boyacá. ICN 10296, 10294, 33638, 10299, 10297: Tunja, Boyacá; and 23 adults from the collection of the University of Kansas Biodiversity Institute: KU 169521–169546: Vado Hondo, Boyacá.

Diagnosis.—*Dendropsophus luddeckei* can be acoustically differentiated from *D. labialis*. It has calls of significantly shorter duration than *D. labialis*, higher pulse rate, and fewer pulses per call (see statistics in text and Table 3). At the molecular level, *D. luddeckei* differs from *D. labialis* by 13 fixed nucleotide differences in 12S–16S, 30 in cytochrome oxidase I (CO1), 27 in cytochrome b (cytb), and 5 in POMC (Guarnizo et al., 2009).

Description of the holotype.—An adult male, SVL 34.7 mm; body of medium width; head about as wide as body; head as wide as long, HW/HL 1.06, widest at level of

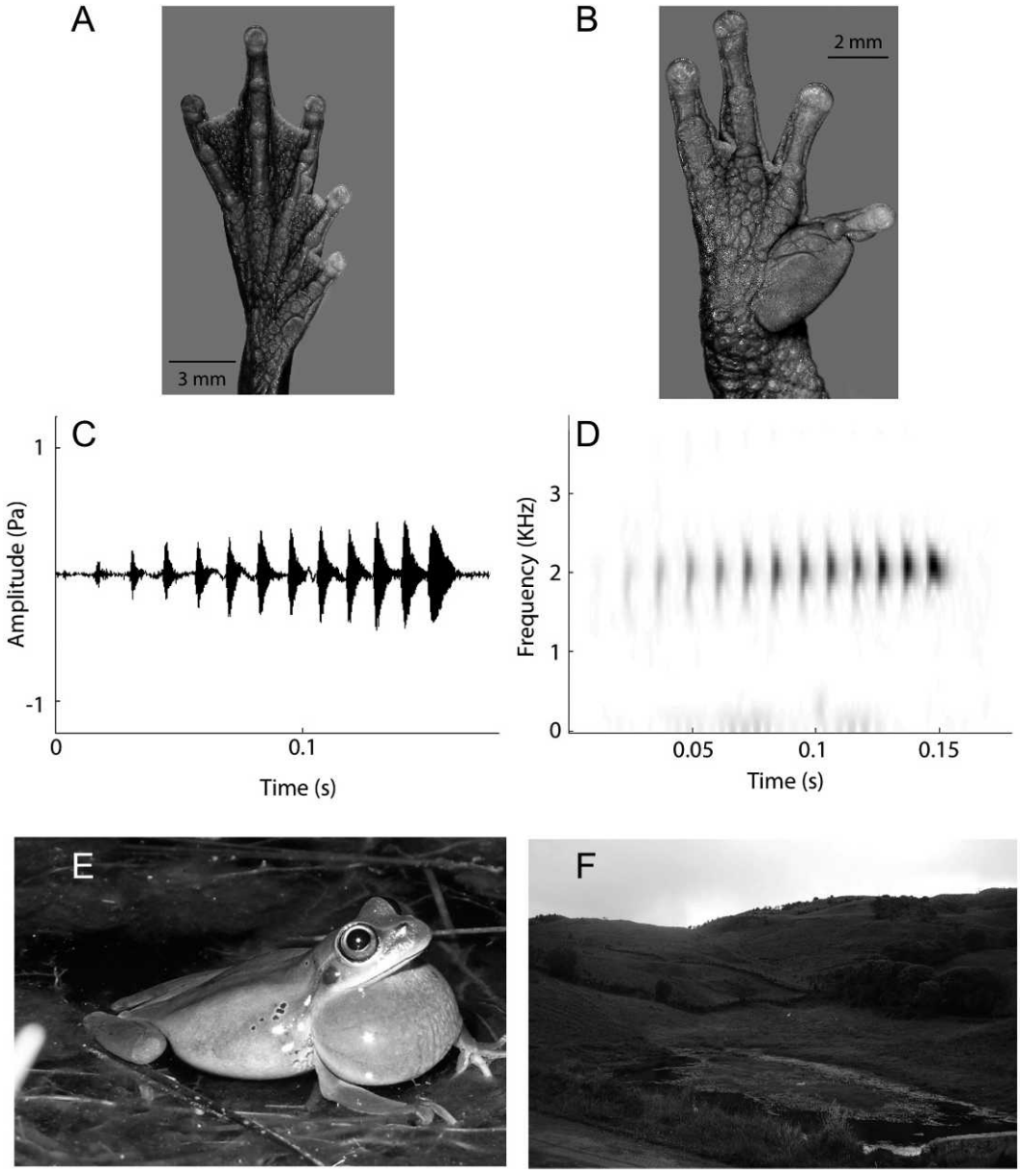

Fig. 7.—Right foot (A), and right hand (B) of paratype (KU 169536; ventral view), oscillogram (C), sonogram (D), holotype (ANDES-A 0603) (E), and typical habitat (F), of *Dendropsophus luddeckei*. Pa = pascal, KHz = kilohertz. Photos (A) and (B) by A. Berrío, (E) by C. Escallon, and (F) by C. Guarnizo.

tympanum; head nearly rounded in dorsal view, snout rounded in lateral profile; eye–nostril distance shorter than diameter of eye, E–N/ED 0.69; canthus rostralis concave in dorsal view, rounded in section; loreal region flat; lips thin, not flared; internarial region slightly concave; nostrils slightly protuberant, directed anterolaterally. Interorbital area flat, IOD/ED 2.11, IOD/HW 0.87; eyes of medium size and moderately protuberant, ED/HL 0.43, ED/HW 0.41. Palpebral membrane transparent. Supratympanic fold conspicuous, slightly

obscuring the posteroventral edge of tympanum; tympanic annulus contacting supratympanic fold; tympanum moderately large, distinct, directed dorsolaterally and separated from eye by a distance equal to half of the width of the tympanum; diameter of tympanum about one-half diameter of eye, diameter TD/ED 0.55. Arm short and robust, lacking an axillary membrane; ulnar fold very weak, consisting of few tubercles, extending from base of hand almost to elbow; fingers short with rounded discs slightly wider than finger; relative length of fingers I, II, IV, III; fingers bearing lateral fringes; subarticular tubercles slightly distinct, low in profile, not bifid except for distal subarticular tubercle of digit IV; subarticular tubercles rounded in ventral view, distal subarticular tubercles more prominent than proximal tubercles, most prominent on digit IV; supernumerary tubercles small; palmar tubercles indistinct; inner metacarpal tubercle low, small, flat; outer metacarpal tubercle smaller; nuptial pad large and prominent, strongly delineated by surrounding skin, extending from base of thumb, narrowing toward distal subarticular tubercle; webbing absent between fingers I and II; webbing basal between fingers II and III, and III and IV. Hind limbs of moderate length; shank robust; TL/SVL 0.52; heels not in contact when hind limbs are held perpendicular; inner tarsal fold distinct, extending from base of inner metatarsal tubercle almost to heel; outer tarsal fold absent; calcars and heel tubercles absent; toes short, bearing small round discs, slightly smaller than discs on fingers, approximately same width as toes, relative lengths of toes I, II, V, III, IV; toes bearing lateral fringes; subarticular tubercles moderately large, round in ventral view, rounded in profile; distal subarticular tubercles of toes IV and V bifid, supernumerary tubercles absent; outer metatarsal tubercle small, indistinct; inner metatarsal tubercle distinct, elliptical in ventral view, flat in profile, in contact with inner tarsal fold and medial fringe of first toe; toes almost fully webbed, webbing moderately thick; webbing extensive between toes III, IV, and V. Skin of all dorsal surfaces smooth; skin of flanks with scattered low warts; skin in axilla, groin, and on anterior surface of thighs smooth; skin of throat, chest, belly, and ventral surfaces of thighs coarsely areolate, skin of tibia and concealed surface of tarsus smooth. Cloacal opening with slight dorsal flap, directed posteriorly at upper level of thighs; cloacal sheath distinct; cloacal tubercles slightly distinct, scattered. Tongue cordiform, filling buccal cavity, barely free on posterior end, posterior margin slightly notched; dentigerous processes of vomers prominent, in two slightly angled series, each bearing three–four teeth, narrowly separated medially; choanae of moderate size, lateral to and narrowly separated from vomers; vocal slits moderately long, extending from midlength of tongue, almost reaching to angle of jaws; vocal sac single, median, subgular covering almost all of the chin.

Measurements of the holotype (mm).—Snout–vent length 34.7, tibia length 18.2, foot length 14.3, head length 9.8, head width 10.4, interorbital distance 9.1, eye–nostril distance 3.0, eye diameter 4.3, tympanum diameter 2.4, eye–tympanum distance 1.8. Measurements of the paratypes are given in Table 3.

Coloration of holotype in life.—Dorsum bright green; throat pale green, chest and belly grey with multiple pale blue spots; extremities with pale grey and fewer pale blue spots; iris golden.

Coloration of holotype in preservative.—Gray dorsum with faint black spots. Ventral pale gray with cream spots. Venter with pale cream spots. Legs display similar color as arms, having fewer cream spots than chest and throat.

Variation.—In the paratype series, individuals from higher elevations (Aquitania and Paramo de la Rusia) are larger, on average, than individuals from lower elevations, replicating the phenomenon observed in the sister species *D. labialis*, where there is a strong correlation between elevation and body size (Amézquita, 1999). The small sample size of females prevents analysis of size dimorphism; however, the available adult female specimens are larger than males. One from Sativanorte is 50.0 mm, and from Paramo de la Rusia 49.6 mm). There is geographic variation within and between populations in the dorsal coloration of individuals in life, which may be various shades of pale green, grading into dark brown. Dorsal coloration is lost in preserved specimens, but not the pattern of dorsal

markings, which display extensive variation among individuals.

Distribution and ecology.—*Dendropsophus luddeckei* is a highland Andean species found northeast of the cities Chiquinquirá, Boyacá (5°10′16.21″N, 73°40′05.52″W; datum = WGS84) and Choconta, Cundinamarca (5°10′16.21″N, 73°40′05.52″W), reaching Departamento de Norte de Santander in Colombia. One of the few hylid species known from high elevations, its elevational range goes from 2000 m (Villa de Leyva, Boyacá) to 4100 m (Serranía del Cocuy, Boyacá). It is abundant and easy to find near temporary or permanent ponds, as well as very humid places in urban areas. Males have been seen calling near large roads or highly populated areas. Even though *D. luddeckei* has nocturnal reproductive activity, it is a heliothermic species and thermoregulates in open areas during the day.

Etymology.—This species epithet is a patronym in recognition of Professor Horst Lüddecke Dr. Rer. Nat. For more than 30 yr, he produced important contributions to the areas of behavior, physiology, and biogeography of high Andean anurans of Colombia, especially *D. labialis*. He encouraged and served as a model to many generations of Colombian biologists.

All nominal species that are now junior subjective synonyms of *D. labialis* (Peters)—*Hyla creolica* Werner 1899, *Hyla servalina* Werner 1899, *Hyla vilsoniana* Cope 1899, *Hyla gularis* Werner 1916, *Hyla vilsoniana krausi* Hellmich 1940, *Hyla labialis labialis* (see Cochran and Goin, 1970), and *Hyla labialis krausi* (see Cochran and Goin, 1970)—were described from holotypes that geographically correspond to the Southern Clade (Cochran and Goin, 1970). No nominal species are based on holotypes from within the range of the Northern Clade. There exists the possibility that some of the holotypes that are reported to originate from Bogotá in fact were collected from localities further north, but there is no direct evidence for this.

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Appendix

Box plot showing the effect of type of cross on larval survival in *Dendropsophus labialis*. Figure obtained from Ulloa (2003) with her permission. The localities from the Northern Clade were 14 and 19 (Table 1). The localities from the Southern Clade were 25, 27 (Table 1) and another two that do not appear in Table 1: Suesca (05°00′30.60″N, 73°46′37.92″W) and Mondoñedo (04°41′0.38″N, 74°15′49.00″W). The horizontal line within the gray boxes indicates the median. Above and below the median are the upper and lower quartiles, respectively. The error bars indicate the smallest and largest observations, and the circles indicate outliers.