Bioacoustic and genetic divergence in a frog with a wide geographical distribution

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The study of intraspecific variation of acoustic signals and its relationship with genetic divergence is important for understanding the origin of divergence in communication systems. We studied geographical variation in the acoustic structure of advertisement calls from five populations of the four-eyed frog, *Pleurodema thaul*, and its relationship with the genetic divergence among these populations. By analyzing temporal and spectral parameters of the advertisement calls, we report that the signals of northern, central, and southern populations have remarkable differences between them. A phylogeographical analysis from a mitochondrial DNA fragment demonstrated three phylogenetic groups coincident with those found with the bioacoustics analysis. Furthermore, bioacoustic and genetic distances show significant correlations after controlling for geographical distance. These results suggest that behavioural divergence among populations of *P. thaul* has a phylogenetic basis, supporting three evolutionary units within this species, as well as prompting the exploration of divergence processes in the sound communication system of this species. © 2013 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2013, ••, ••–••.

ADDITIONAL KEYWORDS: acoustic communication – advertisement call – *Pleurodema thaul* – variation.

INTRODUCTION

Animal communication signals have an important role in species divergence, promoting reproductive isolation and speciation (Coyne & Orr, 2004). These signals were originally considered as invariant traits promoting the recognition of conspecifics and discrimination of heterospecifics (Foster & Endler, 1999). However, in recent decades, variation of behavioural traits involved in communication within specific taxa has been a topic of extensive research, mainly in the context of mate choice and sexual selection (Campbell, 1972; Bateson, 1983; Endler, 1993; Andersson, 1994; Mitani, Hunley & Murdoch, 1999;

Irwin, 2000). In particular, variation of behavioural characters across geographical distribution has been the subject of studies aiming to establish the degree of divergence among populations (Coyne & Orr, 1998, 2004; Stafford, Nieukirk & Fox, 2001; Quispe *et al.*, 2009) or the relevance of adaptations to different environmental conditions (Hunter & Krebs, 1979; Boughman, 2002; van Dongen *et al.*, 2010). Studies on the concomitant divergence of behavioural and genetic characters have yielded contrasting results, supporting both correspondences between the diverging traits (Päckert *et al.*, 2004; Irwin, Thimgan & Irwin, 2008; Toews & Irwin, 2008) and a lack of association between the two domains (MacDougall-Shackleton & MacDougall-Shackleton, 2001; Christianson, Swallow & Wilkinson, 2005; Pröhl *et al.*, 2006, 2007; Ruegg *et al.*, 2006).

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Covariation between genetic and behavioural traits has been explained mainly on the basis of direct relationships between genetic divergence and geographical distances among populations (i.e. isolation-by-distance: Sokal, 1988; Ramachandran *et al*., 2005) or between genetic divergence and temporal separation among taxa (i.e. molecular clock hypothesis: Kumar, 2005; Ho *et al*., 2007; Ho, 2008; Weir & Schluter, 2008). These processes result predominantly in intra- and interspecific divergence, respectively. On the other hand, the lack of association between genetic and behavioural traits has been attributed to the polygenic nature of behavioural traits (Wimer & Wimer, 1985; Coyne & Orr, 1998; Ritchie & Phillips, 1998; Rosenthal & García de León, 2006) or to the effects of selection on call divergence (Griffith & Sheldon, 2001; Relyea, 2001; Sih, Bell & Chadwick, 2004).

Anurans are animals in which calling behaviour is crucial for mating: males produce advertisement calls that attract females and, in some species, promote the establishment of territories (Gerhardt & Huber, 2002). Geographical variation of call structure has been studied in a number of anuran species, and its occurrence has been related to factors such as differences in body size (Castellano, Giacoma & Dujsebayeva, 2000; Giacoma & Castellano, 2001; Castellano *et al*., 2002) or differences in the size of laryngeal structures (McClelland, Wilczynski & Ryan, 1996, 1998; Boul & Ryan, 2004). Clinal variation of advertisement calls related to geographical distance between populations has been reported for different anurans (Nevo & Capranica, 1985; Wilczynski & Ryan, 1999; Castellano *et al*., 2000; Giacoma & Castellano, 2001; Bernal, Guarnizo & Lüddecke, 2005; Pröhl *et al*., 2006, 2007); however, studies exploring relationships between call structure and genetic divergence have shown contrasting results (for a direct relationship, see Funk, Cannatella & Ryan, 2009; for a lack of relationship, see Pröhl *et al*., 2006, 2007). A correlation between call structure and genetic divergence would support the importance of phylogenetic history, whereas the absence of such relationship points to the influence of the acoustic or physical environment on call divergence.

Pleurodema thaul (*Leiuperidae*) is the anuran having the most extensive geographical distribution in Chile (Ortiz & Diaz-Paez, 2006), ranging from the Atacama desert (27°06′S, 69°53′W) (Correa *et al*., 2007) to the Patagonian region (45°24′S, 72°42′W) (Fig. 1A) (Veloso, 2006; Correa *et al*., 2007), encompassing a latitudinal range of over 2200 km and 20° in latitude. In addition, this species has one of the largest altitudinal distribution ranges in Chilean anurans, ranging from 0 to about 3000 m a.s.l. (Formas, 1979; Correa *et al*., 2007, 2010). This anuran exhibits an important phenotypic and genetic variation along its latitudinal range. Duellman & Veloso (1977) contributed morphological, life-history, and cytogenetic evidence for the existence of two lineages (northern-central and southern) of *P. thaul* in Chile. Victoriano *et al*. (1995) reported three stocks, namely, northern, central, and southern, based on analysis of allozymic variation. Recently, Correa *et al*. (2008), based on mitochondrial DNA sequences, have also proposed the existence of three latitudinal lineages.

Pleurodema thaul has a prolonged breeding season, from July to December, during which males call from the water surface in slow flowing creeks, pools, and lagoons, perched on emergent vegetation, with their vocal sacs inflated. Its advertisement call consists of a long sequence of pulses, each of which has duration of approximately 50 ms and contains intrapulse amplitude modulations. The spectrum has a dominant frequency centered at approximately 2 kHz (Penna & Veloso, 1990; Solís, 1994; Penna, Velásquez & Solís, 2008).

In the present study, we analyzed both advertisement call and genetic variation in populations of *P. thaul* encompassing an extensive latitudinal range in Chile, aiming to test the hypothesis that the divergence in both traits has followed similar patterns. Such correspondence could result from restricted gene flow among populations because of geographical distance and isolation. Alternatively, selection imposed by environmental factors might cause calls to diverge, as reported for other anurans (Nevo, 1973; Castellano *et al*., 2000; Castellano *et al*., 2002; Gerhardt & Huber, 2002). In *P. thaul*, environmental factors diverge considerably among populations exposed to conditions ranging from extreme northern desert settings to southern temperate forests,

The analysis of the variation in the acoustic structure of the advertisement calls and genetic distances among populations of *P. thaul* would provide the basis for further explorations on the divergence in the sound communication system of this species along its latitudinal distribution and on the implications of this variation for intra- and intersexual selective processes.

MATERIAL AND METHODS
STUDY SITES

We recorded the advertisement calls of males of *P. thaul* of five populations, encompassing more than 1500 km of the extensive latitudinal geographical distribution range of the species. Recordings were conducted from July to December 2008 and from September to November 2009. The populations and
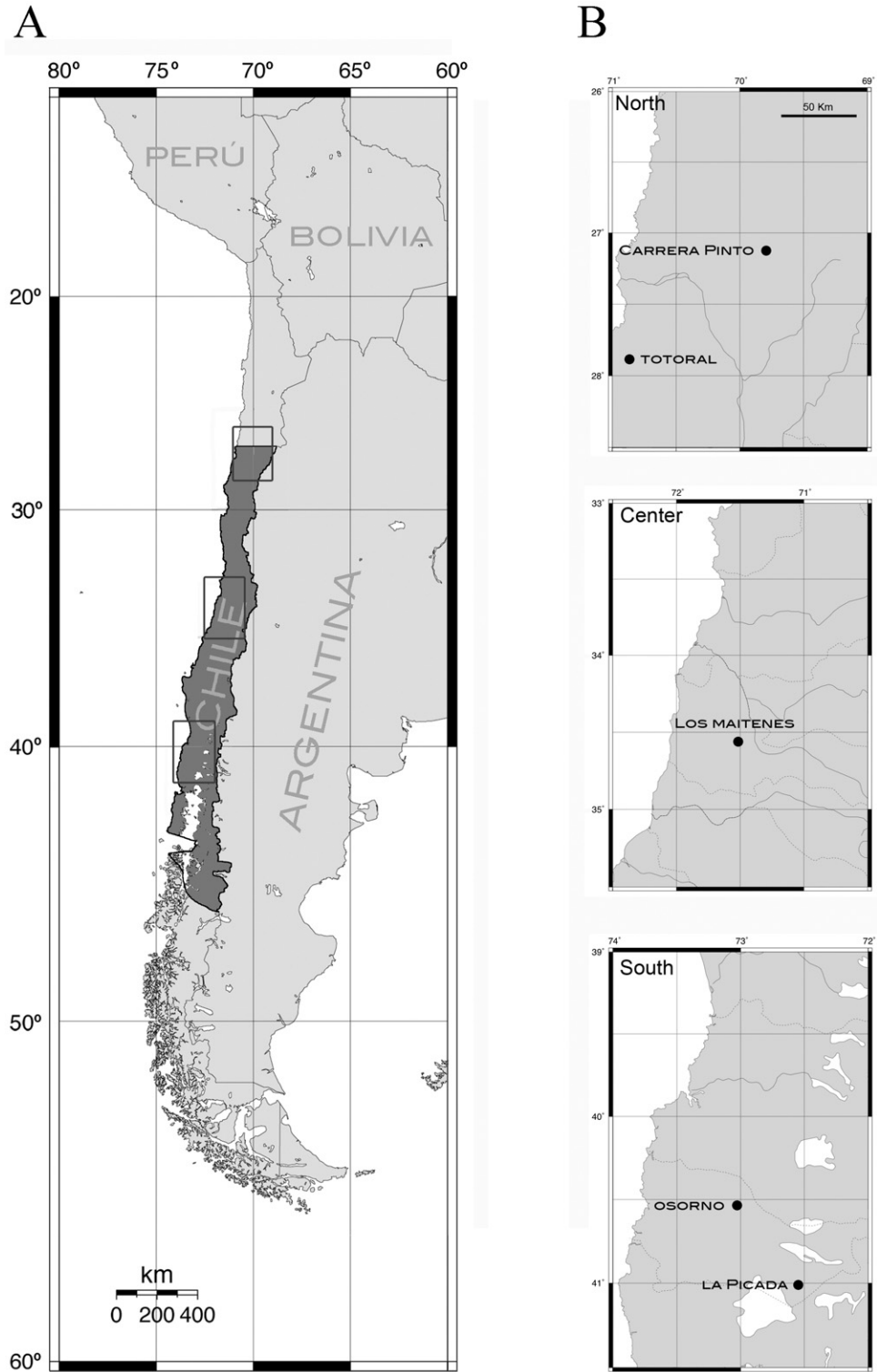
Figure 1. A, distribution of *Pleurodema thaul* in Chile. Rectangles indicate insets shown in (B). B, localities where recordings of advertisement calls of *P. thaul* were carried out.

the number of subjects studied from north to south were: Carrera Pinto (27°06′S, 69°53′W; $N = 13$), Totoral (27°54′S, 70°56′W; $N = 27$), Los Maitenes (34°41′S, 71°26′W; $N = 17$), Osorno (40°35′S, 73°03′W; $N = 23$), La Picada (41°00′S, 72°33′W; $N = 17$) (Fig. 1). The latitude and longitude of each population were recorded using the Global Positioning System (Garmin eTrex Vista H; Garmin International, Inc.). This distribution provided a sampling of advertisement calls at three latitudinal levels.

RECORDING AND ANALYSIS OF ACOUSTIC SIGNALS

The advertisement calls were recorded with a directional microphone (Sennheiser ME66, Electronic GmbH and Co., KG) positioned 0.2–0.5 m from the calling subjects, which were typically perched on the water surface among emergent vegetation, with their vocal sacs inflated. The calls were recorded on a Sony TC D10 PROII digital tape recorder (Sony Electronics, Inc.) or a Tascam HD-P2 digital recorder. The recordings were carried out from 21.00 h to 04.00 h and, after each recording, we measured both air and water temperature with a digital thermometer (Digi-Sense 8528-20; Cole-Parmer Instrument Company). These temperatures were used instead of body temperature because animal handling can affect measurements. Subjects were captured to measure body weight (Pocket Pro Balance; Acculab; ±0.01 g) and

snout–vent length (SVL) (Digital Calliper; Traceable; ±0.001 mm).

The acoustic recordings obtained with a Sony TC D10 PROII digital tape recorder were digitized with a Macintosh computer (G4 Power PC) with PEAK, version 2.52 (Bias, Inc.) at a sampling rate of 44.1 kHz, using an anti-aliasing filter (FT6-2; Tucker-Davis Technologies, Inc.) and an analogue–digital interface (Motu 828; Mark of the Unicorn). The recordings obtained with the Tascam HD-P2 digital recorder were carried out at the same sampling rate and directly downloaded to the computer. The WAV files generated were filtered (high-pass filter, cut-off: 200 Hiltz) using RAVEN PRO, version 1.3 (Laboratory of Ornithology, Cornell University). Calls were analyzed with custom-built scripts in MATLAB, version 7.5 (MathWorks). The scripts implemented analysis in the time and frequency domains, allowing temporal variables to be extracted from oscillograms: call duration (CD), number of pulses (NP), pulse duration (PD), inter-pulse interval duration (ID), pulse rate (PR), and modulation depth (MD). In addition, the dominant frequency (DF) in a pulse from approximately the mid point of each call was analyzed (Fig. 2). To obtain the six temporal parameters, the signal envelope of the entire call was computed as the modulus of the analytical signal obtained from the Hilbert transform of the time series. By applying an amplitude threshold to the envelope superimposed
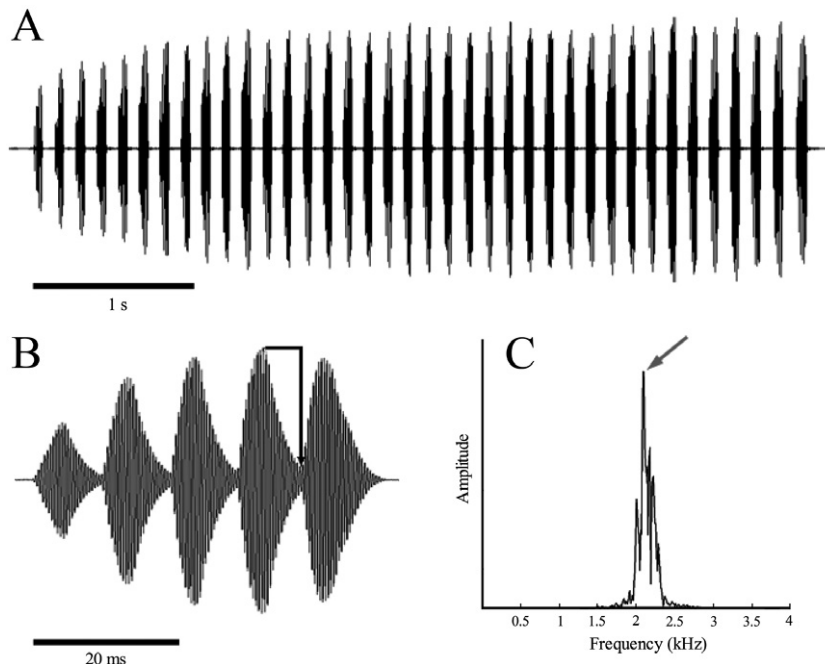

Figure 2. A, oscillogram of an advertisement call of a male of *Pleurodema thaul* from Los Maitenes (central population). B, expanded central pulse of the call shown in (A) (arrow indicates the modulation depth). C, power spectrum of the pulse shown in (B), indicating the dominant frequency.

on the original signal, a logical vector defining the presence (1) or absence (0) of pulsed signals was automatically created. The transform produced was visually inspected and errors were corrected manually if necessary. From this vector, each call was segmented in its corresponding pulses, the initial and final times of which were used to obtain the first five temporal variables. The sixth temporal variable, modulation depth, corresponded to the ratio of the difference between the maximum and minimum envelope amplitude and the maximum envelope amplitude within a pulse, expressed as a percentage. The dominant frequency was obtained as the peak of the fast Fourier transform computed for a central pulse. At least five calls were analyzed for each individual and the mean for each variable was calculated. Because advertisement calls of anurans are affected by environmental and morphological variables (Gerhardt & Huber, 2002), we corrected some temporal variables by water temperature and the dominant frequency by SVL. Water temperature was chosen because this parameter represents the temperature at which the individual is calling. On the other hand, SVL showed a high correlation with body weight (Pearson's correlation: $r = 0.967$; $P < 0.0001$), although the first one was chosen because it showed less variation. Pearson's correlations were carried out to study the relationship between the environmental and morphological variables (predictors) and the temporal variables and dominant frequency, respectively. We corrected those acoustic variables that shown significant correlations with its corresponding predictor (see Supporting information, Table S1). *Sensu* Pröhl *et al.* (2007), regression coefficients were calculated between each acoustic variable and water temperature or SVL, to adjust the acoustic variables to the total means of water temperature and SVL. The equation used for the corrections was: $Y_{corr} = Y - (b \times X_{measured}) + (b \times X_{mean})$, where Y_{corr} is the corrected call variable, Y is the original value of an acoustic variable, b is the regression coefficient, $X_{measured}$ is the water temperature or SVL measured after of each recording, and X_{mean} is the mean value of each of the seven acoustic variables for all populations. These corrected values of the acoustic variables were used for all subsequent analyses.

PHYLOGEOGRAPHICAL ANALYSIS AND GENETIC DISTANCES

We obtained tissue samples from a total of 44 males of *P. thaul* for which advertisement calls were recorded: Carrera Pinto ($N = 10$), Totoral ($N = 10$), Los Maitenes ($N = 10$), Osorno ($N = 9$), and La Picada ($N = 5$). All material was deposited in the herpetological collection of the University of Chile (DBGUCH) (see Supporting information, Table S2). All individuals used in the study were treated using procedures approved by the Ethics Committee of the University of Chile and CONICYT, based on the recommendations of the US National Research Council.

Total DNA of each individual was isolated from a toe using a method of salt extraction (modified from Jowett, 1986). A mitochondrial DNA fragment that extends from the ribosomal *12S RNA* gene to the ribosomal *16S RNA* gene was amplified. The primers utilized were: 1216LN (5′-CCAAYACGTCA GGTCAAGGTG-3′, modified from L2751), 1216H (5′-TGATTACGCTACCTTYGCACGGT-3′, modified from Hedges16H10), 16Sar-L (5′-CGCCTGTTTATCAAAAA CAT-3′) and 16Sbr-H (5′-CCGGTCTGAACTCAGATC ACGT-3′), all reported by (Goebel, Donnelly & Atz, 1999). The reaction mixture for amplification included 3 mM MgCl$_2$, 0.6 mM of each dNTP, 0.12 µM of each primer 1216LN and 1216H (0.2 µM of each primer 16Sar-L and 16Sbr-H), 1.25 U of *Taq* polymerase (Invitrogen) and 50–100 ng of total DNA. The thermal profiles for PCR were: 94 °C for 3 min, followed by 37 cycles of 94 °C for 35 s, 56 °C for 45 s, and 72 °C for 90 s for primers 1216LN and 1216H (35 cycles of 94 °C for 30 s, 58 °C for 45 s and 72 °C for 45 s for the primers 16Sar-L and 16Sbr-H), and a final extension at 72 °C for 10 min.

PCR products were sequenced in both directions at Macrogen, Inc. using an ABI3730XL automatic DNA sequencer (Applied Biosystems). The DNA sequences of individuals were inspected using BIOEDIT, version 7.0.7.0 (Hall, 1999). Sequences were aligned with CLUSTALX, version 1.81 (Thompson *et al.*, 1997), using the default parameters and alignments were inspected visually.

The genetic analyses performed were: (1) Neighbour-joining (NJ) using MEGA, version 5 (Tamura *et al.*, 2011) and (2) Bayesian inferences (BI) using MrBayes, version 3.1.2 (Ronquist *et al.*, 2011). In this case, the best model of sequence evolution obtained with JMODELTEST, version 0.1.1 (Posada, 2008), under the Bayesian information criterion, was TIM2+I. For this analysis, we ran 10 000 000 generations with a burn-in period of 10%. For all analyses, we used *Pleurodema bufoninum* as outgroup, considering that it is the sister group of *P. thaul* (Faivovicha *et al.*, 2012). Finally, a network was constructed using a median-joining haplotype network with NETWORK, version 4.1 (http://www.fluxus-engineering.com; Bandelt, Forster & Rohl, 1999).

STATISTICAL ANALYSIS

We used coefficients of variation (CV) to explore the dispersion of each call variable at three levels: intra-

individual, intra-population, and inter-population. Intra-individual CVs for each variable were calculated as the (SD/mean) × 100 for all calls recorded from each subject. Intra-population CVs for each variable were calculated from the means and SDs obtained for individuals of a given population. Inter-population CVs for each variable were calculated from the means and SDs obtained for the five populations considered in the present study. From the results of intra-individual CVs in each population, we classified the acoustic variables as static or dynamic. We used the univariate general linear model (GLM) to study the variation of the acoustic parameters, morphological parameters, and temperature among populations. A stepwise discriminant analysis was carried out with all of the acoustic variables measured to obtain the discriminant functions and the original acoustic variables that showed the highest correlations with these discriminant functions. We used Jackknife sub-sampling to evaluate classification success. All *a posteriori* multiple comparisons were performed with Tukey's tests.

We assessed genetic distances using MEGA, version 5 (Tamura *et al*., 2011), building a genetic distance matrix that grouped sequences by locality. All positions containing gaps and missing data were eliminated. We calculated the *p*-distance for statistical purposes. The bioacoustic distance matrix was built from Manhattan distances between the means of all variables of advertisement calls, whereas the geographical distance matrix was built from georeferences taken in each population. A Mantel test and a partial Mantel test with 10 000 permutations were performed to study the correlation between the bioacoustic, genetic, and geographical distances (i.e. 5×5 matrices). The hypothesis used for all Mantel tests and partial Mantel tests was $r < > 0$. GLMs and stepwise discriminant analysis were carried out using STATISTICA, version 8.0 (StatSoft, Inc.). The Mantel test and partial Mantel test were performed using XLSTAT 2011 (Addinsoft SARL).

RESULTS

ENVIRONMENTAL AND MORPHOLOGICAL VARIABLES

Water and air temperatures measured after recording the advertisement calls, and the weight and SVLs of individual males, showed significant differences among the five populations considered in the present study (univariate GLM: weight: $F_{4,92} = 107.021$, $P < 0.001$; SVL: $F_{4,92} = 136.307$, $P < 0.001$; T_{air}: $F_{4,92} = 5.692$, $P < 0.001$; T_{water}: $F_{4,92} = 2.749$, $P = 0.033$) (Fig. 3A). In general, subjects from the northern populations had a lower body weight and body size relative to subjects from the central and southern populations.

Body weight differed significantly among all populations, with the exception of the two northern populations (Tukey's test: $P = 0.092$). Air temperatures differed among populations: values in the northernmost population of Carrera Pinto were significantly lower compared to the four other populations (Tukey's test: $P < 0.05$ for all comparisons), in addition, water temperature in Osorno was significantly lower than in La Picada (Tukey's test: $P = 0.036$).

GEOGRAPHICAL VARIATION IN THE ADVERTISEMENT CALLS

Overall, we recorded advertisement calls of 97 males of *P. thaul* at the five localities considered in the present study. Figure 3B shows the variation in the acoustic variables that were measured. The analysis of dispersion using the CVs of these variables at three levels showed that, at the intra-individual level, the variables CD, NP, PD, and ID had large CVs, most often above 8.0, whereas the variables, PR, MD, and DF had small CVs, generally below 8.0 (Table 1). These results prompted us to classify the first group as dynamic variables, and the second group as static variables. However, this classification is not strict because some variables, in particular PR and MD, had CVs lower or higher than 10.0, depending on the population analyzed. Because intra-individual variation of advertisement calls is species-dependent, we decided to modify the classification range used by Gerhardt (1991). We considered dynamic variables as those having a coefficient of variation > 8, whereas those with a coefficient of variation < 8 were classified as static. The general trend described for intra-individual variation was similar both at the intra- and inter-population level.

All seven variables analyzed for the advertisement call of *P. thaul* showed significant differences among populations (univariate GLM: CD: $F_{4,92} = 27.326$, $P < 0.001$; NP: $F_{4,92} = 31.621$, $P < 0.001$; PD: $F_{4,92} = 3.728$, $P < 0.001$; ID: $F_{4,92} = 23.460$, $P < 0.001$; PR: $F_{4,92} = 31.460$, $P < 0.001$; MD: $F_{4,92} = 42.898$, $P < 0.001$; DF: $F_{4,92} = 56.063$, $P < 0.001$). A clear variation pattern comprising larger CD, NP, and MD in the northern and central populations relative to those of the two southern populations was observed. By contrast, DF did not differ between northern and southern populations but was significantly higher in the central population of Los Maitenes (Tukey's tests: $P < 0.05$) (Fig. 3B).

A stepwise discriminant analysis performed on the temporal variables of the calls enables discrimination between the populations (Wilks' $\lambda = 0.017$, $F_{28,311} = 23.217$, $P < 0.001$). The first two discriminant axis explained most of the variation among populations (CV1 = 59.8%, CV2 = 33.1%) (Table 2). The
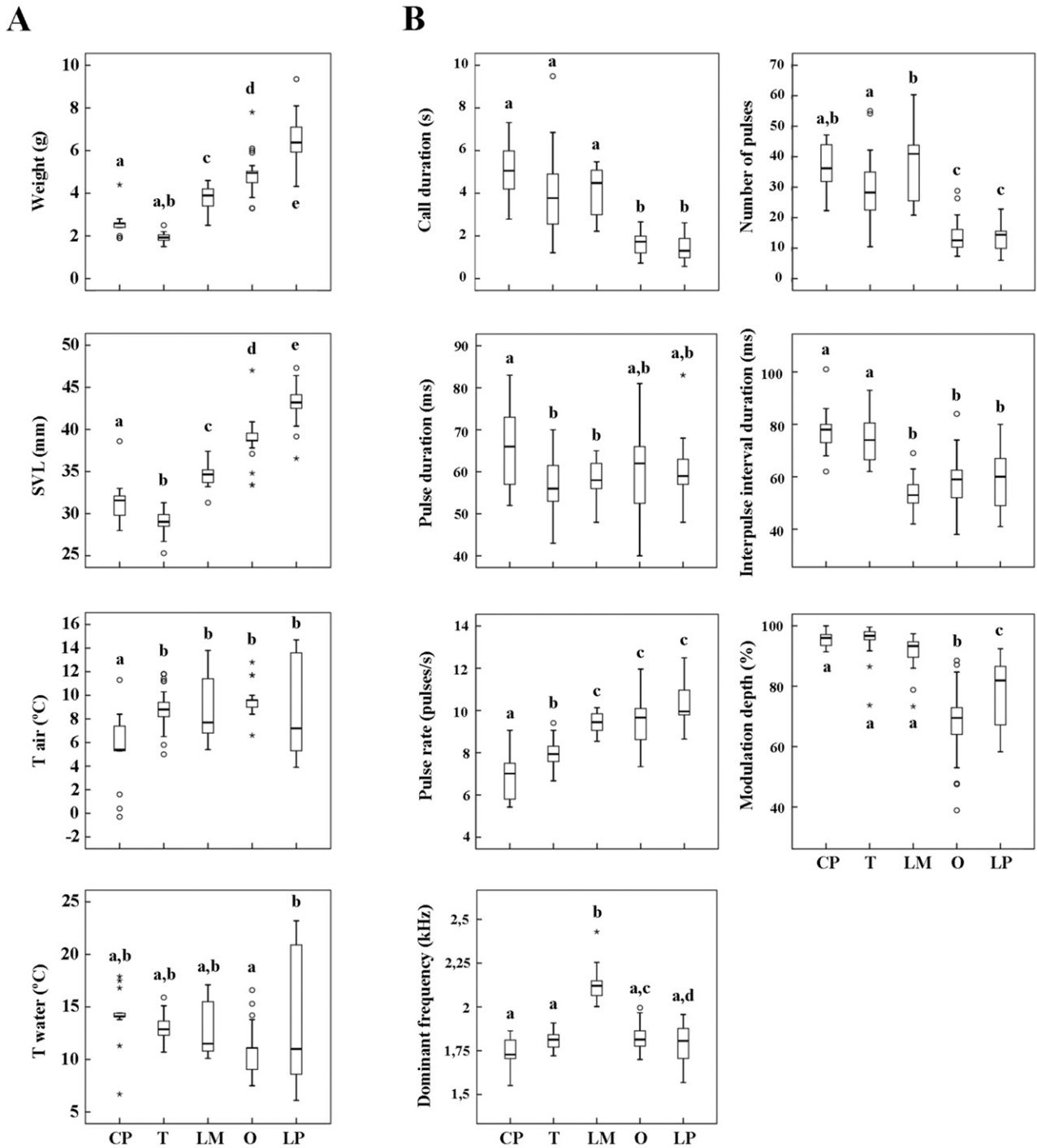
Figure 3. Box plot of all variables measured. A, morphological [weight and snout–vent length (SVL)] and environmental (air and water temperature) variables for the five populations studied (CP, Carrera Pinto; T, Totoral; LM, Los Maitenes; O, Osorno; LP, La Picada). B, acoustic variables of advertisement calls of frogs from the five populations considered in the present study. Different lowercase letters inside each graph indicate significant differences.

Table 1. Coefficients of variation for acoustic variables in five populations of *Pleurodema thaul* in Chile

Analysis level	Population	CD	NP	PD	ID	PR	MD	DF
Intra-individual	Carrera Pinto	46.39	42.39	13.18	19.84	10.82	2.43	2.01
	Totoral	40.81	37.49	11.82	12.15	7.09	2.52	2.44
	Los Maitenes	39.44	37.74	6.78	13.25	5.85	3.29	1.86
	Osorno	37.85	33.98	10.20	9.85	4.77	15.96	1.90
	La Picada	36.98	32.63	10.22	8.85	6.10	12.09	2.34
	Mean	40.29	36.85	10.43	12.79	6.93	7.26	2.11
Intra-population	Carrera Pinto	27.65	23.99	14.69	12.58	17.76	3.26	5.07
	Totoral	47.67	39.15	11.46	12.70	8.47	5.37	2.83
	Los Maitenes	27.72	30.88	7.11	13.25	5.10	7.13	4.94
	Osorno	32.80	40.73	16.65	16.92	12.88	18.61	4.26
	La Picada	44.66	30.38	12.50	18.25	10.13	13.87	6.50
	Mean	36.10	33.03	12.48	14.74	10.87	9.65	4.72
Inter-population		58.39	53.71	13.78	20.38	16.28	16.80	8.45

CD, call duration; NP, number of pulses; PD, pulse duration; ID, inter-pulse interval duration; PR, pulse rate; MD, modulation depth; DF, dominant frequency.

Table 2. Canonical loading, eigenvalues, and explained variance percentage of each canonical variable from discriminant analysis

	CV1	CV2	CV3	CV4
Call duration	−0.398	−1.035	1.093	−0.216
Number of pulses	1.005	0.725	−1.079	0.562
Pulse duration	−0.856	0.062	−0.903	0.790
Interpulse interval duration	0.267	0.466	−0.675	−0.299
Pulse rate	−0.867	−0.140	−1.266	−0.114
Modulation depth	0.624	−0.037	−0.651	−0.183
Dominant frequency	−0.042	−1.028	0.225	−0.168
Eigenvalues	6.159	3.414	0.532	0.202
Explained %	59.753	33.123	5.160	1.964

structure matrix, which correlates the discriminant functions with the original variables, indicates that MD was the variable with the highest correlation with CV1 ($r = 0.524$) and DF had the highest correlation with CV2 ($r = -0.843$) (Fig. 4C, Table 2), with these comprising the two variables explaining the largest proportion of inter-population variation of the advertisement calls of *P. thaul*. CV1 allowed us to discriminate two groups: a northern-central group (Carrera Pinto, Totoral, Los Maitenes) and a southern group (Osorno, La Picada), whereas CV2 highlighted the differences between Los Maitenes and the other populations. In addition, the discriminant analysis showed that 81.4% of the recorded subjects were correctly classified as belonging to their population of origin. Errors in the classification occurred mostly within each geographical zone, with only one exception that classified one subject as belonging to a population from another geographical region (a frog from Carrera Pinto that was assigned to La Picada). The percentage of correct classification decreased to 76.3% when we used the Jackknife sub-sampling strategy (Table 3).

GENETIC VARIATION BETWEEN POPULATIONS

The primers produced two partially superimposed fragments of approximately 1300 bp (1216LN and 1216H) and 600 bp (16Sar-L and 16Sbr-H). The alignment of both fragments results in a total length of 1734 nucleotide sites. All sequences were deposited in GenBank (GenBank accession numbers: JN559413–JN559456; see Supporting information, Table S2).

Coincidentally three groups were obtained using NJ and BI: a group comprising individuals from Carrera Pinto and Totoral; a second group comprising individuals from Los Maitenes; and a third group
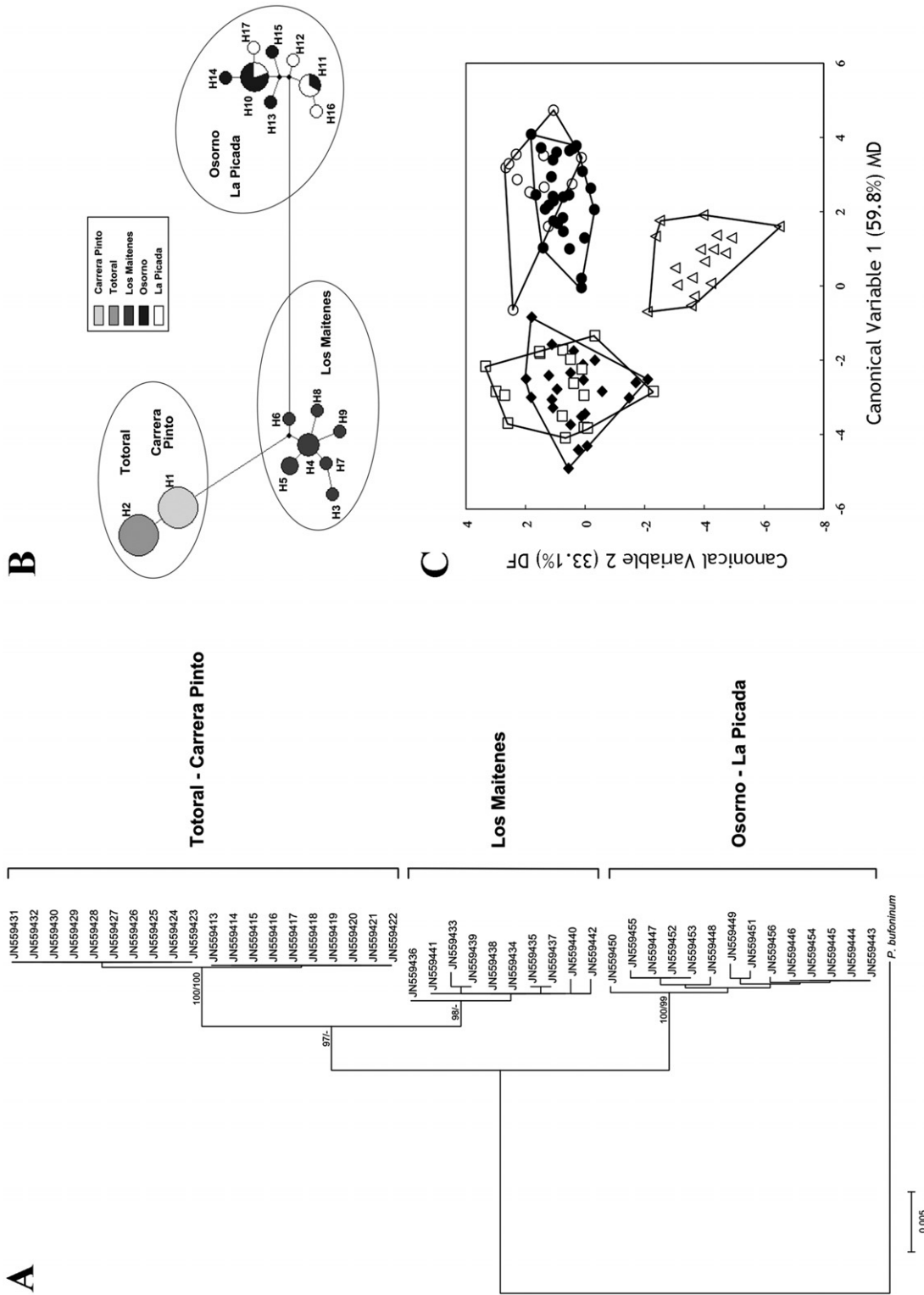
Figure 4. A, phylogeographic relationships of *Pleurodema thaul* populations, obtained with Neighbour-joining (NJ) and Bayesian inference (BI). Numbers on each node indicate bootstrap estimations for NJ and credibility values for BI. B, median-joining network of *P. thaul* from five populations. C, stepwise discriminant analysis preformed on all acoustic variables of the advertisement calls of *Pleurodema thaul* populations. The first two canonical variables explained almost 93% of variation among calls of different populations. The explained variance for CV1 was mainly associated with modulation depth and the explained variance for CV2 was mainly associated with dominant frequency (see text). Empty circles, Carrera Pinto; filled circles, Totoral; triangles, Los Maitenes; squares, La Picada; diamonds, Osorno.

Table 3. Stepwise classification matrix

	Carrera Pinto	Totoral	Los Maitenes	Osorno	La Picada	Total
Carrera Pinto	8 (7)	4 (5)	0 (0)	0 (0)	1 (1)	13
Totoral	5 (6)	22 (21)	0 (0.0)	0 (0)	0 (0)	27
Los Maitenes	0 (0)	0 (0)	17 (17)	0 (0)	0 (0)	17
Osorno	0 (0)	0 (0)	0 (0)	17 (16)	6 (7)	23
La Picada	0 (0)	0 (0)	0 (0)	2 (4)	15 (13)	17

Values correspond to the number of individuals classified with respect to each population. Numbers in parentheses correspond to the same classification using the Jackknife sub-sampling strategy.

comprising individuals from La Picada and Osorno. Most of the nodes had high bootstrap support values (NJ) and high values of credibility (BI) (Fig. 4A). The network analysis showed the same pattern as that observed with the NJ and BI analyses (Fig. 4B).

A Mantel test performed between the bioacoustic and geographical distance matrices showed no significant correlation between these distances (Mantel test: $r = 0.457$, $P = 0.189$) with this correlation being maintained when controlled by genetic distance (partial Mantel test: $r = -0.593$, $P = 0.068$). Both geographical and genetic distances also showed a high correlation (Mantel test: $r = 0.887$, $P < 0.001$) and this relationship was maintained after controlling by bioacoustic distance (partial Mantel test: $r = 0.908$, $P < 0.001$). Similarly, bioacoustic and genetic distances were highly correlated (Mantel test: $r = 0.727$, $P < 0.015$), although this relationship persisted when controlled for by the geographical distance (partial Mantel test: $r = 0.784$, $P = 0.023$).

DISCUSSION

Both advertisement calls and genetic markers of *P. thaul* show a remarkable variation along the latitudinal gradient. Phylogeographical analysis shows that the populations considered in the present study are grouped into three lineages (Fig. 4A, B). Similarly, the discriminant analysis that considered all acoustic variables classified the populations into three bioacoustic groups (Fig. 4C). The large congruence between the bioacoustic and phylogeographical analyses indicates that the divergence observed at the behavioural level among populations of *P. thaul* has a phylogenetic basis, supporting three evolutionary units within this species.

Our results support the idea that the structure of acoustic signals is closely related to genetic differences among populations. Relationships between bioacoustic traits and genetic differences have also been reported in another frog (Amézquita *et al*., 2009; Funk *et al*., 2009). By contrast, other studies have found that variation in advertisement calls is associated with geographical rather than with genetic distances (Ryan, Rand & Weigt, 1996; Pröhl *et al*., 2006, 2007). The high correlation between bioacoustic and phylogenetic data indicates that the divergence in the sound communication system of this anuran is highly consolidated, and also shows that signals are reliable indicators of the genetic distance among populations, as shown to occur in other vertebrates, including primates (Thinh *et al*., 2011; Meyer *et al*., 2012). This correspondence is also supported by the high correlation found in the Mantel test between bioacoustic and genetic distance even after it is controlled for geographical distance. This correlation suggests that divergence among populations of *P. thaul* has resulted mainly from genetic drift and the effects of isolation, rather than selection.

The advertisement call of *P. thaul* exhibits a large variation along the extended latitudinal range of this anuran species in Chile. Both temporal and spectral variables showed differences among the populations that were studied. In particular, intra-individual analysis showed that the variables with a higher variation were call duration, number of pulses, pulse duration, and inter-pulse interval duration, whereas pulse rate, modulation depth, and dominant frequency had a restricted variation at this level. We classified the first group of variables as dynamic and the second as static Gerhardt (1991). However, this classification is a simplification, with static and dynamic categories representing the ends of a continuum (Gerhardt, 1991; Pröhl, 2003; Reinhold, 2009). The original classification considered a CV of 5 as the limit between both categories but, because variation of these parameters is highly species-dependent, we used a value of 8, which accommodates better to the variation observed in our data. These two kinds of variables have been considered to play different roles in communicative processes: dynamic variables are under energy constraints and thus serve as potential honest indicators of male condition. By contrast, static variables are subject to morpho-physiological constraints, which likely act as indicators of constitutional differences between populations,

contributing information relevant for mate recognition (Gerhardt, 1991; Castellano & Giacoma, 1998; Zimmitti, 1999; Castellano *et al.*, 2000, 2002). Intra-population variation in the static variables of the advertisement call (PR, MD, and DF) was larger than intra-individual variation, suggesting that these variables could be used in individual recognition. The dynamic variable PD also showed mostly larger intra-population relative to intra-individual coefficients of variation. The dynamic variables CD, NP, and ID showed larger intra-individual relative to intra-population variation, and therefore are not well suited for individual recognition, being more likely to denote the physiological state or disposition of individuals with respect to sustaining a confrontation. Similar relationships have been found for properties of vocalizations in other frogs (Gerhardt, 1991; Opazo *et al.*, 2009) and other vertebrates (Lengagne, Luaga & Jouventin, 1997; Charrier, Mathevon & Jouventin, 2003). However, to evaluate possible hypothesis for this geographical variation in signal structure, we would need to carry out phonotaxis tests with females and evoked vocal response experiments with males, searching for preferences for calls of different populations of *P thaul*.

The considerable inter-population variation observed in variables of the advertisement call and the accuracy of classification of individuals into their corresponding populations is consistent with a latitudinal divergence of the advertisement call in this species and allows us to identify calls of the populations sampled along the geographical range of *P. thaul*. Advertisement calls of northern and central populations had large MDs, long CDs, and large NPs, relative to the southern populations (Figs 3, 4C). From the stepwise discriminant analysis performed, MD and DF arise as the acoustic variables accounting for most of the variation among the advertisement calls of different populations. However, variation shown by acoustic parameters among populations does not follow a clinal pattern. Thus, the remarkable differences in the acoustic parameters between calls of different populations of *P. thaul* found in the present study are more consistent with the existence of three bioacoustic groups within this species (northern, central, and southern groups) differing in discrete acoustic parameters rather than in a clinal mode.

By contrast to the divergence in temporal patterns between the northern-central and southern populations, the call DF of the central population of Los Maitenes was higher than in the northern populations, despite the larger body size of frogs from this site. This lack of correspondence between DF and body size contrasts with a number of studies reporting an inverse relationship between these two variables across different populations (Castellano *et al.*, 2000, 2002; Giacoma & Castellano, 2001). In other anurans, DF has shown a relationship with the size of laryngeal structures rather than body size (McClelland *et al.*, 1996, 1998; Boul & Ryan, 2004). Studies of laryngeal morphology of the populations along the latitudinal distribution of *P. thaul* are needed to provide more information about such a possible anatomical determinant.

We have no evidence that relates the patterns of variation of the call of *P. thaul* along its geographical distribution with environmental variables of either a biotic or abiotic nature. Relationships between call structure and abiotic variables have been reported for the North American frog *Acris crepitans* (Ryan, Cocroft & Wilczynski, 1990; Wilczynski & Ryan, 1999). In Chile, northern and central populations of *P. thaul* inhabit arid and Mediterranean climates, respectively, which are relatively similar, in contrast to the humid temperate climate of southern populations (INE, 2008). Northern environments have lower humidity and vegetation coverage, factors that favour the propagation of high frequencies (Wiley, 2009) and, instead, frogs from these populations produce calls with lower frequencies than those in the central zone. However, amplitude modulation depth is a variable that could relate to abiotic environmental differences between the northern-central and the southern populations. In habitats with dense vegetation coverage, a smearing of amplitude modulations is likely to occur and acoustic signals of anurans and birds inhabiting forest environments have less amplitude modulation than species from open habitats (Ryan *et al.*, 1990; Wiley, 2009). The lower amplitude modulation depth of the calls of the southern populations of *P. thaul* relative to northern and central populations is concordant with this tendency.

In addition to abiotic factors, call divergence may also have been driven by biotic factors, among which differences in sound environments are of special relevance. One particular example is the phenomenon of reproductive character displacement, originally restricted to the divergence of behavioural reproductive traits among related syntopic species. This notion has been recently extended by Hoskin & Higgie (2010) to any species interactions that produce divergence in mating traits. Applying this concept to *P. thaul*, differences in temporal structure between the northern-central and southern populations could be related to differences in the biotic sound environment between the two regions. The number of anuran species calling in the breeding sites of *P. thaul* in the northern and central localities is much more restricted than in the southern populations (Penna & Veloso, 1990; N. Velásquez, unpublished data). Under such interferences, it would be favourable for south-

ern *P. thaul* males to emit shorter calls, which are less likely to be masked by concurrent signals produced by other frogs.

Our results obtained from a bioacoustic and genetic analysis provide support for the existence of three evolutionary lineages within *P. thaul*, supporting the grouping proposed by Victoriano *et al*. (1995) and Correa *et al*. (2008), instead of the two lineages proposed by Duellman & Veloso (1977). Overall, the evidence obtained in the present study prompts the need for studies aiming to establish the taxonomic status of distinct lineages within this anuran species along its geographical distribution. Our results also prompt behavioural studies aiming to assess the consequences of geographical variation in advertisement call structure for vocal interactions among males and the phonotactic responses of females. Studies conducted in this perspective should contribute to our understanding of the processes driving divergence in the sound communication system of *P. thaul* and other vertebrates with extensive geographical distributions.

ACKNOWLEDGEMENTS

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Coefficient of Pearson's correlation (*r*) and the regression coefficient (*b*) between water temperature and temporal variables and between snout–vent length (SVL) and dominant frequency for the individuals of the five populations of *Pleurodema thaul*.

Table S2. Locations, voucher, and GenBank accession numbers of the specimens used for the genetic analysis.