
Discordance in body size, colour pattern, and advertisement call across genetically distinct populations in a Neotropical anuran (*Dendropsophus ebraccatus*)

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Patterns of intraspecific geographic variation in morphology and behaviour, when examined in a phylogenetic context, can provide insight into the microevolutionary processes driving population divergence and ultimately speciation. In the present study, we quantified behavioural and phenotypic variation among populations from genetically divergent regions in the Central American treefrog, *Dendropsophus ebraccatus*. Our fine-scale population comparisons demonstrated regional divergence in body size, colour pattern frequencies, and male advertisement call. None of the characters covaried with phylogenetic history or geographic proximity among sampled populations, indicating the importance of highly localized selection pressures and genetic drift in shaping character divergence among isolated regions. The study underscores how multiple phenotypic characters can evolve independently across relatively small spatial scales. © 2009 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2009, 97, 298–313.

ADDITIONAL KEYWORDS: acoustic signal – biogeography – Central America – discordant character evolution – gene flow – microgeographic differentiation – phenotype.

INTRODUCTION

The geographic distribution of phenotypic diversity among populations provides insights into the historical roles of drift and selection, as well as the potential for population diversification and speciation (Foster & Endler, 1999; Panhuis *et al.*, 2001; Gray & McKinnon, 2006; Hoffman *et al.*, 2006). Phenotypic differences can lead to divergent lineages when populations are geographically isolated or subject to strong localized selection (Dobzhansky, 1940; Thorpe *et al.*, 1995; Hoekstra, Krenz & Nachman, 2005; Pröhl *et al.*, 2006). Thus, to fully understand the relationship between phenotypic differentiation and genetic divergence, variation should be examined in light of historical and geographic factors. Vicariance, isolation by distance, and local adaptation can all influence

survival and reproductive success within populations, and in turn contribute to the evolution of particular phenotypes (Lougheed *et al.*, 2006).

Traditionally, variation has been measured along three axes: morphology, behaviour, and genetics. Although not always evolving in the same direction or at the same rate (Marroig & Cheverud, 2001; Wiens & Penkrot, 2002), patterns of covariation among these characters demonstrate the relative roles of gene flow, vicariance, and adaptation in shaping spatial and temporal diversification (Lu & Bernatchez, 1999; McKay & Latta, 2002; Thorpe & Stenson, 2003; Gübitz, Thorpe & Malhotra, 2005; Nosil & Crespi, 2006). In the present study, we quantified intraspecific variation in body size, colour pattern frequency, and male advertisement call in a Neotropical frog, and interpreted our results in light of the phylogenetic history of the study populations and their distribution relative to barriers to gene flow in Central America.

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Body size is relatively conserved within and among anuran species (Cherry, Case & Wilson, 1978; Green, 1993); however, this trait does vary with environmental gradients in habitat, elevation or latitude (Berven, 1982; Morrison & Hero, 2003; Rosso, Castellano & Giacoma, 2004). For example, larger body size in the cricket frog (*Acris crepitans*) affords higher resistance to desiccation in arid climates (Nevo, 1973). Body size can also be plastic, varying with environmental factors such as temperature, humidity, or predator pressure that influence larval development rates (Camp, Marshall & Austin, 2000; Touchon & Warkentin, 2008). Measuring variation in body size across historically divergent lineages allows us to infer whether population differences are explained by geographic isolation alone, or whether localized selection (and/or plasticity) contribute to fine-scale population variation.

Colour pattern polymorphisms and male advertisement calls are often heritable and subject to selection (Endler, 1992; Ryan, Rand & Weigt, 1996; Kingston, Rosenthal & Ryan, 2003; Hoekstra, Drumm & Nachman, 2004; Hoffman *et al.*, 2006). Species are polymorphic when individuals of the same sex and age display colour or pattern variants that are genetically inherited and for which expression is minimally sensitive to body condition or environment (Roulin, 2004). The evolutionary processes that maintain polymorphisms differ; reductions in gene flow can lead to phenotypic divergence among ecologically distinct populations, whereas disruptive and frequency-dependent selection more often drive diversification within populations (Gray & McKinnon, 2006; Rueffler *et al.*, 2006). Likewise, male advertisement calls can diverge as a result of environmental adaptation (West-Eberhard, 1983; Ryan & Wilczynski, 1991; Panhuis *et al.*, 2001; Couldridge & van Staaden, 2004) but, because calls function in species recognition and mate choice, they can also evolve through sexual selection (Arak, 1983; Forester & Czarnecki, 1985; Ryan & Rand, 1990; Ryan & Wilczynski, 1991; Ryan, 1999). Given the numerous pathways for signal divergence, it is not surprising that differentiation in calls is often observed at the population level (Ryan *et al.*, 1996; Foster & Endler, 1999) as well as among species (Blair, 1958; Sanderson, Szymura & Barton, 1992; Roberts & Wardell-Johnson, 1995; Parsons & Shaw, 2001; Smith *et al.*, 2003). At both scales, behavioural and morphological characters can retain evolutionary and phylogenetic signal, and thus reflect the order of lineage divergence (de Queiroz & Wimberger, 1993; Foster & Endler, 1999; Wiens & Penkrot, 2002; Price, Friedman & Omland, 2007).

The hourglass treefrog, *Dendropsophus ebraccatus*, is an ideal species in which to examine covariation among phenotypes and genetic differentiation across

isolating barriers. A recent study of genetic variation among populations in Costa Rica and Panama revealed deeply divergent lineages corresponding to five biogeographic regions (Robertson, 2008; Robertson, Duryea & Zamudio, 2009). This species is polymorphic in colour pattern, with all morphs present in most populations across its range (Duellman, 1970). Similarly, the advertisement call of this species is highly stereotyped, with low levels of variation among individuals within populations (Wells & Schwartz, 1984). To determine whether high genetic divergence among populations was concordant with call and phenotypic character diversity, we quantified geographic variation in male advertisement calls, colour pattern, and body size across multiple *D. ebraccatus* populations. We analysed behavioural and morphological variation in the context of population phylogenetic history and the complex biogeographic landscape of lower Central America to test hypotheses about the origin and maintenance of population diversity.

Our study had three goals: (1) to quantify variation in body size (S), colour pattern (P), and male advertisement call (C); (2) to determine whether each character varied with genetic and geographic distance; and (3) to detect covariation among characters after accounting for the phylogenetic history of sampled populations. We used a hierarchical analysis to quantify and compare variation in size, pattern, and advertisement calls within and among populations while taking into account both phylogenetic and geographic distance (Fig. 1). Phenotypic variation that is concordant with phylogenetic and geographic distance indicates the importance of historical geographic isolation in diversification (Grady & Quattro, 1999; Richards & Knowles, 2007). Conversely, discordance in phenotypic and phylogenetic patterns implicates the role of genetic drift and local selection regimes in driving phenotypic divergence among populations (Schneider & Moritz, 1999; Schneider *et al.*, 1999).

MATERIAL AND METHODS

STUDY SPECIES AND FIELD SAMPLING

The hourglass treefrog, *D. ebraccatus*, is distributed from Mexico to northern Colombia (Duellman, 1970). In lower Central America, the distribution of populations is limited by the Cordillera de Talamanca, a mountain range that extends over 200 km from Central Costa Rica to Central Panama (Kohlmann, Wilkinson & Lulla, 2002) and divides the lowland fauna into Caribbean and Pacific regions (Savage, 2002) (Fig. 2). During the breeding season, male *D. ebraccatus* congregate in temporary ponds and call to attract mates (Savage, 2002). Two congeners with

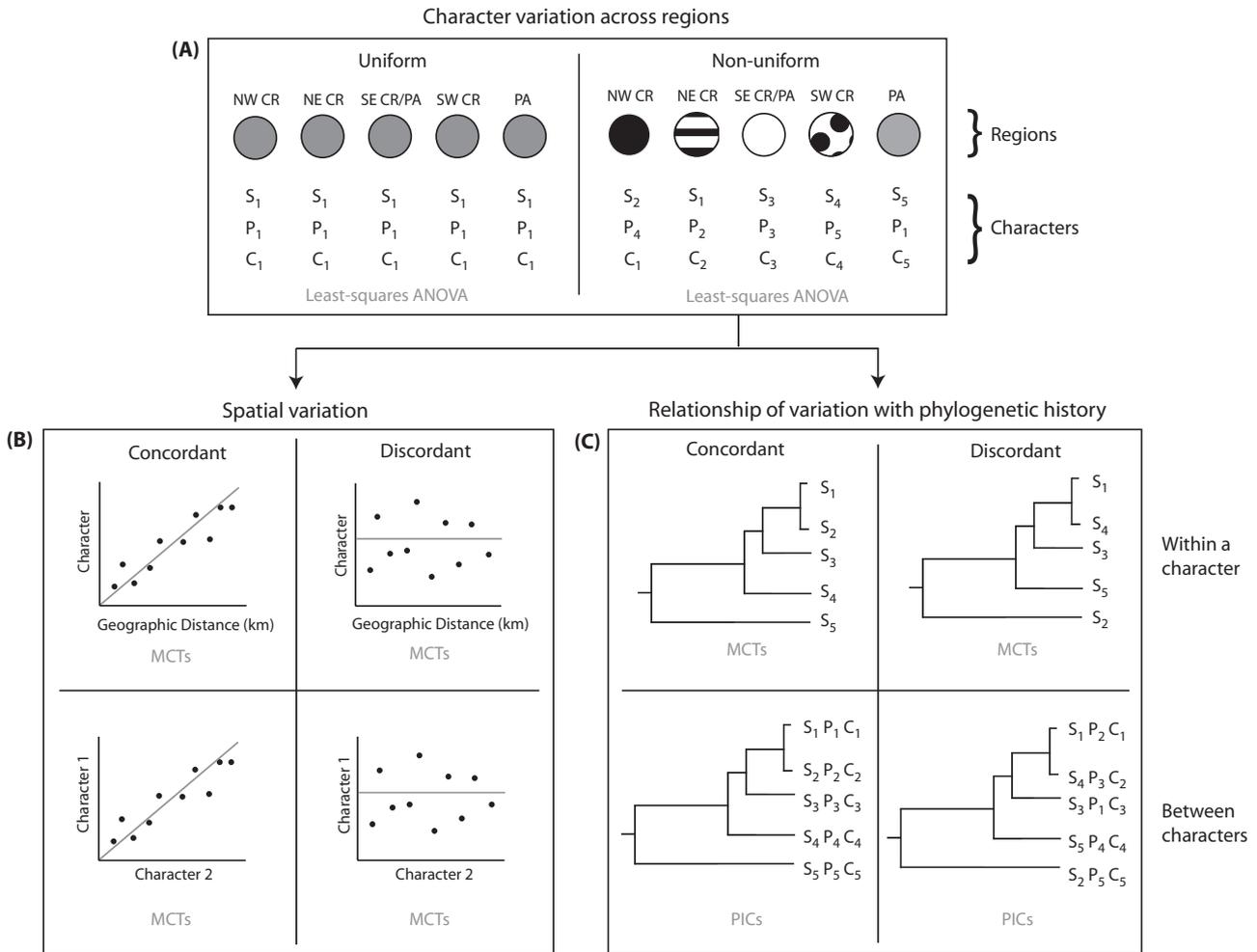


Figure 1. Heuristic diagram of hierarchical tests and methods for quantifying the distribution of phenotypic and genetic variability in *Dendropsophus ebraccatus*. At the most basic level (A), variation in any character can be either uniform or non-uniform across regions. If populations are not uniform, quantifying how characters vary (B) spatially and (C) with respect to the phylogenetic history of populations, allows us to infer microevolutionary determinants of this diversification. In each case we examined within and among regional variation in body size (B), colour pattern (P), and advertisement call (C). Subscripts represent different states for each of those characters.

similar calls, *Dendropsophus microcephala* and *Dendropsophus phlebodes*, breed syntopically with *D. ebraccatus* in many, but not all populations, suggesting possible selection for unique species-recognition signals in a complex acoustic environment (Schwartz & Wells, 1984; Savage, 2002; Wollerman & Wiley, 2002).

We measured morphological and behavioural phenotypic variation at 13 populations of *D. ebraccatus* in Costa Rica (CR) and Panama (PA; Fig. 2). A previous population-level phylogenetic analysis of this species revealed deep genetic divergences among five geographic regions (Robertson, 2008; Robertson *et al.*, 2009): Northeastern Costa Rica (NE CR), Southeastern Costa Rica and Panama (SE CR/PA), Northwestern Costa Rica (NW CR), Southwestern Costa Rica

(SW CR) and Central Panama (PA; Fig. 2). We conducted field surveys of all regions during the breeding seasons (May–August) of 2003, 2004, and 2005. At each sampling site we captured 8–49 adults and collected data on body size [snout–vent length (SVL), mm] and colour pattern. This species exhibits sexual dimorphism for size but not pattern (Duellman, 1970), therefore we included only males in analyses of body size but retained colour pattern data from both sexes. We documented colour pattern with digital photographs of each individual taken with a Nikon Coolpix 5700 digital camera. We also recorded male advertisement calls at each locality using a Sennheiser ME66/K6 directional microphone and either a Marantz PMD100 or Sony TCD-D7 hand-held digital audio recorder. We recorded vocalizations from a dis-

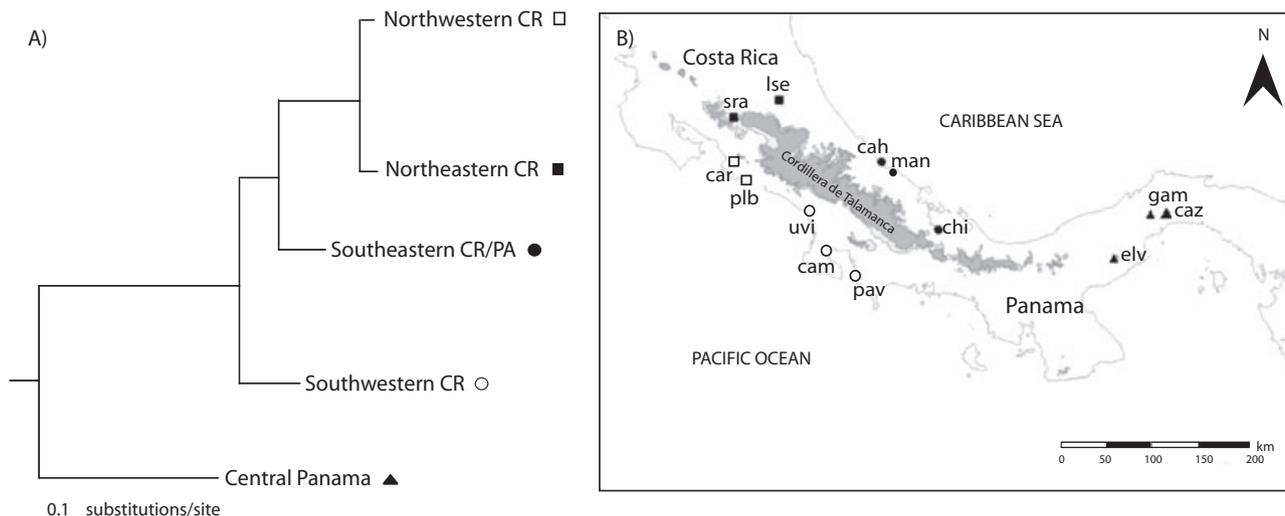


Figure 2. The phylogenetic and spatial framework for our study of phenotypic variation in the hourglass treefrog, *Dendropsophus ebraccatus*. A, a recent topology inferred from Bayesian analysis of mitochondrial DNA genes (Robertson, 2008; Robertson *et al.*, 2009) showed deep phylogenetic divergence among monophyletic lineages of populations in five regions. B, we sampled phenotypic data from 13 populations of *Dendropsophus ebraccatus* representing those five genetically differentiated regions.

tance of approximately 2–10 m from calling individuals. On average, we recorded 31 males at each site (range = 7–82) and avoided duplicate recordings by moving linearly through each site during sampling. Most individuals were released at site of capture. Voucher specimens and photographs have been deposited at the Cornell University Museum of Vertebrates (CUMV; CU 14029) and the Museo de Zoología, Universidad de Costa Rica (UCR 19102).

COLOUR PATTERN VARIATION

Dendropsophus ebraccatus obtains its common name from variegated yellow, gold, and brown blotches and spots on its dorsum, which can resemble an hourglass. Duellman (1970) surveyed 485 individuals from populations throughout the range and identified ten pattern variants ranging from uniformly yellow–tan to the hourglass-shape pattern (Duellman, 1970). For the present study, we classified colour pattern into four categories that encompass variation in our sampled populations: (1) hour spots; (2) hour; (3) spots; and (4) plain (Duellman, 1970) (Fig. 3). Preliminary analyses from a breeding trial indicate that these colour patterns are genetically inherited (J. Robertson & J. Touchon, pers. comm.).

CHARACTERIZING CALLS

We digitized recordings at a sampling rate of 44 100 pts s⁻¹, and visualized calls using the software RAVEN, version 1.2.1, (Charif, Clark & Fristrup,

2004). Calls with the highest signal-to-noise ratios were selected for further analyses. We characterized only one call per individual and on average 20 individuals from each study site. Each call consists of a main note, the crek, followed by zero, one, two or more shorter notes, called clicks (Fig. 4). We analysed only the first click for each male because the number of clicks varies with male density, and thus is not a reliable indicator of geographic variation (Wells & Schwartz, 1984). Using both the waveform and spectrogram views, we measured five temporal and spectral properties: low frequency (Hz; the lowest frequency of the dominant harmonic), high frequency (Hz; the highest frequency of the dominant harmonic), maximum frequency (Hz; the frequency at which the amplitude, or intensity, is highest; Charif *et al.* 2004), and time (length of note in seconds). We also measured the internote interval in seconds (INI, time between crek and click; Fig. 4).

STATISTICAL ANALYSIS

Body size, colour pattern, and advertisement call

We used one-way analysis of variance (ANOVA) to compare differences in body size (SVL) among populations, among regions, between low (0–300 m) and mid-elevation (≥ 300 m) sites, and between Caribbean and Pacific populations. We used a least-squares ANOVA to estimate the interaction effect of elevation and geographic location on body size. For colour pattern data we compared the frequency distribution of morphs within and among regions using χ^2

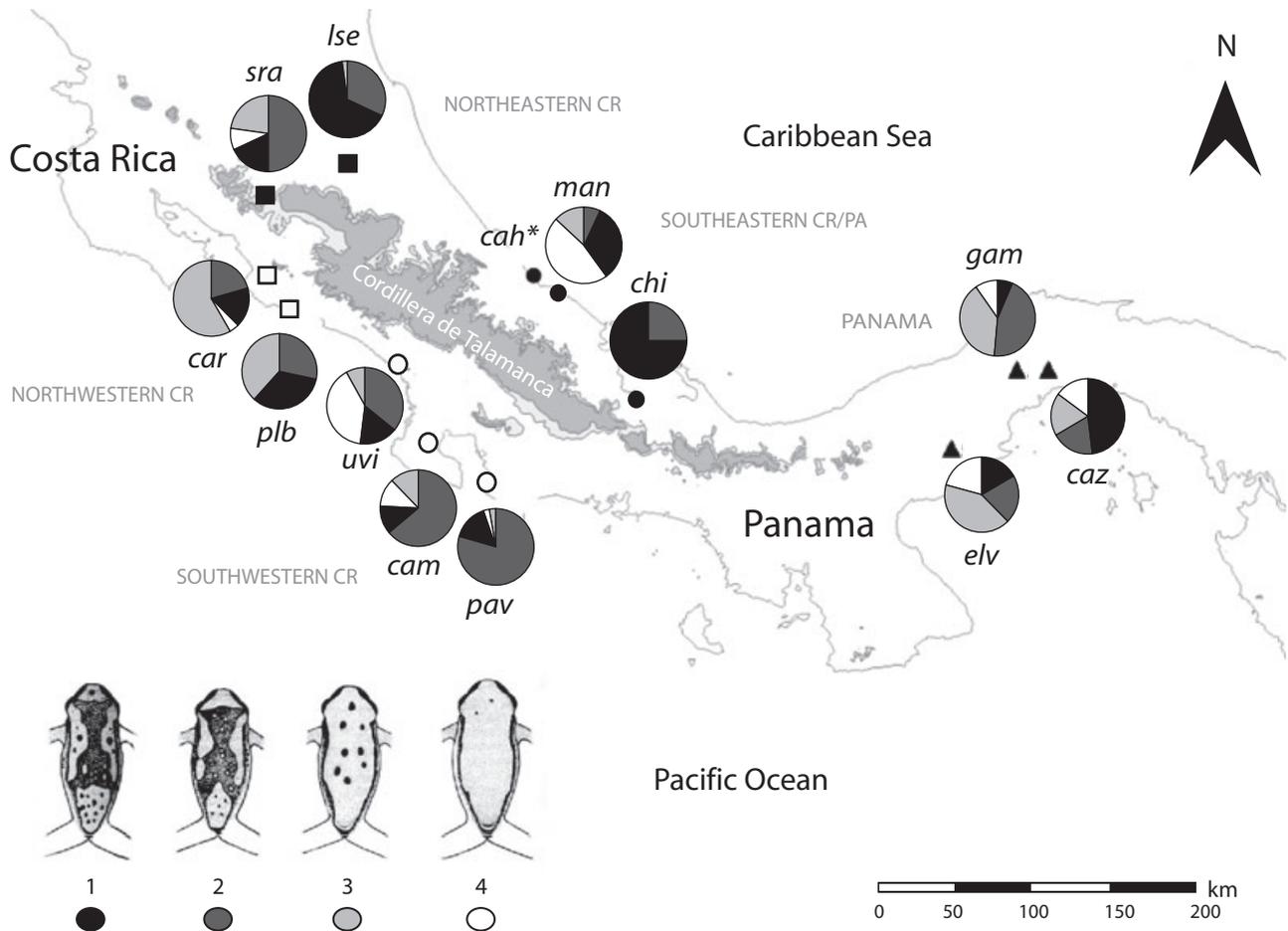


Figure 3. Frequency distribution of the four colour pattern variants in populations of *Dendropsophus ebraccatus*: 1, hour spots (spotted with an hourglass shape); 2, hour (hourglass shape with no spots); 3, spots; and 4, plain (Duellman, 2001). We did not obtain pattern data for population Cahuita (cah*), nor body size and call data for populations Manzanillo (man), Uvita (uvi), and Campo (cam), although data are available for all three characters for remaining populations (see Supporting information, Appendix S1).

contingency tests. We determined the significance of the χ^2 using Fisher's exact test to account for small sample sizes (R Statistical Package, version 2.7.0, R Development Core Team, 2005; see Supporting information, Appendix S1).

We tested for regional differentiation in call parameters (see Supporting information, Appendix S2) using standard least squares ANOVA tests. To adjust for body size and temperature variation among regions, SVL and elevation were included in the model effects. Because we lacked temperature measurements from many sites, elevation (m) was used as a proxy to account for correlated climate variables. To determine overall variation of each note (crek and click) across regions, we created composite crek and click variables derived from principal components analysis of all five parameters measured. The first principal component axis explained 52.2%

of the variation in the crek and 48.9% variation in the click. Within this first eigenvector, crek time (s) and click time (s) had the lowest eigenvalues, and thus explained the least amount of variation. Accordingly, the composite crek and click indices were derived only from the spectral properties (low, high, and max frequencies) of each call, weighted by the first principal component eigenvalue loadings for these properties. We used these composite variables in addition to the five measured call parameters in standard least squares ANOVA tests (Table 2). For significant ANOVA tests among regions, we conducted pairwise post-hoc tests using Tukey (HSD) tests in JMP, version 7 (SAS Institute Inc., 2007). In cases where Tukey tests failed to detect pairwise differences, we used Sheffé's multiple comparison tests to infer combinations of regions that differed significantly at each call parameter.

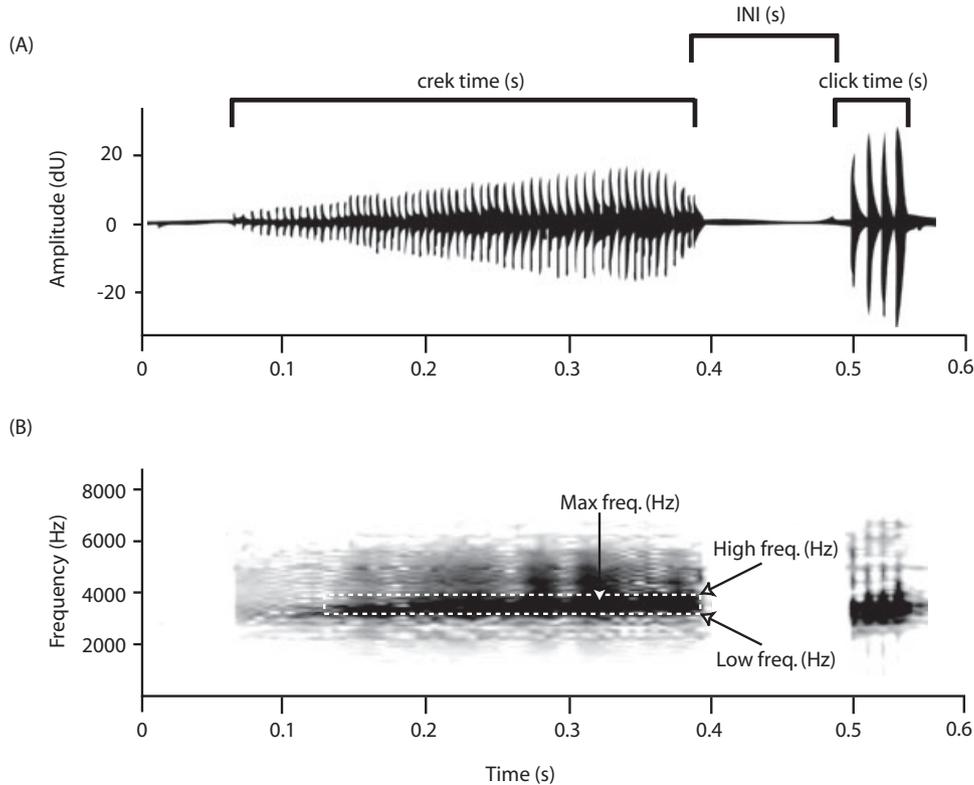


Figure 4. Waveform (A) and spectrogram (B) of a typical *Dendropsophus ebraccatus* call, a crek followed by one click. This diagram illustrates the temporal and spectral acoustic properties measured in our analysis. INI, internote interval.

Matrix correspondence tests: genes, phenotype and geographic distance

We conducted matrix correspondence tests (MCT) to examine the association of genes, phenotype and behaviour among populations. First, we tested whether behavioural and phenotypic characters varied with geographic and genetic distance. Second, we tested for an association between phenotypic and behavioural characters across populations (Fig. 1). MCTs use repeated randomization and permutation to test for the correlation between two distance matrices by comparing the individual pairwise distance for each parameter (Manly, 1986). Randomized values provide a null distribution with which to test the hypothesis of no association. Significance values were determined by comparing the observed and expected Z-statistic, generated by 10 000 permutations in the R Statistical Package, version 2.7.0 (R Development Core Team, 2005).

For colour pattern and body size, we constructed Euclidian distance matrices in the R Statistical Package. A colour pattern matrix was derived from the frequency distribution of all morphs within populations. For the genetic dissimilarity matrix among populations, we estimated pairwise patristic distances using TreeEdit, version 1.0a10 (Rambaut & Charles-

ton, 2001) derived from the Bayesian mitochondrial phylogeny for our sampled populations (Robertson, 2008; Robertson *et al.*, 2009). We represented geographic distance as the linear distance between populations calculated around the Cordillera de Talamanca, beginning with the southern-most population Cerro Azul (caz), in Panama, extending northward along the Caribbean versant, around the northern edge of the Cordillera de Talamanca, and then southward along the Pacific versant, ending at the population Pavones (pav) in Southwestern Costa Rica (Figs. 2, 3). This measure of geographic distance around the Cordillera de Talamanca extends 921 km, and reflects effective population distances given the barrier to gene flow between Caribbean and Pacific lowlands imposed by the mountains (Robertson, 2008; Robertson *et al.*, 2009). Finally, we constructed pairwise Euclidian distance matrices for each of four call parameters (composite crek, composite click, INI, crek time).

Phylogenetic independent contrasts

We used phylogenetic independent contrasts (PICs) to test for the association of body size, colour pattern frequency, and advertisement call parameters across populations while accounting for evolutionary relationships among populations (Felsenstein, 1985).

Standardized linear contrasts were estimated using CAIC, version 2.6.2 (Purvis & Rambaut, 1995). Branch lengths were determined using TREEEDIT, version 1.0a10 (Rambaut & Charleston, 2001), based on the phylogeny of mitochondrial DNA (mtDNA) haplotypes (Robertson, 2008; Robertson *et al.*, 2009), and their adequacy validated according to the procedures described by Garland, Harvey & Ives (1992). Branch lengths required log transformation, but were first multiplied by a common factor to ensure lengths greater than 2 units (as required by the CAIC program, Purvis & Rambaut, 1995). Population averages of body size (SVL) and call parameters (composite creek, composite click, and INI) were used as continuous variables. To represent the distribution of colour pattern as a continuous variable, we included percentages of three of the four most common colour pattern categories: hour, hour spots, and plain. We tested for a significant correlation between phenotypic characters by performing least-squares linear regressions of contrasts forced through the origin (Pagel, 1993). We used the CRUNCH procedure in CAIC as all of our variables were continuous. Because the colour pattern variables are based on percentage data and therefore invariably

correlated, we also performed multivariate analyses of pattern and call variables.

RESULTS

BODY SIZE, COLOUR PATTERN, AND ADVERTISEMENT CALL

Body size varied significantly among regions ($F_{4,281} = 8.00$, $P < 0.0001$) and among populations within NE CR ($F_{1,64} = 10.77$, $P = 0.0017$), SE CR/PA ($F_{2,21} = 11.77$, $P = 0.0004$), and SW CR ($F_{2,74} = 8.33$, $P = 0.0005$). The population with the mean largest individuals, Chiriqui Grande (chi; mean \pm SD = 26.75 ± 1.63), and the population with the mean smallest individuals, Manzanillo (man; mean \pm SD = 23.63 ± 1.35) both occurred within SE CR (Table 1). Frogs were slightly larger at higher elevations ($F_{1,284} = 3.96$, $P = 0.0475$) and in Pacific versant populations ($F_{1,284} = 23.53$, $P < 0.0001$). However, no significant interaction was found between elevation and geographic location relative to the Cordillera de Talamanca ($P = 0.794$).

All four colour patterns occurred in all regions (Fig. 3) but the relative frequency of each pattern

Table 1. Variation in male body size [snout–vent length (SVL), mm] for 13 populations of *Dendropsophus ebraccatus* sampled from five regions in Costa Rica and Panama

Region	N	Population	SVL (mean \pm SD)	GIS (Lat, Long, El)*
Northeastern CR	45	lse	24.24 \pm 1.18	10.4327, -84.0080, 37
	21	sra	25.19 \pm 0.84	10.2335, -84.5287, 638
Total	66		$F_{1,64} = 10.77$, $P = 0.0017$	
Southeastern CR/PA	3	cah	23.83 \pm 1.49	9.7406, -82.8593, 21
	8	chi	26.75 \pm 1.63	8.9460, -82.1571, 21
	13	man	23.63 \pm 1.35	
Total	24		$F_{2,21} = 11.77$, $P = 0.0004$	
Northwestern CR	31	car	25.70 \pm 0.88	9.7257, -84.5314, 385
	22	plb	25.83 \pm 1.47	9.5188, -84.3774, 23
Total	53		$F_{1,51} = 0.17$, $P = 0.68$	
Southwestern CR	26	pav	24.99 \pm 0.89	8.4204, -83.1069, 37
	27	uvi	25.74 \pm 1.09	
	24	cam	24.70 \pm 0.99	
Total	77		$F_{2,74} = 8.33$, $P = 0.0005$	
Central Panama	20	elv	25.35 \pm 1.28	8.6299, -80.1159, 866
	24	gam	24.62 \pm 1.33	9.1231, -79.6930, 51
	22	caz	24.49 \pm 0.96	9.1673, -79.4193, 638
Total	66		$F_{2,63} = 3.044$, $P = 0.055$	
Among regions	286		$F_{4,281} = 8.00$, $P < 0.0001$	

*Geographic coordinates (GIS) are latitude (Lat), longitude (Long), and elevation (El, in metres).

Body size was significantly different among all regions and among populations within northeastern and southeastern Costa Rica.

Values in bold represent significant differences in male body size among and within regions.

Table 2. Variation in male advertisement calls of *Dendropsophus ebraccatus* from five genetically divergent regions in Costa Rica and Panama

	Crek*				Click*				INI*		
	Low frequency (Hz)	High frequency (Hz)	Maximum frequency (Hz)	Time (s)	Composite crek†	Low frequency (Hz)	High frequency (Hz)	Maximum frequency (Hz)		Time (s)	Composite click†
Northeast	2618.10 ^{a,b}	3385.80 ^a	3093.23 ^a	0.14 ^b	5234.52 ^a	2613.87 ^a	3369.46	3066.92 ^a	0.034 ^a	5209.57 ^a	0.095 ^{a,b}
Northwest	2528.26 ^{a,b}	3108.49 ^b	2878.18 ^b	0.15 ^{a,b}	4896.21 ^b	2505.28 ^b	3103.04	2814.41 ^b	0.028 ^{a,b}	4835.34 ^b	0.070 ^b
Southeast	2708.91 ^a	3346.26 ^a	3072.85 ^{a,b}	0.14 ^b	5247.47 ^a	2763.87 ^a	3306.29	3096.75 ^{a,b}	0.039 ^{a,b}	5260.35 ^{a,b}	0.12 ^{a,b}
Southwest	2574.82 ^{a,b}	3409.28 ^a	3069.56 ^a	0.19 ^a	5209.16 ^a	2773.76 ^a	3375.78	3037.73 ^{a,b}	0.020 ^{a,b}	5266.08 ^{a,b}	0.11 ^a
Panama	2486.44 ^b	3353.79 ^a	3062.06 ^{a,b}	0.14 ^b	5127.49 ^{a,b}	2600.78 ^a	3437.82	3079.64 ^{a,b}	0.019 ^b	5252.18 ^{a,b}	0.10 ^{a,b}
<i>F</i> ₍₆₎	9.18	5.91	4.68	12.31	6.65	5.00	2.37	2.67	5.4	2.54	4.36
<i>P</i> -value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.072	0.0167	< 0.0001	0.023	0.0004

*For each call parameter, regions with superscript letters, a or b, are significantly different based on post-hoc comparisons, after corrections for multiple tests.

†Composite crek and click variables are weighted combinations of low, high and maximum frequencies. Standard least-squares analyses of variance show high among region variation for all but one call parameter. Standard least square means for each region are adjusted for average male snout-vent length and elevation.

Bolded *P*-values highlight call parameters that varied among regions. INI, internote interval in seconds.

varied significantly among regions ($P < 0.0001$; see Supporting information, Appendix S1). Within four of the five regions, we also detected significantly different pattern frequencies among populations (see Supporting information, Appendix S1). Three populations (lse, chi, and plb) did not contain all four morphs; in those cases, the absent morphs were spots, plain or both (Fig. 3). The dominant morph varied in each population with no clear geographic signal among sampled populations (Fig. 3).

All call parameters, except the high frequency for click, varied significantly among regions (Table 2). Post-hoc tests indicated that call parameters showed different patterns of regional differentiation (Table 2). Regional variation in the composite crek and click variables corroborated these results and further distinguished the male advertisement calls of northeast and northwest populations (Table 2). The composite crek in NW CR differed from all other regions, whereas the northeast and northwest populations were significantly different from each other in composite click (Table 2).

MATRIX CORRESPONDENCE TESTS:

GENES, PHENOTYPE AND GEOGRAPHIC DISTANCE

Geographic and genetic distances were strongly correlated in MCT ($r = 0.8177$, $P = 0.0001$; Table 3). In contrast, neither call, nor pattern or body size varied significantly with geographic or genetic distance. We also found no significant association among those variables in pairwise MCTs (Table 3). Therefore, MCTs reveal complete discordance among characters and also between phenotype and patterns of genetic divergence.

PHYLOGENETIC INDEPENDENT CONTRASTS

Independent contrasts showed no significant correlations between body size and colour pattern frequency or advertisement call parameters (Table 4), even when accounting for phylogenetic similarities among sister lineages. Likewise, univariate regression analyses between call parameters (composite crek, composite click, and INI) and pattern types (% hour spots, % hour, and % spots) were not significant, nor were multivariate regression analyses between each call character and the frequency distribution of pattern variables (Table 5).

DISCUSSION

The fine-scale analysis of phenotypic variation conducted in the present study revealed significant regional divergence in body size, colour pattern, and advertisement calls in hourglass treefrog populations

Table 3. Matrix correspondence tests of the association between geographic distance (km), mitochondrial DNA patristic distance (DNA), call parameters (composite crek, composite click, and INI) and colour pattern (pattern) across ten populations of *Dendropsophus ebraccatus*

Matrix <i>x</i>	Matrix <i>y</i>	<i>r</i>	<i>P</i> *
km	DNA	0.8177	0.0001
	Composite click	-0.0737	0.6587
	Composite crek	0.2664	0.0594
	INI	0.0386	0.4043
	Crek time	-0.136	0.8170
	Pattern	-0.041	0.5756
	SVL	-0.144	0.7986
DNA	Composite click	0.0006	0.4073
	Composite crek	0.1777	0.1301
	INI	0.1039	0.3079
	Crek time	-0.0853	0.6528
	Pattern	-0.1009	0.7215
	SVL	-0.0730	0.6463
Pattern	Composite crek	0.0823	0.3275
	Composite click	0.092	0.2680
	INI	-0.155	0.7016
	Crek time	-0.123	0.7610
	SVL	0.1711	0.1922

**P*-values are Bonferroni corrected for multiple comparisons (adjusted *P* = 0.003, for a table wide significance level of $\alpha = 0.05$).

The single bold *P*-value indicates a significant association between geographic and phylogenetic (patristic) distances among populations.

INI, internote interval in seconds; SVL, snout-vent length.

in Costa Rica and Panama, indicating more variation than previously documented (Duellman, 1970; Wells & Schwartz, 1984). Most importantly, our analyses show that, although phenotypic characters are variable across populations and biogeographic regions, phenotypic divergences are not predicted by phylogenetic or geographic distance, thus indicating a role for non-neutral processes in the diversification of these traits. The widespread discordance between the history of lineages and the evolution of phenotype indicates that other microevolutionary mechanisms such as localized selection (Sandoval, 1994; Sandoval & Nosil, 2005; Rosenblum, 2006; Richards & Knowles, 2007), genetic drift (Hoffman *et al.*, 2006; Edh, Widen & Ceplitis, 2007; Abbott *et al.*, 2008), or both (Slatkin, 1985), must be contributing to patterns of phenotypic diversification among populations. Quantifying the relative strength of selection and drift could be achieved through temporal sampling of changes in morph frequencies within populations (Gillespie & Oxford, 1998; Abbott *et al.*, 2008); com-

Table 4. Independent contrast tests of correlation between body size (SVL), advertisement call, and colour pattern among populations of *Dendropsophus ebraccatus* in Costa Rica and Panama

Variables		SVL		
		<i>F</i>	d.f.	<i>P</i> *
Call	Composite crek	0.733	1,7	0.420
	Composite click	0.704	1,7	0.429
	INI	3.829	1,7	0.091
Pattern	% hour	0.249	1,7	0.633
	% hour spots	0.243	1,7	0.637
	% spots	1.036	1,7	0.272

*Probability values for bivariate linear regression analyses of independent contrasts of snout-vent length (SVL) and call variables (composite crek, composite click, and INI) and pattern (% hour, % hour spots, and % spots). INI, internote interval in seconds.

parison of neutral and 'selected' markers (Tordoff, Pettus & Matthews, 1976; Orr, 1998; McKay & Latta, 2002; Hoekstra *et al.*, 2004; Hoffman *et al.*, 2006); or tests of specific habitat and ecological factors associated with trait expression (Hoekstra *et al.*, 2005; Rosenblum, 2006; Richards & Knowles, 2007). Our single sample of phenotypic variation and use of neutral markers for phylogenetic analyses cannot distinguish between drift and selection in the present study. However, the traits that we measured are known to evolve, at least partially, due to both mechanisms in amphibians. The phenotypic characters we measured demonstrated unique patterns of regional diversification, indicating that each trait evolves independently. Possible selective drivers of morphological and behavioural phenotypes include climatic and microhabitat variation (Hoffman & Blouin, 2000; Gray & McKinnon, 2006), diverse predator assemblages (Endler, 1992), and geographic variation in mate choice (Irwin, Thimgan & Irwin, 2008). We discuss each phenotypic character in light of our knowledge of the natural history, possible selective regimes experienced by hourglass treefrogs in our sampled populations, and possible stochastic evolutionary processes that may have shaped diversity.

Adult body size in anurans can evolve due to changes in the local environment (Ashton, 2002; Morrison & Hero, 2003), genetic drift in isolated populations (Knopp *et al.*, 2007) or natural selection (Laugen *et al.*, 2003; Gübitz *et al.*, 2005). Thus, the discordance between body size and phylogeny that we detected in *D. ebraccatus* is not entirely surprising, and likely reflects the multiple processes shaping body size in different populations and regions. Adult body size in

Table 5. Independent contrast tests of the correlation between advertisement call parameters and colour pattern in populations of *Dendropsophus ebraccatus* in Costa Rica and Panama

Variables	Univariate*			Multivariate†		
	<i>F</i>	d.f.	<i>P</i>	<i>F</i>	d.f.	<i>P</i>
Composite creak						
% hour	0.870	1,6	0.387	0.003	1,6	0.957
% hour spots	0.030	1,6	0.869	0.023	1,6	0.888
% spots	0.383	1,6	0.559	0.057	1,6	0.824
Composite click						
% hour	0.360	1,6	0.571	0.623	1,6	0.474
% hour spots	0.024	1,6	0.882	0.884	1,6	0.400
% spots	1.042	1,6	0.347	1.262	1,6	0.324
INI						
% hour	0.255	1,6	0.632	7.25	1,6	0.055
% hour spots	0.640	1,6	0.454	7.24	1,6	0.055
% spots	0.779	1,6	0.412	5.32	1,6	0.082

*Probability values for bivariate linear regression analyses of independent contrasts of call variables (composite creak, composite click, and INI) with snout–vent length (SVL) and pattern (% hour, % hour spots, and % spots).

†Partial probability values for multivariate regression analyses.

INI, internote interval in seconds.

anurans depends on time to and size at metamorphosis, age of maturity, growth rate before and after maturity, and longevity (Berven, 1982; Smith, 1987; Friedl & Klump, 1997; Rosso *et al.*, 2004) and, although these life history characteristics are under genetic control, they also are influenced by environmental conditions (Lu, Li & Liang, 2006; Navas, 2006). We detected a trend of larger hourglass treefrogs in populations above 300 m, consistent with findings from other studies that frogs at mid to high elevations are larger at all life stages (Morrison & Hero, 2003). Populations on the Pacific versant were also larger in body size, indicating that environmental factors other than elevation also contribute to body size differences. Temperature (Ashton, 2002) and aridity (Nevo, 1973) regulate body size in other anuran species, and ecological factors such as differences in food abundance, predation, and interspecific competition may vary regionally throughout our study area. Quantifying geographic variation in environmental variables at the scale of our sampled populations may reveal local determinants of body size.

Colour pattern polymorphisms can be maintained by selection favouring multiple variants within populations (Hoffman & Blouin, 2000; Merilaita, 2006; Vercken *et al.*, 2007; Dijkstra, Seehausen & Groothuis, 2008). Although field studies rarely measure selection on anuran colour polymorphisms directly, it can be inferred based on studies showing nonrandom survival of morphs under different conditions (Merrell & Rodell, 1968; Dapkus, 1976) or fluctua-

tations in morph frequency in natural populations (Jameson & Pequegnat, 1971; Wentz & Phillips, 2005; Woolbright & Stewart, 2008). The occurrence of all *D. ebraccatus* colour pattern morphs in all regions could be explained by selective maintenance of all polymorphisms in these genetically isolated areas. However, the significant regional and population-level differences in colour pattern frequencies indicate that either the selective advantage of morphs varies among populations or that genetic drift causes stochastic changes in morph frequency despite similar selective regimes. Our data show evidence of both mechanisms: the absence of up to two colour morphs in three disjunct populations could represent stochastic losses. However, our data also suggest that selection contributes to colour pattern diversification. Population genetic analyses of these same populations show limited gene flow across regions but some gene flow among populations within regions (Robertson, 2008; Robertson *et al.*, 2009); under this scenario, we would expect selection–drift equilibrium to result in similar morph frequencies within regions. By contrast, we found significant phenotypic differences even among populations within regions, suggesting that strong, highly localized selection also contributes to the observed patterns.

The adaptive role of dorsal pattern in *D. ebraccatus* is unknown; their patterns could be cryptic and facilitate predator avoidance (Cott, 1940; Endler, 1992; Duellman, 2001) or they could be conspicuous and play a role in mate choice (Summers *et al.*, 1999;

Gomez & Thery, 2007). A comparative analysis of dorsal colour pattern among Central American Dendropsophini frogs lends some evidence that this character evolved in response to environmental and/or predator pressures rather than as a species recognition signal (Robertson, 2008). Colour pattern may still be sexually selected in this species, but those analyses indicate that the deeper history of colour pattern diversification in this clade was due to natural selection and not mate choice or conspecific recognition (Robertson, 2008). Laboratory studies have tested predation rates as a function of colour pattern in other polymorphic frogs and found that predators attacked cryptic (background-matched) morphs less often (Tordoff, 1980; Morey, 1990). The hourglass treefrog exhibits bright background colours that are disrupted by the hourglass or spotted contrasting pattern, possibly serving as cryptic coloration in their highly heterogeneous environments (Duellman, 1970; Merilaita, Lyytinen & Mappes, 2001; Merilaita & Lind, 2005; Schaeffer & Stobbe, 2006). However, for other brightly coloured and conspicuous anurans, such as poison arrow frogs (Dendrobatidae), bold colour patterns may serve to protect individuals from predation through aposematism (Summers & Clough, 2001; Darst, Cummings & Cannatella, 2006). We do not know whether the bright coloration in *D. ebraccatus* is aposematic because the toxicity of their skin secretions to potential predators is unknown.

Frequency-dependent predation can exert selection that favours the maintenance of polymorphisms within a population (Ayala & Campbell, 1974; Bond & Kamil, 2002; Gray & McKinnon, 2006). Under this form of selection, predators consume common morphs at higher frequencies, permitting rare morph persistence (Cott, 1940; Punzalan, Rodd & Hughes, 2005). The dominant morph varied significantly among our sampled populations, a pattern expected if frequency dependent selection is operating independently in each population (Gillespie & Oxford, 1998; Merilaita, 2006). Further studies will be necessary to elucidate the adaptive significance of colour pattern in our focal species, but the geographic differentiation in pattern frequencies is suggestive of selection-mediated maintenance of this polymorphism.

Male advertisement calls serve two primary functions in anurans: species recognition and mate choice (Backwell & Jennions, 1993; Ryan & Rand, 1993; Foster & Endler, 1999). These two roles are not independent and both contribute to patterns of geographic variation, reproductive isolation and, ultimately, speciation (West-Eberhard, 1983; Ryan & Wilczynski, 1991). Past studies of hourglass treefrog calls have focused either on range wide characterization (Duellman, 1970) or intrapopulation variation in light of sexual selection and acoustic interference (Wells &

Schwartz, 1984; Backwell & Jennions, 1993; Wollerman, 1998, 1999; Wollerman & Wiley, 2002). Our population level analyses of call variation demonstrated that, although spectral and temporal properties of calls do vary among regions, this variation was not associated with geographic proximity or the evolutionary relationship of populations. These results are inconsistent with other studies that demonstrated congruence between genetic distance and differentiation of advertisement calls (Ryan *et al.*, 1996; Phillips & Johnston, 2008). A regional study of vocalizations in the green toad (*Bufo viridis*) showed opposing patterns of diversification of two call types, demonstrating that release calls have evolved through random processes of genetic drift, but that advertisement calls have undergone directional selection (Castellano *et al.*, 2002). Given the strong genetic divergence observed among regions for the hourglass treefrog, several scenarios could explain the uncorrelated changes in male acoustic signals, including genetic drift in isolated populations; differences among regions in environmental characteristics affecting calls (Baker & Logue, 2003; Slabbekoorn, 2004); and/or differences in local female preferences for calls.

Local differences in community structure and environmental conditions can promote divergence among geographically-isolated populations (Morton, 1975; Baker & Logue, 2003; Slabbekoorn, 2004; Baker, 2006). Anurans often breed in dense aggregations and the presence of congeners with similar calls can select for different male advertisement signals that minimize interference through partitioning of acoustic call space (Drewry & Rand, 1983; Ryan *et al.*, 1996; Foster & Endler, 1999). Wells & Schwartz (1984) found that *D. ebraccatus* females more readily approach males whose calls were not masked by other calling conspecifics or males of *D. microcephala*, a common syntopic congener. Throughout its range, the hourglass treefrog is syntopic with up to six congeners (Savage, 2002), making mate recognition a possible explanation for divergence among regions. Dissimilarity in predator communities could also lead to variability in *D. ebraccatus* advertisement calls throughout its range. Ryan, Tuttle & Rand (1982) demonstrated that, as male túngara frog calls become more complex, they become more attractive to both conspecific females and frog-eating bats. Further investigations into the microgeographic differences in congener and predator populations across the range of *D. ebraccatus* are needed to better understand the biotic factors shaping call variation in this species. Divergence in call characteristics can also result due to multiple, inter-related environmental variables that vary at small spatial scales. The physical environment can attenuate calls because sound waves are

scattered and absorbed by the surrounding ground, water, air, and vegetation (Wiley & Richards, 1978; Richards & Wiley, 1980). Thus, anuran calls may evolve to maximize habitat-specific transmission (Kime, Turner & Ryan, 1999). Clearly, multiple selective forces may contribute to call diversification in hourglass treefrogs and, although we cannot eliminate the possibility that drift alone has stochastically fixed variation in geographically-isolated regions, the important roles of frog acoustic signalling in mate choice and attraction suggest that selection plays some role in the variation observed.

Anurans are ideal taxa for spatial studies of character divergence (Lougheed *et al.*, 2006). Their relatively low dispersal abilities and high breeding site fidelity contribute to population and regional-level differentiation at small spatial scales (Newman & Squire, 2001; Beebee, 2005; Lougheed *et al.*, 2006). The present study has revealed regional phenotypic differentiation in morphology and behaviour that was not concordant with the phylogenetic history of hourglass treefrog populations. This finding indicates that character evolution has been largely independent of the historical processes that caused genetic differentiation among regions. Our data suggest that highly localized selection pressures, in combination with genetic drift and reduced gene flow, likely resulted in regional diversification in body size, colour pattern frequency, and male advertisement call. The fine-scale uncorrelated genetic, morphological, and behavioural variation in this study underscores that these characters may be especially prone to diversification in anurans, even over very small geographic scales.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Population and regional variation in colour pattern frequencies among populations of *Dendropsophus ebraccatus*. Contingency analyses showed significant variation among all regions and among populations within all regions, except for Northwestern Costa Rica.

Appendix S2. The mean \pm SD of call parameters (not adjusted for body size or elevation) measured from ten populations of *Dendropsophus ebraccatus*.

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Corrigendum

09 December 2009

Drs. Joshua J. Schwartz, Kent D. Wells, and Michael Reichart kindly pointed out that we might have included aggressive calls, in addition to advertisement calls, in our analyses. This suggestion was based on the length of the example call presented in Figure 4, and also differences in the mean creek length between our dataset and their previously published dataset¹ for the Gamboa, Panama population.

We reanalyzed our Gamboa population dataset and determined that of the 82 calls recorded, 20 were ‘squeaky’ with a high pulse rate (indicating a possible aggressive call²). We removed these from the analyses and found the mean \pm std dev for creek length to be 130 ms \pm 0.034; CV = 26.4%. This is not significantly different than our previous mean of 125 ms \pm 0.03 (Type III t-test; $t=0.760$, $P=0.45$). To be cautious, three additional calls were removed due to background noise and the analyses were run a third time ($N = 59$, creek mean \pm std dev = 126 ms \pm 0.027, CV = 21.5%). Again, this final data set was not significantly different than our original dataset (Type III t-test; $t=0.139$, $P=0.89$). Based on re-analyses of all Gamboa recordings, we feel comfortable that the means reported in our study were not significantly biased by measurement error, or that inclusion of possible aggressive calls significantly changed the results or interpretation of this study.

The example call for *D. ebraccatus*, presented in Figure 4 shows all the variables measured in our study. This call was chosen based on the clarity of the signal. However, the call is abnormally long (325 ms) and has a high pulse rate (180 pulses/s), indicative of an aggressive call. While this call was not analyzed in our study, to prevent confusion we present a clarified figure legend below.

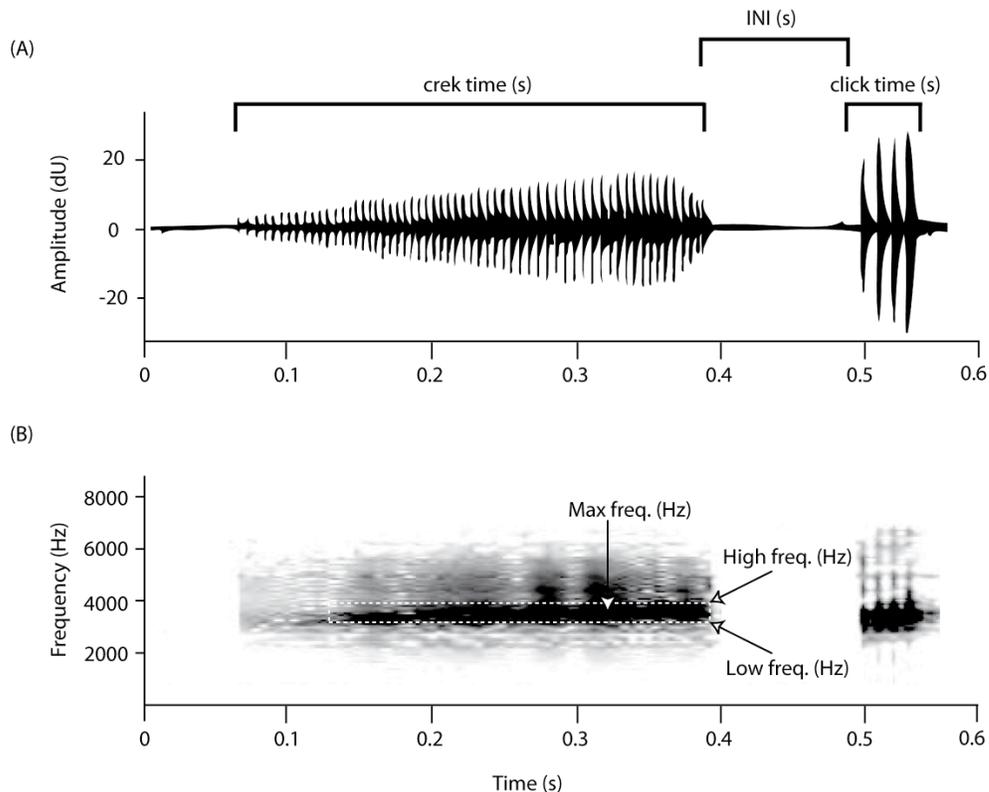


Figure 4. Waveform (A) and spectrogram (B) of a typical *Dendropsophus ebraccatus* aggressive call, a creek followed by one click. Advertisement calls are similar in structure but often shorter in length. This diagram illustrates the temporal and spectral acoustic properties measured in our analysis. INI, internote interval.

¹Wells KD, Schwartz JJ. 1984. Vocal communication in a neotropical treefrog, *Hyla ebraccata*: Aggressive calls. *Behaviour* 91: 128-145.

²Wells KD, Greer BJ. 1981. Vocal responses to conspecific calls in a neotropical Hyliid frog, *Hyla ebraccata*. *Copeia* 1981: 615-624.