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ORIGINAL ARTICLE

Spatial and temporal patterns of phenotypic variation in a Neotropical frog

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ABSTRACT

Aim Studies of the spatial and temporal patterns of phenotypic diversity help to elucidate the fine-scale evolutionary and ecological mechanisms underlying geographical differentiation. The red-eyed tree frog, *Agalychnis callidryas*, is a widespread Neotropical frog that exhibits a broad range of polymorphism of coloration and flank-stripe pattern. The goal of this study was two-fold: first, to investigate the stability of polymorphisms over a 38-year period; and second, to evaluate biogeographical hypotheses of diversification between lower Central American populations through quantification of phenotypic diversity on a fine geographical scale.

Location This study was conducted at 12 sites across four biogeographical regions in Costa Rica and Panama.

Methods We quantified colour, categorized flank-stripe pattern from digital photos taken during field sampling, and measured body size for each individual. We compared the regional frequency of each flank-stripe pattern in 2005 with the frequency distribution from a previous study of the same sites in 1967 using logistic regression analyses. We determined the geographical signal of leg coloration by employing linear discriminant function analyses to generate a classification matrix based on covariance similarities, and by comparison of the average hue values within and between regions.

Results We found a temporal shift in the frequency of flank-stripe patterns in three of four regions over 38 years. Based on measures of leg coloration, the frequency distribution of flank-stripe patterns and body size, we conclude that *A. callidryas* populations are easily distinguishable at a regional scale.

Main conclusions *Agalychnis callidryas* exhibits regional differentiation in all phenotypic traits measured in this study, supporting the role of three major biogeographical barriers to gene exchange. We found evidence of a putative contact zone between polytypic regions in Costa Rica. In addition, we report temporal instability of the relative frequency of stripe patterns located on the flanks. The ecological and evolutionary mechanisms that may underlie this variation include sexual selection and avoidance of predators.

Keywords
***Agalychnis,* Anura, Central America, coloration, Costa Rica, Panama, polymorphism, polytypic, temporal instability.**

INTRODUCTION

Spatial and temporal patterns of phenotypic diversity and the underlying ecological and evolutionary processes that produce them provide important insights into the biogeographical history of a species (Grinnell, 1924; Endler, 1973; Velez & Feder, 2006). All taxa exhibit some level of individual or population variation, but some species are highly polytypic

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across their range, or exhibit geographical clines in body size, behaviour, coloration and ornamentation (Nevo, 1973; Gray, 1983; Brooks & Endler, 2001; Storz *et al.*, 2001). Phenotypic diversity may vary between regions due to demographic factors (e.g. effective population size), diversifying selection, historical biogeography or the isolating effects of restricted gene flow (Grinnell, 1924; Pyburn, 1961a; Endler, 1973; Hairston, 1979). Tracking this diversity over time allows us to observe the relative stability of these demographic, selective or migration forces acting upon populations (Grinnell, 1924; Pyburn, 1961a; Holt *et al.*, 2004; Blanco *et al.*, 2005; Prieto *et al.*, 2005; Grant & Grant, 2006). Studies of spatial and temporal variation provide insights into taxon diversification and, ultimately, processes leading to speciation (Irwin *et al.*, 2001).

Intraspecific phenotypic variation can be distributed either within or between populations. Approximately 5% of anurans exhibit within-population phenotypic diversity (Hoffman & Blouin, 2000); the relative frequency of known polymorphisms varies between sites depending on ecological and local factors, driven primarily by predator–prey relationships (Pyburn, 1961b; Hoffman & Blouin, 2000; Ray & King, 2006). Variation within a population also depends on genetic drift, migration and/or selection favouring balancing polymorphisms (Lenormand, 2002; Eakley & Houde, 2004). Some anurans exhibit between-population variation where all individuals in a population/region are relatively monomorphic for a single phenotype but phenotypes vary between regions. Evolutionary mechanisms driving between-population variation include genetic isolation (D'Anatro & Loureiro, 2005) and local differences in natural or sexual selection (Hairston, 1979; Hoekstra *et al.*, 2004; Velez & Feder, 2006), including crypsis favouring behavioural background matching (Kettlewell & Conn, 1977; Gillis, 1982; Morey, 1990). Temporal instability of the relative frequency of polymorphisms is predicted from sudden changes in effective population size (usually population bottleneck), migration patterns or ecological conditions that change the direction of selection.

Here, we investigate patterns of phenotypic diversity in the red-eyed tree frog, *Agalychnis callidryas* Cope 1862 (Anura: Phyllomedusa: Hylidae), a species distributed from Central Mexico to Colombia, which exhibits striking regional differentiation in flank and leg coloration (Savage & Heyer, 1967; Duellman, 2001). *Agalychnis callidryas* is one of a few Neotropical frogs to exhibit low within-population variation but high between-population variation (Summers *et al.*, 2003; Richards & Knowles, 2007). These colour differences are sufficiently dramatic that north-eastern and western Costa Rican populations were once considered different species (Funkhouser, 1957; Savage & Heyer, 1967). The red-eyed tree frog exhibits sexual dimorphism in size (females are larger and heavier), but no sexual dimorphism in coloration or pattern. Phenotypic diversity in this species was studied almost 38 years ago (Savage & Heyer, 1967), permitting a study of the geographical distribution of phenotypic diversity and its stability over time.

In this study, we quantify the current geographical distribution of three phenotypic traits in populations of *A. callidryas* in Costa Rica and Panama and examine temporal variation in one of the traits, flank-stripe pattern, after 38 years. These study populations represent 25% of the geographical range of the species, yet contain all of the known colour variation, thus providing an excellent opportunity to examine spatial and temporal processes acting at a fine geographical scale. We examine differences in flank-stripe pattern, colour differentiation, and body size between focal populations.

We evaluate geographical patterns of phenotypic variation in light of the complex topographic landscape of Central America. Plate tectonics and the formation of the Cordillera de Talamanca, the mountain range extending along the Central American continental divide, have played an important role in the geological history of Central America, in particular in Costa Rica and Panama (Kohlmann *et al.*, 2002; Savage, 2002). We investigated three putative biogeographical breaks (Cordillera de Talamanca, Limón and the Osa Peninsula) and one contact zone (north-east–north-west) in Costa Rica and Panama to understand the distribution of phenotypic diversity in our focal species. The Cordillera de Talamanca, approximately 3 Myr old, extends 400 km along the length of Costa Rica and western Panama (Kohlmann *et al.*, 2002; Savage, 2002). This mountain range asserts a strong barrier to gene exchange between Caribbean and Pacific populations for other terrestrial amphibians and reptiles (Zamudio & Greene, 1997; Crawford, 2003; Zeh *et al.*, 2003; Weight *et al.,* 2005). The second putative biogeographical break occurs between populations on either side of the Golfo Dulce, which has separated the Osa Peninsula and the Burica Peninsula for the last 2 Myr (Kohlmann *et al.*, 2002). Finally, an off-shore Caribbean coral reef influences the distribution of genetic and ecological diversity of marine organisms near Limón, Costa Rica (Kohlmann *et al.*, 2002; Fig. 1). However, the nature of the biogeographical break is poorly understood for terrestrial organisms. Limón coincides with species distribution limits for some amphibians and beetles (Kohlmann *et al.*, 2002; Savage, 2002) and we test the hypothesis that it may also act to isolate *A. callidryas* populations.

We also investigated a putative contact zone that occurs west of the Talamanca Mountains at the junction of three younger, non-contiguous mountain ranges: Cordillera de Guanacaste, Cordillera Central and Cordillera de Tilarán. Due to low-elevation passes between the north-east and north-west regions, it is possible that low levels of historical gene flow connected these two regions (Savage & Heyer, 1967).

Our fine-scale sampling across these biogeographical features allows us to investigate the isolating effect of barriers and the potential for homogenizing gene flow across a contact zone. Specifically, our three objectives were: (1) to examine differences in phenotypic variation relative to hypothesized biogeographical barriers that may have promoted regional differentiation, (2) to test covariation between microevolutionary/ecological traits and large biogeographical patterns through temporal and spatial sampling, and (3) to review the generality of the patterns of

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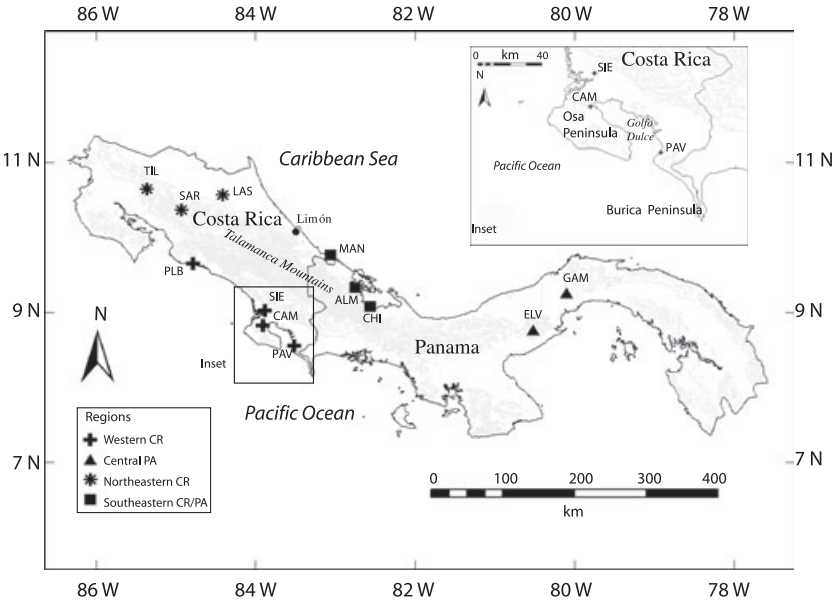

Figure 1 Sampling localities for *A. callidryas* Cope 1862 from four regions in Costa Rica (CR) and Panama (PA): north-eastern CR (NE CR), south-eastern CR/PA (SE CR/PA), western CR, and central PA. The grey shaded region shows topographic relief. Sites sampled in 2005 are close to localities sampled in a previous study of polymorphism (Savage & Heyer, 1967). The inset shows a detailed view of south-western CR. See Table S1 for sample locality abbreviations.

differentiation in *A. callidryas* using comparisons with patterns reported for other taxa in this region.

MATERIALS AND METHODS

Study sites

We evaluated the geographical variation of individuals sampled from 12 populations in four regions throughout Costa Rica (CR) and Panama (PA): north-eastern CR, south-eastern CR/PA, western CR, and central PA (Fig. 1, see Table S1 in Supplementary Material: regional nomenclature follows Savage & Heyer, 1967). Populations in the western CR region occur along the Pacific versant of the Talamanca Mountains. All other populations occur along the Caribbean, including all central PA populations which are located on the Caribbean side of the continental divide (GIS data; Table S1). Seven of the sampled populations are the same as those sampled by Savage and Heyer, allowing for a direct comparison between sites over a 38-year period. An additional five populations in close proximity to the Savage and Heyer sites (within 8–20 km) were included in the temporal comparisons.

Sampling sites were selected to test four biogeographical hypotheses of regional differentiation in the red-eyed treefrog. We compare diversity patterns between the Pacific and Caribbean regions to test the isolating role of the Cordillera de Talamanca (Fig. 1). For the second biogeographical barrier (Gulfo Dulce), we sampled populations from both the Osa and Burrica peninsulas (Fig. 1). Finally, we compared north-east and south-east CR/PA populations to test a putative break at Limón, CR. To investigate a possible contact zone between north-east and north-west CR populations, we sampled two mid-elevation sites situated along this potential corridor (Til and SaR).

We conducted field surveys during part of the breeding season (May–August) in 2004 and 2005. Data for both years were combined into a single data set (2005). At each sample site, we captured adult males and females, collected body size data (snout–vent length, SVL), flank-stripe pattern and coloration. We documented flank-stripe pattern and coloration by taking digital photographs of every individual using a Nikon Coolpix 5700 against a background black–white–grey card for colour standardization (photographs available upon request, archived at Cornell University Museum of Vertebrates (CUMV)). We photographed each individual in four positions to capture the full range of body coloration: posterior surface of the thighs, ventral surface and both the left and the right side of the body with the legs and arms outstretched. One to three individuals from eight populations were preserved as vouchers and deposited at CUMV (14093, 14206–08, 14210–11, 14228, 14230, 14231–33) and the University of Costa Rica, San José (19100–101, 19213). All other individuals were released at the capture sites.

Flank-stripe pattern

Agalychnis callidryas shows bright, contrasting flank stripes, usually white to pale yellow, overlaying the background colour. We implemented the scoring system designed by Savage & Heyer (1967) to categorize individuals as possessing one of five flank-stripe pattern types: A, AB, B, BC and C (Fig. 2). In 2005, we discovered frogs with two novel combinations of the three basic types; for these individuals, we modified the Savage and Heyer protocol and categorized them as AC or ABC (Fig. 2). Individuals sampled in both time periods often exhibited different patterns on the two sides of their body (Savage & Heyer, 1967). Due to high rates of asymmetry, we analysed only the left side of the body.

Measuring coloration

In life, red-eyed tree frogs are bright green dorsally, have large red eyes and orange-red feet and hands. Coloration of flanks

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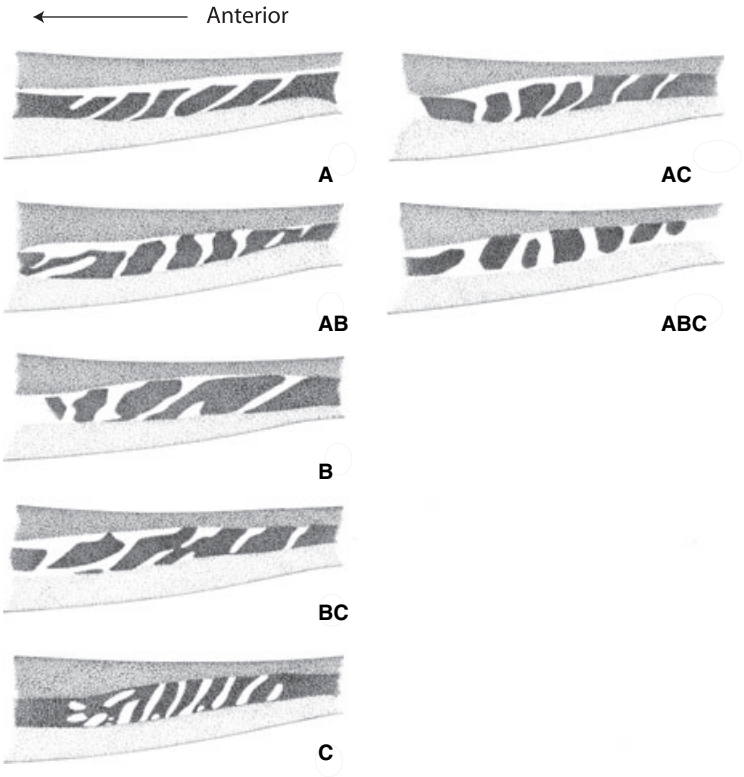

Figure 2 Variation of flank pattern in *A. callidryas*. Individuals with pattern A have a horizontal line connecting all vertical stripes; in pattern B, the vertical stripes are disconnected, and each is 'T' shaped; in pattern C the disconnected vertical stripes have no 'T' shape. Individuals with a combination of these three basic pattern types are characterized as AB (both A and B stripes) or BC. This study identifies two novel pattern types, ABC and AC, not observed by Savage & Heyer (1967).

and limbs is very similar for an individual in most regions, thus we only measured and report leg colour in this study.

Many studies of coloration use spectral reflectance (Summers *et al.*, 2003; Hofmann *et al.*, 2006; Vercken *et al.*, 2006), which yields precise measures of hue, saturation and brightness at focal points of interest. This technique yields highly accurate results for quantifying colour, especially when hue does not vary within an individual. The greatest advantage of our method of quantifying colour is that we are able to accurately measure multiple hues occurring across the entire surface area of an organism, as opposed to subsampling colour patches. The use of the grey-card colour standard provides necessary and sufficient standardization to accurately differentiate hue. In addition, digital photography is increasingly utilized to document animal coloration (Stevens *et al.*, 2007; Richards & Knowles, 2007; J. Touchon & K. Warkentin, personal communication); digital cameras are a cost-effective alternative for quantifying colour variation and are easily used in field conditions.

Photographs of each individual were imported into Adobe Photoshop CS version 8 to correct for ambient light colour correction by reference to a black–white–grey standard (QPcard 101) in the background of every photograph. The colour-corrected photographs were then imported into ImageJ (version 10.2) for analyses. We measured colour as 'hue' in the HSB (hue, saturation, and brightness) domain because it became evident from preliminary testing that having a one-dimensional measure of colour (i.e. hue) was sufficient to distinguish populations.

The number of dominant leg colours of *A. callidryas* varies regionally; individuals from some populations are virtually monochromatic (e.g. blue), others contain two dominant colours (e.g. blue and orange), while others contain a continuum of multiple hues (e.g. reddish blue to greenish blue). To avoid a sampling bias, we therefore selected the entire posterior surface of the leg in ImageJ (as opposed to focal subsampling) to acquire a frequency histogram of the number of pixels for each hue (0–255), corresponding to 8-bit hue values of 360. We were careful to exclude sampling the green portion of the leg common to all individuals (see Fig. 3 for example). We transformed the ImageJ hue data (which range from 0–255) to the more conventional standard measure of hue with a range of 0–360. Because of the broad range of leg coloration in the red-eyed tree frog, we divided the 360° colour spectrum into eight equal colour bins, each spanning 45° (Fig. 3). Each colour bin has a central hue, surrounded by a gradient of neighbouring hues. The eight colour bins in this study are named according to the central hue for that bin (measured in degrees): red (337.5 to 22.5), orange (22.6–67.5), yellow (67.6–112.5), green (112.6–157.5), light blue (157.6–202.5), dark blue (202.6–247.5), purple (247.6–292.5) and violet (292.5–337.5). The standard hue definition of pure red is zero, therefore the red bin spans 22.5° on each side of 0°. Our transformations from the ImageJ data also eliminated any

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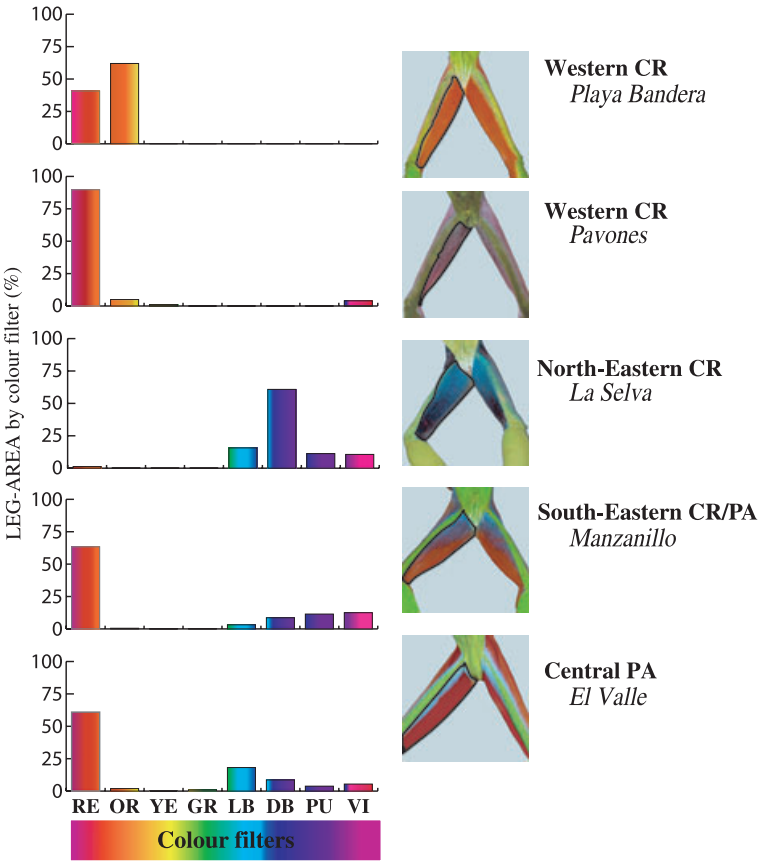

Figure 3 Colour polymorphism in the posterior surface of the thigh of *A. callidryas* from four regions throughout Costa Rica and Panama. Western CR exhibits a clinal change in coloration, therefore we provide photo images of the northern-most population (Playa Bandera) and the southern-most population (Pavones). For each photograph, the corresponding LEG-AREA histogram obtained from hue analyses in ImageJ is transformed to a traditional 360 colour range. A thin black line outlining the thigh shows colour selection analysed in ImageJ. Histogram bars are coloured based on the corresponding 360 colour range. Colour bins are: red (RE), orange (OR), yellow (YE), green (GR), light blue (LB), dark blue (DB), purple (PU), violet (VI). The exact numerical range for each colour bin is given in the text.

bright white overexposed (blown-out) regions of the photograph that come from light reflection from the wet body of the frog.

Statistical analyses

Flank stripe

We measured both temporal and spatial variation in flank-stripe pattern diversity. For the temporal analyses, we tested whether the relative frequency of each pattern differed between 1967 and 2005 within each region using a χ^2 contingency test in JMP (Version 5.1.2). Due to low sample sizes in some categories, we performed an exact test for a measure of significance of the χ^2 test (StatXact Version 4, 1998, Cytel Software Corporation, Cambridge, MA, USA). Because the 1967 study did not contain AC or ABC phenotypes, we analysed temporal patterns in two ways: first, we excluded AC and ABC individuals from 2005 for a direct comparison with the 1967 data set; second, we considered the possibility that ABC and AC patterns were actually present in 1967 but scored as AB or A, respectively. We repeated the analyses with those individuals in the study but rescored accordingly.

For the spatial analyses, we tested whether the distribution of flank-stripe patterns differed both within and between regions sampled in 2005 (using χ^2 contingency tests and exact test for correction of low sample sizes). In addition, we applied these analyses to the 1967 data set to test for patterns of differentiation between regions sampled in the Savage & Heyer (1967) study.

Coloration

We tested for differences in leg colour within and between regions sampled in 2005. We analysed leg coloration in two ways. First, we measured the percentage of leg area in each colour bin, hereafter referred to as LEG-AREA; second, we compared the mean population hue for each of eight colour bins. To test for population and regional differences in LEG-AREA, we used linear discriminant analysis, which compares each individual with the group multivariate mean (JMP Version 5.1.2). A classification matrix shows the number of individuals correctly assigned to source populations and the number of individuals misclassified to an alternative population, based on LEG-AREA alone. We used χ^2 tests to determine whether individual assignment was random with respect to source populations for each population and each region. Accurate assignment indicates that LEG-AREA has diagnostic value for regional identification and differentiation. In some cases, LEG-AREA was insufficient to unambiguously assign individuals to the correct population and/or region. For these cases, we compared the average hue among regions using Kruskal–Wallis nonparametric comparison of means implemented in JMP Version 5.1.2. We used discriminant

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analysis to test the independence of flank pattern and leg coloration. We tested the correlation between flank pattern and leg coloration (using chi-square contingency tests and exact tests for correction of low sample sizes) by categorizing leg colour into four bins: blue, blue and orange, orange, purple. We also used discriminant function analysis to test whether flank pattern is predicted for each individual (regardless of population of origin) based on LEG-AREA of blue, orange and purple.

Body size

We compared average male and female body size (SVL) among regions using Kruskal–Wallis nonparametric test of means. The 1967 study includes measures of regional (but not individual) body size. Therefore, we could not directly compare the two data sets, but can comment on the stability of a generalized pattern.

RESULTS

Flank stripe

Temporal pattern

The regional distribution of flank-stripe patterns has changed significantly over the last 38 years in all regions except western CR (significance of exact test of χ^2: $P = 0.0902$; north-eastern CR, $P < 0.001$; south-eastern CR/PA, $P < 0.001$; central Panama, $P < 0.001$; Fig. 4). Repeating those analyses with ABC/AC individuals rescored as AB and A, respectively, did not change those results. Changes in this phenotype over time include a shift in the frequency of the dominant flank pattern: A to AB in north-eastern CR; AB to BC in south-eastern CR/PA; AB to C in Panama. In addition, we observed the loss and gain of patterns, including a gain of AB in western CR, a gain of BC in north-eastern CR, a gain of C and loss of A in the south-eastern CR/PA and loss of A and B in Panama. Therefore, although populations continue to be significantly distinct based on flank-stripe patterns, these patterns are not static, even over relatively short time frames.

Spatial pattern

In 2005, all regions were distinguished from each other by different dominant pattern(s) ($\chi^2_{\text{d.f.}=18} = 384.08$, $P < 0.001$): pattern C in western CR; pattern AB in north-eastern CR; BC in south-eastern CR/PA; and a high proportion of both BC and C in central Panama (Fig. 4). Western CR is readily distinguishable from the other three regions based on the near fixation of pattern C and the absence of novel types ABC and AC, which are present in all other regions. These results are similar to our analyses of between-region variation in the 1967 data set ($P < 0.001$).

We detected differences in flank pattern between the two central Panama populations ($\chi^2_{\text{d.f.}=2} = 23.458$, $P < 0.001$), but no differences between populations within the other three regions: western CR ($\chi^2_{\text{d.f.}=3} = 6.36$, $P = 0.095$); north-eastern CR ($\chi^2_{\text{d.f.}=6} = 6.324$, $P = 0.38$), south-eastern CR/PA ($\chi^2_{\text{d.f.}=6} = 11.409$, $P = 0.076$).

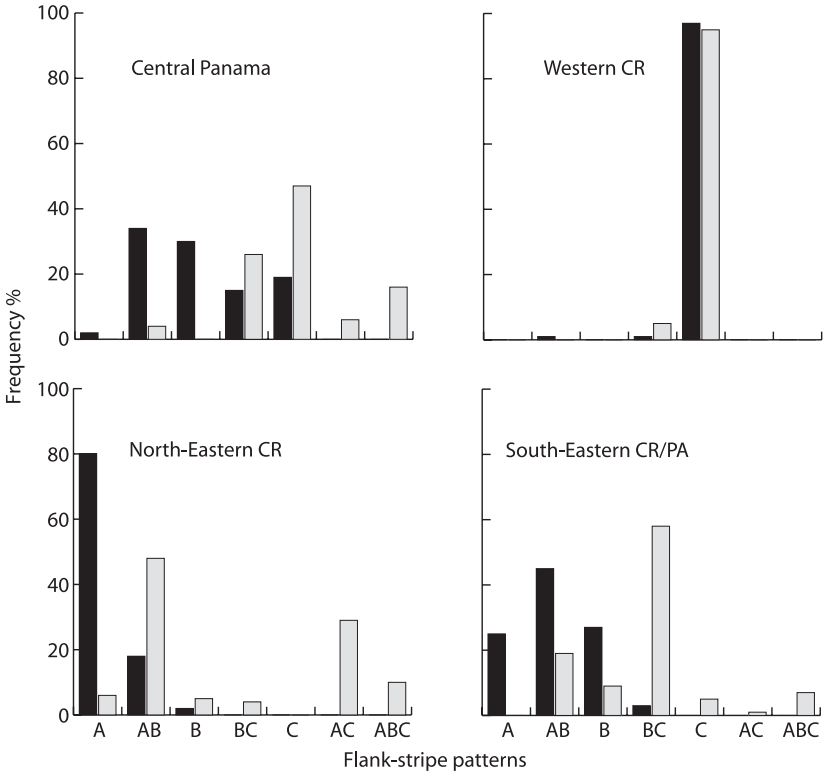

Figure 4 Temporal and spatial variation in the flank-stripe pattern in *A. callidryas* across four regions, sampled in 1967 (black bars) and 2005 (grey bars). All regions except western CR show temporal change in flank-stripe frequencies. Sample sizes are provided in Supplementary Table S1.

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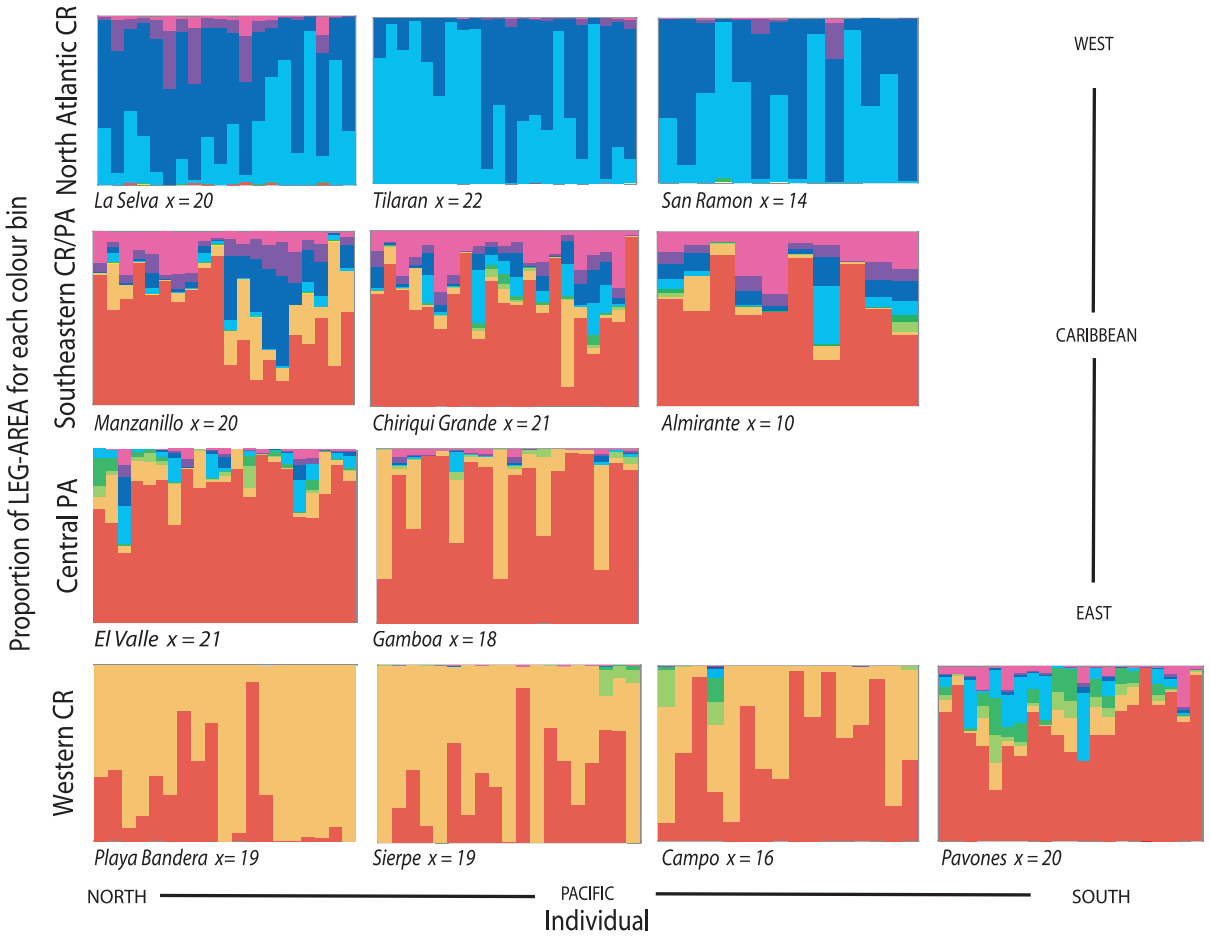

Figure 5 The proportion of the leg (measured as % pixels) assigned to each of eight colour bins (LEG-AREA) for 12 populations of *A. callidryas*. For each population, individuals are aligned along the horizontal axis. LEG-AREA for each individual is represented in a stacked, vertical histogram. Populations are arranged in a west to east direction for the three Caribbean regions and in a north to south direction for the Pacific region. Sample sizes (x) are provided for each population.

Coloration

Among-region variation

Our results show regional differentiation in leg coloration. The only individuals in the study with completely blue legs were found in north-eastern CR (Fig. 5). Individuals with bi-coloured legs (red/orange and blue; Fig. 5) occurred in two regions, south-eastern CR/PA and central PA. Western CR contains the only populations with solely red/orange-legged individuals.

We observe a west–east transitional change in coloration among populations along the Caribbean coast; the western-most site contains individuals with completely blue legs while individuals from the easternmost populations have primarily red/orange legs with some light blue coloration (Fig. 5). Between these two regions, individuals exhibit a near equal mix of red/orange and dark blue coloration on their legs.

Discriminant function analyses on LEG-AREA resulted in classification of most individuals to the source region ($\chi^2_{\text{d.f.}=16} = 530.82$, $P \leq 0.001$; Table 1). Discriminant function analyses classified north-eastern CR individuals correctly in all cases (Table 1). Some individuals from western CR were misclassified as central PA, and vice versa. Individuals from south-eastern CR/PA were assigned incorrectly to central PA, but with no reciprocity in misclassification (Table 1). To distinguish between these regions (with misclassified individuals), we compared the average regional hue in each of eight colour bins. First, we compared Pavones (western CR population with a high misclassification rate) with three regions: south-eastern CR/PA, central Panama and the other populations in western CR. We found that Pavones was distinguishable from the others based on three colour bins: red ($\chi^2_{\text{d.f.}=3} = 101.11$, $P < 0.001$), orange ($\chi^2_{\text{d.f.}=2} = 49.23$, $P < 0.001$), and violet ($\chi^2_{\text{d.f.}=3} = 27.38$, $P < 0.001$; Table 2). Next we compared western CR (excluding Pavones) and central Panama and found average hue differences in two colour bins: red ($\chi^2_{\text{d.f.}=1} = 16.27$, $P < 0.001$) and yellow ($\chi^2_{\text{d.f.}=1} = 7.96$, $P = 0.004$; Table 2). Finally, a comparison between south-eastern CR/PA and central PA revealed differences in average red ($\chi^2_{\text{d.f.}=1} = 30.07$, $P < 0.001$), light blue ($\chi^2_{\text{d.f.}=1} = 19.33$, $P < 0.001$) and dark blue ($\chi^2_{\text{d.f.}=1} = 11.12$, $P < 0.001$) bins (Table 2).

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Table 1 Classification matrix based on discriminant function analyses of LEG-AREA; the number of individuals sampled from population *i* on the vertical axis, into population *j* on the horizontal axis. The dark grey shaded boxes show the number of individuals correctly assigned to the source population; the light grey bars show correct assignment to source region. The unshaded boxes show the number of incorrectly assigned individuals. Total sample size per population, *n*. See Table S1 for population abbreviations. SE, south-eastern; NE, north-eastern.

	Population *j*											
	SE CR/PA			Central PA		Western CR				NE CR			
Population *i*	Alm	ChG	Man	ElV	Gam	Cam	Sie	PlB	Pav	LaS	Til	SaR	*n*
SE CR/PA													
Alm	4	2	2	1	1	0	0	0	0	0	0	0	10
ChG	6	6	5	1	2	0	0	0	1	0	0	0	21
Man	5	1	9	2	1	0	0	1	0	1	0	0	20
Central PA													
ElV	0	0	2	11	5	1	0	0	2	0	0	0	21
Gam	0	0	0	4	10	2	1	2	0	0	0	0	18
Western CR													
Cam	0	0	0	0	7	3	2	3	1	0	0	0	16
Sie	0	0	0	0	1	4	5	9	0	0	0	0	19
PlB	0	0	0	0	3	0	2	14	0	0	0	0	19
Pav	1	1	2	1	2	0	0	0	13	0	0	0	20
NE CR													
LaS	0	0	0	0	0	0	0	0	0	9	7	4	20
Til	0	0	0	0	0	0	0	0	0	0	11	11	22
SaR	0	0	0	0	0	0	0	0	0	2	5	7	14

Table 2 The mean regional (bold) and population hue for eight colour filters (each colour filter spans 45°, see text). See Table S1 for population abbreviations and Fig. 3 for sample sizes. Colour bin abbreviations: RE, red; OR, orange; YE, yellow; GR, green; LB, light blue; DB, dark blue; PU, purple; VI, violet.

	RE	OR	YE	GR	LB	DB	PU	VI
Western CR	**10.89**	**33.77**	**83.89**	**138.93**	**171.50**	**223.38**	**270.66**	**321.92**
PlB	20.67	26.48	–	–	–	–	–	–
Sie	16.71	32.05	79.63	129.08	174.99	216.57	268.56	318.28
Cam	14.70	30.62	79.53	137.26	174.71	224.42	267.84	314.57
Pav	−3.93	41.76	86.75	141.99	169.89	224.93	271.36	327.31
North-eastern CR	**−10.31**	**46.52**	**90.62**	**145.13**	**194.74**	**213.90**	**259.59**	**312.08**
LaS	−11.06	47.84	90.38	146.38	195.25	217.35	262.46	311.6
Til	−12.04	48.82	88.73	141.16	193.99	211.54	257.77	312.71
SaR	−5.08	42.09	92.18	145.98	195.18	212.66	256.86	312.58
South-eastern CR/PA	**6.54**	**30.42**	**89.80**	**140.28**	**186.70**	**225.90**	**269.50**	**321.70**
Man	9.95	27.80	92.88	143.13	189.63	226.63	267.85	320.02
ChG	4.60	30.47	89.66	140.40	183.71	224.68	270.52	322.68
Alm	3.83	35.56	86.07	136.54	187.15	227.00	270.68	323.03
Central PA	**13.26**	**31.75**	**86.66**	**138.20**	**178.37**	**223.40**	**270.90**	**321.71**
ElV	12.76	32.70	86.95	140.18	177.75	221.31	269.74	321.98
Gam	13.82	30.69	86.23	135.32	179.29	226.72	271.04	323.01

The association between flank pattern and leg coloration varies regionally (Fig. 6): flank pattern C (which occurs primarily in western CR) contains mostly orange-legged individuals, whereas flank pattern A (north-eastern CR) is observed with only blue-legged individuals. However, the other flank patterns co-occur with three of four leg colour types. There is a correlation between leg colour and flank pattern when considering all individuals in the study ($R^2 = 0.479$, $\chi^2_{\text{d.f.}=18} = 368.52$, $P < 0.001$). This correlation is largely due to the near fixation of flank pattern C in western CR (dominated by orange legs; Fig. 6). We repeated this analysis after removing individuals with flank pattern C and found a weaker correlation ($R^2 = 0.271$, $\chi^2_{\text{d.f.}=8} = 91.409$, $P < 0.001$). Overall, these findings are consistent with results from the discriminant function analyses which correctly assigned individuals to flank pattern based on LEG-AREA ($\chi^2_{\text{d.f.}=16} = 361.59$, $P < 0.001$).

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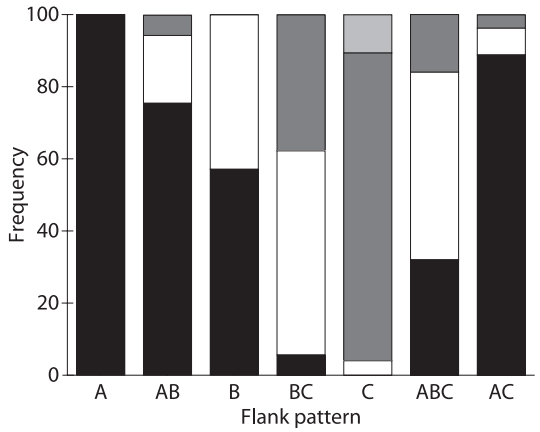

Figure 6 The proportion of each of four leg colour bins for seven flank-pattern types in *A. callidryas* individuals. Leg colour bins are as follows: blue (black bars), blue and orange (white bars), orange (dark grey bars), violet (light grey bars). Leg colour is positively correlated for flank pattern ($P < 0.001$).

Within-region variation

We detected among-population differentiation in coloration for all regions. This is most evident in western CR, where populations exhibit a north–south cline in coloration (LEG-AREA: $\chi^2_{\text{d.f.}=9} = 77.54$, $P = 0.001$; Fig. 5): individuals have red/orange legs in Playa Bandera; Sierpe contains red/orange/green-legged individuals; Campo contains red/orange/green/light blue-legged individuals; and Pavones (the most southerly western CR site) contains individuals with coloration in almost all colour bins (Fig. 5).

All north-eastern CR populations contain individuals with blue leg coloration; however, La Selva is distinct from San Ramon and Tilarán in both analyses of LEG-AREA ($P < 0.001$) and average hue in two colour bins, dark blue ($\chi^2_{\text{d.f.}=2} = 14.28$, $P = 0.0008$) and purple ($\chi^2_{\text{d.f.}=2} = 12.06$, $P = 0.0024$; Table 2). South-eastern CR/PA populations contain individuals with bi-coloured legs (red/orange and blue); based on LEG-AREA, most individuals were correctly assigned to their source population ($\chi^2_{\text{d.f.}=4} = 23.77$, $P < 0.001$; Table 1). Further, we detected fine-scale hue differences between south-eastern CR/PA populations in five colour bins: red ($\chi^2_{\text{d.f.}=2} = 11.84$, $P = 0.002$), orange ($\chi^2_{\text{d.f.}=2} = 10.84$, $P = 0.004$), yellow ($\chi^2_{\text{d.f.}=2} = 7.69$, $P = 0.02$), light blue ($\chi^2_{\text{d.f.}=2} = 9.38$, $P = 0.009$) and violet ($\chi^2_{\text{d.f.}=2} = 7.6$, $P = 0.02$; Table 2). Central PA populations are distinct from each other based on both LEG-AREA ($\chi^2_{\text{d.f.}=1} = 9.14$, $P = 0.01$; Table 1) and mean hue value in the dark blue colour bin ($\chi^2_{\text{d.f.}=2} = 6.12$, $P = 0.013$; Table 2).

Body size

Male body size varies regionally ($\chi^2_{\text{d.f.}=3} = 158.06$, $P < 0.0001$); individuals are biggest in north-eastern CR, smallest in Panama and of similar intermediate size in south-eastern CR/PA and western CR (Table S1). Unfortunately, we could not obtain the raw body size data from the 1967 study to compare SVL over the course of 38 years. Savage & Heyer (1967) report that male body size is (similarly) largest in north-eastern CR but of smaller, equal size in the other three regions. However, without raw data we cannot conclusively comment on any directional change in body size in these regions. Female body size in 2005 also varied regionally ($\chi^2_{\text{d.f.}=2} = 28.51$, $P < 0.0001$), but exhibited a slightly different pattern from the males. Although females are also largest in north-eastern CR, they are of intermediate body size in central Panama and smallest in western CR (Table S1). We lack body size data for females from central Panama to include in the analysis.

DISCUSSION

Our data corroborate the patterns of phenotypic divergence among *A. callidryas* in the four Central American biogeographical zones observed in previous studies (Savage & Heyer, 1967; Duellman, 2001). In addition to the flank pattern and body size variation already noted, we show that this variation is also observed in the geographical distribution of leg coloration. Regions are easily differentiated based on a combination of LEG-AREA and average hue value across eight colour bins. We also detected fine-scale geographical variation in coloration between some populations within each region. Temporal analyses of the flank-stripe pattern show significant changes in regional composition over 38 years in all areas except western CR.

Most anuran colour patterns are genetically inherited (Pyburn, 1961b; Hoffman & Blouin, 2000). Amphibian skin coloration is controlled primarily by two groups of pigment cells, melanophores and chromatophores (Hoffman & Blouin, 2000). Colour change over a short period of time (seconds to minutes) is associated with physiological changes due to temperature, humidity and ambient light, whereas changes over generations (e.g. due to natural selection) is typically associated with changes between greys and browns (Hoffman & Blouin, 2000). For *A. callidryas*, the shade of green on the dorsum can change rapidly with light exposure due to intracellular transport of pigment cells (Schliwa & Euteneuer, 1983). However, the coloration along the flanks, thighs and upper arms does not change with environmental cues (JMR personal observation). Therefore, we consider colour and pattern as variable, genetically inherited traits because it is unlikely that the full-colour spectrum differentiation between *A. callidryas* populations is due to local environmental factors.

Overall, north-eastern CR populations are distinguished by their large body size, blue legs and dominant flank-stripe pattern A. South-eastern CR/PA populations are intermediate in size, have bi-coloured legs (orange and dark blue) and a dominant B stripe. Central Panamanian frogs are small, have predominantly orange legs with a small percentage of light blue and contain flank-stripe patterns B and C. Western CR populations are of intermediate body size, exhibit a

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north–south clinal change in coloration and are unique in being nearly monomorphic for flank-stripe pattern C.

Two of the phenotypic traits measured in this study are correlated (leg coloration and flank pattern). The strength of the correlation varies for different flank patterns. For example, flank patterns A and C are tightly correlated with coloration, but the same is not true for other patterns (Fig. 6). We suggest that these two traits loosely co-evolve over spatial and temporal scales. In North Atlantic CR, all individuals are fixed for blue legs. While flank pattern A is found only in blue-legged individuals, a large percentage of these individuals with blue legs have alternative flank patterns. Similarly, individuals sampled from western CR exhibit near-fixation for flank-pattern C but with variation in leg coloration (Fig. 5). Thus, one region (North Atlantic) shows a near-fixation for blue leg coloration (with variable flank pattern) while western CR shows near-fixation for flank pattern C (with variable leg coloration).

Spatial variation

Understanding the mechanisms that underlie geographical patterns in phenotypic variation provides insight into the evolutionary history of the species. These mechanisms include genetic isolation, microhabitat adaptations and directional selection via signalling (mate choice, predator–prey relationships; Endler, 1992). Based on the patterns of phenotypic diversity, we apply our knowledge of the natural history of the red-eyed tree frog and the geological history of Central America to discuss the possible mechanisms underlying the spatial patterns of diversity in two parts: biogeography and signalling.

Biogeography

The distribution of genetic or phenotypic diversity is often strongly correlated with landscape history (Prohl *et al.*, 2006) and microhabitat differences (Thorpe & Baez, 1993; García-París *et al.*, 2000). Many anurans, including *A. callidryas*, rely on rainfall for reproduction. Because rainfall patterns and climate vary across regions in Costa Rica and Panama (Holdridge, 1947; Kohlmann *et al.*, 2002), we expect that these differences will reinforce spatial isolation between populations. The highly localized variation in colour pattern in *A. callidryas* may be partially explained by reduced gene flow as a result of the topographic landscape (providing both physical and climatic barriers) of Central America. For the most part, our results corroborate regional genetic differentiation in other taxa (Zamudio & Green, 1997; Crawford, 2003; Zeh *et al.*, 2003; Weight *et al.*, 2005).

We found evidence to support the isolating effect of all three biogeographical barriers (Cordillera de Talamanca, Limón, Osa Peninsula) and one putative contact zone (north-east–north-west) in influencing the distribution of phenotypic diversity in *A. callidryas*. The Cordillera de Talamanca is a strong barrier to gene exchange between Caribbean and Pacific populations for other terrestrial amphibians and reptiles (Zamudio & Greene, 1997; Crawford, 2003) and probably explains the divergence in coloration and flank stripe pattern in *A. callidryas* (Figs 4 & 5).

Both leg coloration and flank pattern differentiate the two regions separated by Limón (Figs 4 & 5) supporting the hypothesis that Limón is a biogeographical break for some terrestrial organisms. The addition of other characters (molecular, behavioural) to this data set will greatly contribute to understanding the nature of this biogeographical break.

Populations in western CR showed the most intra-region variability of all the sampled regions, exhibiting a north–south clinal change in coloration (Fig. 5). The southernmost population, Pavones, containing the most colour polymorphism, is isolated from the other three western CR populations by the Golfo Dulce. Thus, it is possible that Pavones and the other western CR populations evolved in allopatry.

We found evidence to support the hypothesis of a putative contact zone (north-east–north-west) at the junction of three non-contiguous mountains east of the Talamanca Mountains. Savage & Heyer (1967) suggested that low levels of historical gene flow connected these two regions. Our more extensive sampling of flank pattern corroborates that low-elevation mountain passes provide habitat corridors within the physiological tolerance of *A. callidryas*, and facilitate passage for dispersal between these two regions (Fig. 4). For example, the dominant flank pattern observed in Tilarán was AC, a combination of A and C flank stripes. This mid-elevation site may be a site of historical gene flow between north-eastern CR 'A' and western CR 'C' forms, or alternatively, may reflect ancestral polymorphisms. However, breeding studies are required to confirm that AC is a hybrid form. We maintain caution in using flank-pattern analyses alone to make predictions about gene flow patterns because leg coloration clearly distinguishes these two regions.

Climate also acts as a geographical barrier (Grinell, 1914). For example, differences in wind and rain patterns across the Talamanca Mountains alter the climate of eastern, western and central Costa Rica (Holdridge, 1947; Kohlmann *et al.*, 2002). As a result, these regions are very diverse and range from dry, lowland, deciduous forest (Pacific), to cloud forest (along the divide), to hot and wet lowland rainforest (Caribbean). *Agalychnis callidryas* occurs only in wet forest in the Caribbean, and in patches of coastal wet forest in the dry pacific versant, a pattern common in many Central American frogs with affinity to wet forest (Savage, 2002). Thus, migration is further restricted by the dry forest landscape between south-western CR and central Panama.

Signalling

Phenotypic signals used for communication (visual and acoustic) co-evolve with the sensory systems of conspecifics and predators, and are therefore a balance between sexual selection (Endler, 1980, 1992; Tuttle & Ryan, 1981) and natural selection (Hoekstra *et al.*, 2004). Therefore, it is useful to

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consider the type of colour pattern (cryptic and aposematic) and its employment (predator avoidance and/or visual signalling for mate choice) as co-evolutionary forces that shape the directional selection of phenotypic diversity (Endler, 1992; Maan *et al.*, 2004; Endler & Mielke, 2005).

Organisms that possess no inherent chemical defence typically display cryptic coloration and/or behavioural crypsis such that their colour pattern matches a random sample of their environment. Many anurans exhibit cryptic colour polymorphism (Savage & Emerson, 1970; Nevo, 1973; Sazima, 1974; Morey, 1990). We know that this is true for *A. callidryas*: the green dorsal coloration reflects in the infrared range (700–900 nm), perfectly matching leaf reflectance (Schwalm *et al.*, 1977). *Agalychnis callidryas* takes retreat under leaves during the day, thus effectively hiding from diurnal predators. However, the variation we quantified in this study (reds, oranges and blues) is not typically associated with crypsis, and we suggest that the differences in bright coloration or contrasting colour pattern may have evolved as aposematic coloration. Aposematism occurs in organisms that advertise chemical defences (such as distasteful/poisonous toxins) to potential predators through flashy and/or bright colours (Siddiqi *et al.*, 2004). A classic example in anurans is a number of species in the poison arrow family, Dendrobatidae (Summers *et al.*, 2003). One species, *Dendrobates pumilio*, is highly colour polymorphic and utilizes aposematism to avoid diurnal predation (Siddiqi *et al.*, 2004). Similar to poison arrow frogs, phyllomedusine frogs contain noxious skin peptides (Cei & Erspamer, 1966) which stimulate regurgitation by snake predators (Sazima, 1974). Aposemetism in crespuscular amphibians is poorly understood. However, a behavioural study showed reduced predation rates on a brightly coloured crepuscular salamander (*Ensantina e. xanthoptica*; Kuchta, 2005), suggesting that behavioural and vision studies are critical in determining whether crepuscular/nocturnal predators (birds, snakes, spiders) possess the visual system required to identify and discriminate *A. callidryas* from non-toxic prey items. It is possible that *A. callidryas* utilizes a combination of crypsis and aposematism as a defence mechanism.

Sexual selection underlies geographical patterns of diversity and can drive speciation through assortative mating among closely related species and among populations (West-Eberhard, 1983; Masta & Maddison, 2002; Summers *et al.*, 2003, 2004; Siddiqi *et al.*, 2004). We hypothesize that female *A. callidryas* uses both visual and acoustic signals in mate choice: the colourful leg and flank regions with contrasting vertical and horizontal stripes may serve as visual signals, while the male advertisement calls are known as acoustic signals. These two signals (acoustic and visual) may operate together (Masta & Maddison, 2002; Candolin, 2003, 2005; Prohl *et al.*, 2006): the acoustic signals provide information on the location of males within a swamp and the visual signals (colour pattern) form the basis of mate choice at close range. Directional selection for brighter and larger contrasting colour pattern has been observed in other anuran species, including the frog *Hyla squirelli* (Buchanan, 1994), and it is possible that differences in flank-stripe characteristics in *A. callidryas* reflect localized sexual selection pressures.

Temporal variation

We observed a significant shift in the dominant flank-stripe pattern from data collected 38 years ago in three of the four regions (Fig. 4). The only region that remained static was western CR, a region that was and remains monomorphic for flank-stripe pattern C. Temporal studies of changes in genetic and phenotypic variation are uncommon (Barcia *et al.*, 2005; Prieto *et al.*, 2005; Ray & King, 2006). These studies have shown differing results, with some reporting large shifts (Barcia *et al.*, 2005; Prieto *et al.*, 2005) and others observing no change over time (Blanco *et al.*, 2005; Rendell & Whitehead, 2005). In the cases of limited change, the authors have argued that large effective population size limits genetic drift and stabilizes polymorphisms over time (Blanco *et al.*, 2005).

In our study, temporal variation in flank-stripe pattern may reflect directional selection due to changes in mate choice criteria or predator behaviour (Pyburn, 1961b). Alternatively, landscape changes, either natural or anthropogenic, might alter gene flow patterns and effectively swamp localized selection (Lenormand, 2002). The stability of flank-stripe pattern in western CR could be due to large effective population sizes (N_e), or to historical fixation of a single pattern in a region that receives no migrants from other more variable regions. We have no reason to believe N_e is higher in western CR than in other regions. The combination of these two temporal patterns (static in western CR and dynamic in three regions) suggests that *A. callidryas* is not subjected to the same ecological and evolutionary history across its range. We are currently using mitochondrial and nuclear genetic markers to examine historical and current patterns of gene flow among these populations/regions for comparison with phenotypic variation in this species.

CONCLUSIONS

We observe a spatial and temporal change in flank-pattern diversity in three of four regions as well as present-day regional divergence in three phenotypic traits in *A. callidryas*: coloration, flank stripe and body size. The occurrence of colour polymorphisms has been well documented over broad spatial scales (Savage & Heyer, 1967; Duellman, 2001); however, our analyses of population- and regional-level colour and pattern variation reveals a much more complex evolutionary history than previously described.

The characters analysed in this study clearly delineate biogeographical regions and allow us to formulate questions about population dynamics within regions. However, these traits are not intended for phylogenetic analysis; that is, the traits do not offer insight into the evolutionary relationships among regions. We are continuing work in this direction using nuclear and mitochondrial DNA markers.

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The present study detected fine-scale variation in coloration within and between populations, allowing us to consider the action of specific evolutionary processes (selection and gene flow) that operate to maintain differentiation. Based on these patterns of similarity, we expect that our genetic analyses will detect high gene flow between south-eastern CR/PA and Panamanian regions, and restricted gene flow between the two subdivided western CR populations. We propose that landscape features, historical geological processes and asynchronous reproductive seasons limit gene flow between regions and that sexual selection of colour pattern may underlie the phenotypic differentiation observed in this study.

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SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article online:

Table S1 Sampling populations of *Agalychnis callidryas* from four regions in Costa Rica and Panama.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-2699.2007.01824.x

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