

Genetic Diversification, Vicariance, and Selection in a Polytypic Frog

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Abstract

Spatial patterns of heritable phenotypic diversity reflect the relative roles of gene flow and selection in determining geographic variation within a species. We quantified color differentiation and genetic divergence among 20 populations of the red-eyed tree frog (*Agalychnis callidryas*) in lower Central America. Phylogenetic analyses revealed 5 well-supported mitochondrial DNA clades, and we infer from our phylogeny that geographic barriers have played a large role in structuring populations. Two phenotypic characters varied independently among isolated population groups: Flank coloration distinguished Caribbean from Pacific individuals, whereas leg coloration exhibited a more complex geographic pattern. We detected 3 generalized spatial patterns of genetic and phenotypic diversity: 1) phenotypic differentiation in the presence of historical connectivity, 2) phenotypic uniformity across genetically differentiated regions, and 3) codistribution of genetic and phenotypic characters. These patterns indicate that phenotypic diversification is highly regionalized and can result from spatial variation in localized adaptations, geographic isolation, genetic drift, and/or evolutionary stasis. Although the mode of selection underlying color variation was not the focal objective of this study, we discuss the possible roles of natural and sexual selection in mediating population differentiation. Our study underscores the fact that selection gradients vary across relatively small spatial scales, even in species that occupy relatively homogeneous environments.

Key words: *Agalychnis*, biogeography, color pattern, Costa Rica, Panama

The spatial distribution of divergent phenotypes provides insight into the relative roles of natural and sexual selection, gene flow, and vicariance in the diversification of a species (Grinnell 1924; Ford 1971; Slatkin 1985; Brown et al. 1996). Many taxa exhibit some phenotypic variation among individuals within populations, although the spatial distribution of genetic and phenotypic diversity varies substantially among species (Hoffman and Blouin 2000; Gray and McKinnon 2007). For some species, geographic clines in body size (Brown and Thorpe 1991; Storz 2002), behavior (Thompson 1990; Pröhl et al. 2006), color pattern (McDiarmid 1968; Stewart 1974; Hoffman and Blouin 2000; Woolbright and Stewart 2008), life-history traits (Dhondt et al. 1990), or ornamentation (Nevo 1973; Gray 1983; Brooks and Endler 2001a; Storz et al. 2001) result from selection acting on continuous traits across selective gradients (Endler 1973; Storz et al. 2001; Storz 2002), genetic drift, or both (Hoffman et al. 2006). In contrast, far fewer species are highly polytypic across their range, with significant phenotypic variation among but low variation within populations. In those cases, divergence usually results from isolation due to dispersal barriers that can also be reinforced by

local adaptation, sexual selection, and genetic drift (Summers et al. 2003; Fuller et al. 2004; Maan et al. 2006; Boul et al. 2007). These 2 patterns of spatial variation are not exclusive; some species exhibit phenotypic divergence among regions (due to dispersal barriers) as well as clinal variation within regions (Endler 1973).

The interaction between gene flow and localized selection determines the extent of phenotypic differentiation among localities and the rate and direction of phenotypic change over time (Grinnell 1924; Slatkin 1985; Lenormand 2002). For example, high levels of gene flow, either due to dispersal capacity or the absence of isolating barriers, combined with limited localized selection result in widespread genetic and phenotypic homogeneity. In contrast, strong localized divergent selection and geographic isolation can result in highly structured populations (King and Lawson 1995; Boul et al. 2007). Congruence in spatial patterns of variation among multiple traits indicates that trait evolution has been mediated by similar historical microevolutionary processes. Alternatively, discordance between genetic and phenotypic characters could demonstrate

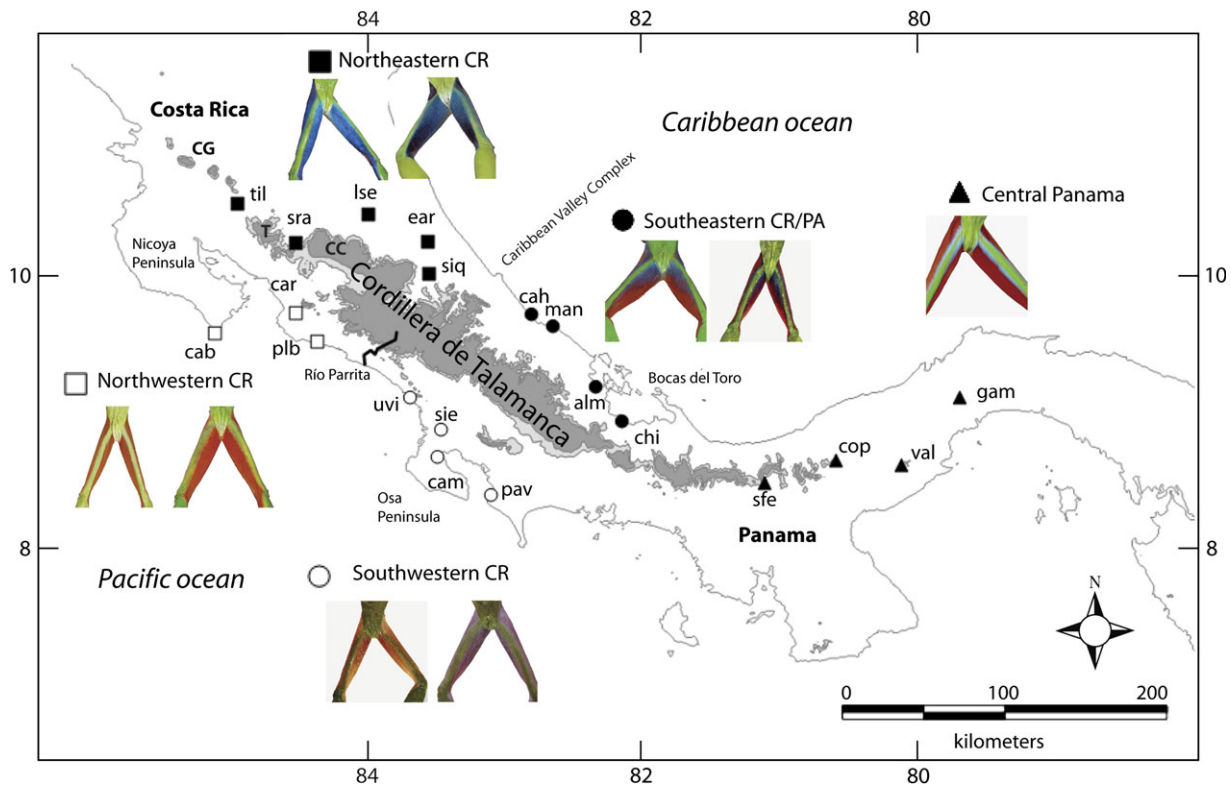


Figure 1. Sampling localities for 20 populations of *Agalychnis callidryas* in five biogeographic regions. The Cordillera de Talamanca isolates the Pacific (open symbols) and Caribbean (dark symbols) versants of CR and PA. The shading of topological relief corresponds to elevation: dark gray (>1300 m), light gray (300 – 1299 m), white (0 – 300 m). Elevations higher than 1300 m exceed the physiological tolerance of *A. callidryas*. Dry forest habitat between Southwestern CR and Central PA prevents the occurrence of red-eyed tree frogs along the Pacific coast of PA. Putative biogeographic barriers tested in this study: Cordillera de Talamanca, Caribbean Valley Complex, Bocas del Toro, Río Parrita, and Osa Peninsula. Also featured are the Cordillera Central (CC), Tilarán mountains (T) and Cordillera Guanacaste (CG). Representative photographs of frogs from each region demonstrate spatial variation in leg coloration.

evolutionary stasis for phenotypic traits despite restrictions in gene flow or from strong localized selection in the presence of gene flow (Endler 1973; Gray 1983; Dallimer et al. 2003; Hoekstra et al. 2005; Jordan et al. 2005; Pröhl et al. 2006; Rosenblum 2006).

The red-eyed tree frog, *Agalychnis callidryas*, is a common neotropical frog broadly distributed from Central Mexico to Colombia (Duellman 2001; Savage 2002); this species is polytypic (Savage and Heyer 1967; Duellman 2001; Robertson JM and Robertson AD 2008) and shows sufficient phenotypic differentiation to distinguish frogs from 5 biogeographic regions in Costa Rica (CR) and Panama (PA) with high accuracy (Robertson JM and Robertson AD 2008; Robertson et al. 2009). Unlike the green dorsum coloration in this species, leg and flank colors do not change with light intensity (Schliwa and Euteneuer 1983) or after adult coloration is acquired. Captive breeding of *A. callidryas* (Gomez-Mestre I, personal communication) indicates that color pattern has a strong heritable component. Thus, although further studies are necessary to rule out any environmental modulation of color or phenotypic

plasticity, the available evidence indicates that color pattern is a reliable character for evolutionary studies.

Previous analyses revealed regional diversity in leg coloration for *A. callidryas* (Robertson JM and Robertson AD 2008); a subsequent study of gene flow patterns among these regions, using polymorphic microsatellite loci, indicated that populations were structured into 5 geographic regions with admixture among some, but not all, neighboring regions (Robertson et al. 2009). Here, we tested whether these 5 geographic regions are associated with historical barriers to gene flow in lower Central America by examining mitochondrial DNA (mtDNA) haplotype diversification and the distribution of 2 phenotypes (flank and leg coloration).

The 5 regions in this study have been shaped by the complex geological history of lower Central America (Holdridge 1947; Kohlmann et al. 2002; Savage 2002; Kohlmann et al. 2007a). Three of these regions occupy the wet Caribbean forest east of the Continental Divide (northeastern CR, southeastern CR, and Central PA), whereas 2 occupy the Pacific slopes of the Continental Divide (northwestern CR, southwestern CR; Figure 1).

Three major landscape features coincide with the geographic borders between regions and are known to structure populations of other Central American taxa (Kohlmann et al. 2002). The exact nature of the other regional breaks is not well understood. Here, we discuss each of 5 putative barriers that we hypothesize contribute to geographic structure among red-eyed tree frog populations. 1) Major montane features include the Cordillera de Talamanca, and 3 smaller volcanic mountain ranges (Tilarán Mountain, Cordillera Central, and Cordillera Guanacaste) that lie along the Continental Divide and isolate Caribbean from Pacific populations. The uplift of the Cordillera de Talamanca occurred approximately 3–5.4 million years ago (Ma) (Coates and Obando 1996; Kohlmann et al. 2002) and has imposed a strong barrier to gene exchange between Pacific and Caribbean populations of terrestrial snakes (Zamudio and Greene 1997), frogs (Crawford 2003), beetles (Kohlmann et al. 2007a), and montane salamanders (García-París et al. 2000). This mountain range extends 400 km along the Central American Continental Divide and reaches its highest point of 3800 m at Cerro Chirripó (Coates and Obando 1996; Kohlmann et al. 2002). The wet tropical forest typical of lower elevations is replaced by cloud forest and dry Páramo above 3100 m (Kohlmann et al. 2002). These higher elevation habitats are inhospitable to *A. callidryas* and likely prohibit movement across the Cordillera de Talamanca. 2) The Osa Peninsula is well known for its high endemism and unique distribution of plants and animals, supporting the geological hypothesis that the Osa Peninsula was an offshore island that drifted into the mainland of CR approximately 2 Ma (Kohlmann et al. 2002). 3) Prior to the formation of the Cordillera de Talamanca, the region of Bocas del Toro experienced a short-lived uplift, approximately 5–7 Ma. Multiple colonization events consistent with the uplift have been documented for other taxa (Zeh et al. 2003; Weigt et al. 2005). This region behaves as a geographic barrier for other anuran taxa (Crawford 2003; Summers et al. 2003; Wang et al. 2008) and has been referred to as the “Bocas Break” (Crawford et al. 2007). 4) Plate tectonic and microplate tectonic activity resulted in the isolation of dry forest in northwestern CR and wet forest in southwestern CR (Holdridge 1947; Kohlmann et al. 2002; Crawford et al. 2007). This divide is important for insect and plant taxa (Kohlmann et al. 2007a, 2007b), but the consequences for anuran population structure is unclear. We refer to this barrier as the Río Parrita because this river drains from the Cordillera de Talamanca into the Pacific Ocean and coincides with the geological history of Pacific coast forest. Although the river may not be a barrier, it marks the geographical location of historical barriers that occurred in that region. 5) The Caribbean lowland forest is primarily wet, lowland tropical forest in northeastern CR and transitions into a series of floodplain valleys that occupy the southeastern CR region (Valle de la Estrella, Valle de Talamanca, and Llanura de Santa Clara [Kohlmann et al. 2002, Figure 1]). The isolation of northeastern and southeastern CR is not well understood for terrestrial, lowland taxa but coincides with the geographic distribution

of some anuran and beetle taxa (Kohlmann et al. 2002; Savage 2002; Hagemann and Pröhl 2007). Here, we refer to this putative barrier as the Caribbean Valley Complex.

In this study, we compared the distribution of variation in flank and leg color with that of genetic lineages across this region to test hypotheses about the role of selection and gene flow in patterns of diversification. Our sampling throughout CR and PA represents approximately 25% of the total geographic range of the species and encompasses all the known color variants (Duellman 2001). Our specific objectives were to test 1) the role of isolation due to geographic distance and/or geographic features in structuring genetic diversity, 2) whether regional color variation could also be explained by the same geographic factors, and 3) the hypothesis that spatial patterns of phenotypic and genetic diversity were concordant.

Material and Methods

Field Sampling

We quantified patterns of genetic and phenotypic variation among 20 red-eyed tree frog populations from 5 biogeographic regions in CR and PA (Figure 1). We expanded on an earlier study of phenotypic variation (Robertson JM and Robertson AD 2008) to include more populations, an additional measure of phenotypic diversity (flank coloration), and an analysis of mtDNA differentiation among the same individuals to serve as a comparative framework for the phenotypic data. We conducted field surveys during the breeding seasons (May–August) of 2003, 2004, and 2005. At each site, we captured 10–26 individuals and collected data on leg and flank coloration and pattern. We photographed every individual using a Nikon Coolpix 5700 against a black–white–gray card for color standardization. For each individual, we photographed the posterior surface of the thighs, left flank, and right flank of the body.

Up to 3 individuals per population were preserved as vouchers and deposited at the Cornell University Museum of Vertebrates (CUMV: 14093, 14206–14211, 14228, 14231–14233) and the University of CR, San Jose, CA (accession numbers: 19100–19101, 19213). All photographs have been archived at the CUMV. Nonvouchered individuals were photographed, toe clipped for genetic material, and released at site of capture.

Population Genetic Variation

We extracted genomic DNA from 125 individuals sampled for this study (Table 1). Toe clips or liver was digested in standard lysis buffer with proteinase K followed by purification using the Qiagen DNeasy Tissue Kit (Qiagen, Valencia, CA) following manufacturer’s protocols. We amplified a fragment of the mtDNA including the partial 16S rRNA, the complete nicotinamide adenine dinucleotide plus hydrogen (NADH) dehydrogenase subunit 1, and the adjacent flanking tRNA^{Met} (hereafter, we refer to this entire amplified sequence as ND1, for simplicity) using

Table 1. Geographic sampling of phenotypic and mtDNA variation for populations of *Agalychnis callidryas*

Region	Province, Country	Population	GIS	Color	DNA	No. Unique Haplotypes	<i>h</i>	Po	π
Northeast CR	Heredia, CR	La Selva	10.4327, -84.0080, 37	20	10	8	0.95 (0.059)	15	0.0034 (0.0021)
	Alajuela, CR	San Ramon	10.2335, -84.5287, 638	14	4	2	0.50 (0.265)	6	0.0026 (0.0020)
	Guanacaste, CR	Tilarán	10.5162, -84.9601, 637	22	6	2	0.73 (0.152)	7	0.0030 (0.0020)
	Heredia, CR	Siquirres	10.0546, -83.551, 574	0	1	—	—	—	—
	Heredia, CR	Universidad de EARTH	10.2368, -83.567, 44	0	6	5	0.93 (0.121)	10	0.0032 (0.0021)
Southeast CR/PA	Limón, CR	Manzanillo	9.6332, -82.6556, 2	26	8	7	0.96 (0.077)	32	0.0074 (0.0043)
	Limón, CR	Cahuita	9.7189, -82.8143, 16	0	3	3	1.00 (0.272)	22	0.0127 (0.0098)
	Bocas del Toro, PA	Almirante	9.1980, -82.3445, 13	10	2	2	1.00 (0.500)	1	0.0008 (0.0012)
	Bocas del Toro, PA	Chiriqui Grande	8.9460, -82.1571, 21	21	6	5	0.93 (0.121)	29	0.0093 (0.0057)
Central PA	Panamá, PA	Gamboa	9.1231, -79.6930, 51	22	6	5	0.93 (0.121)	13	0.0043 (0.0028)
	Coclé, PA	El Copé	8.6299, -80.592, 792	22	7	5	0.85 (0.137)	12	0.0034 (0.0022)
	Coclé, PA	El Valle	8.6299, -80.1159, 866	21	4	3	0.83 (0.222)	4	0.0018 (0.0015)
	Veraguas, PA	Santa Fe	8.5070, -81.1141, 714	17	6	2	0.53 (0.172)	34	0.0157 (0.0094)
Southwest CR	Puntarenas, CR	Sierpe	8.8892, -83.477, 17	19	11	6	0.80 (0.113)	7	0.0017 (0.0012)
	Puntarenas, CR	Campo	8.6909, -83.5013, 35	16	5	4	0.90 (0.161)	5	0.0022 (0.0016)
	Puntarenas, CR	Uvita	9.1235, -83.7011, 26	24	11	9	0.94 (0.065)	82	0.0345 (0.0183)
	Puntarenas, CR	Pavones	8.4204, -83.1069, 37	20	7	4	0.71 (0.180)	4	0.0009 (0.0008)
Northwest CR	Puntarenas, CR	Cabo Blanco	9.5805, -85.1246, 166	18	7	4	0.75 (0.139)	43	0.0097 (0.0056)
	Puntarenas, CR	Carara	9.7256, -84.5313, 385	25	5	2	0.93 (0.121)	10	0.0026 (0.0019)
	Puntarenas, CR	Playa Bandera	9.5188, -84.3774, 23	19	11	8	0.92 (0.066)	14	0.0038 (0.0023)

Sampling information includes province, country, and population with GIS data (latitude, longitude, elevation [m]). The number of individuals sampled for phenotype (leg and flank color, color) and genetic variation (DNA). A summary of within-population diversity of NADH1 sequences: heterozygosity (*h*, with standard error SE), number of polymorphic sites (Po), nucleotide diversity (π with SE). Estimates from the population Siquirres were not calculated (—) due to small sample size, GIS, geographic information system.

primers tmet-frog (5'-TTGGGGTATGGGCCCAAAAG-CT-3'; Wiens et al. 2005) and a primer designed for *A. callidryas* (ACA-Int: 5'-ACGTGATCTGAGTTCAGACCG-3'). Polymerase chain reactions (PCRs) were performed in a total volume of 25 μ l, each containing approximately 100 ng template DNA, 1 \times PCR buffer, 0.75 mM dNTPs, 1.5 mM MgCl₂, 1 μ M primer, and 0.625 U *Taq* polymerase (Roche Diagnostics, Switzerland). PCR conditions consisted of an initial 95 °C denaturation for 5 min; followed by 35 amplification cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 1 min; and a final 5-min extension at 72 °C. We removed unincorporated oligonucleotides and dNTPs with 1 U each of exonuclease I and shrimp alkaline phosphatase with an incubation at 37 °C for 45 min and denaturation at 90 °C for 10 min. We performed cycle sequencing reactions with Big Dye terminator sequencing components according to manufacturer's protocol (Applied Biosystems, Perkin Elmer, Foster City, CA) using the same primers used for fragment amplification. Cycle sequencing reaction conditions were 25 cycles of 96 °C (30 s), 50 °C (15 s), and 60 °C (4 min). We sequenced gene fragments in both directions to resolve any base-calling ambiguities. Products were column purified to remove nonincorporated terminator dye using Sephadex G-50 and electrophoresed on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Electropherograms

were checked by eye and fragments assembled into contiguous sequences using Sequencher 4.1 (GeneCodes, MI).

We aligned ND1 sequences using ClustalW (Thompson et al. 1994) in the MegAlign 6.1.2 program of the Lasergene sequence analysis software (DNASTAR, Inc, Madison, WI). We conducted multiple alignments using the "slow/accurate" option and varied gap costs (4, 8, 10, 15) to identify possible regions of ambiguous homology that might bias phylogenetic analyses (Gatesy et al. 1993). We detected no gaps in our alignment and thus included the entire sequence length in subsequent analyses.

We estimated haplotype diversity (*h*) and nucleotide diversity (π) (Nei 1987) and the number of unique haplotypes in our sample using Arlequin 3.01 (Schneider et al. 2000). Overall genetic differentiation among regions was estimated using pairwise *F* statistics (Reynolds et al. 1983) (Φ_{ST}), and we compared estimates with a null distribution of no difference between regions to test for significance ($\alpha = 0.05$) using 10 000 permutations in Arlequin.

We inferred a Bayesian phylogenetic tree using MrBayes v3.0 (Huelsenbeck and Ronquist 2001). The best fitting model of nucleotide substitution for our data was selected based on the Akaike information criterion as implemented in MrModelTest (Nylander 2004). Our Bayesian analyses

consisted of 2 independent runs of 4 Markov chains, run for 10 000 000 iterations and with sampling every 1000th iteration. We applied default prior distributions in MrBayes with the exception of the α shape (exponential, mean = 1.0) and branch length parameters (exponential, mean = 0.1). We determined the appropriate number of burn-in samples and tested for stationarity of parameter values using trace plots in the software package TRACER (Rambaut and Drummond 2005). Removal of 10% of the initial samples provided ample burn-in in both analyses.

Phenotypic Variation

In life, red-eyed tree frogs are green dorsally and have large red eyes and orange or violet front and hind feet. This species is one of few anuran taxa that exhibits relatively low levels of phenotypic variation within populations but high variation among populations (Hoffman and Blouin 2000; Savage 2002). To human observers, variation in leg and flank coloration among populations is obvious and includes hues of red–orange, yellow, blue, and violet.

We imported photographs of each individual into Adobe Photoshop CS2 (Adobe Systems Incorporated) to correct for ambient light intensity and color by reference to the black–white–gray standard (QPcard 101) in the background of every photograph (Stevens et al. 2007). We quantified color as “hue” in the hue, saturation, and brightness realm because previous analyses of leg coloration confirmed that hue accurately represents variation when saturation values are high (McKenna et al. 1999; Robertson JM and Robertson AD 2008). Saturation is an index of the purity of a color; low saturation values correspond to “muddy” colors because they contain a mixture of all 3 primary colors, whereas highly saturated colors contain only 1 or 2 of 3 complementary colors. We used the Color Picker function in Adobe Photoshop CS2 to measure hue and saturation and conducted a homogeneity-of-variance test within populations to compare the variance in hue for individuals exhibiting high (>30%) and low (\leq 30%) saturation (implemented in JMP ver 7.0; SAS Institute Incorporated, 2006).

We measured leg and flank coloration of 14–26 frogs per population. Dominant leg colors of *A. callidryas* vary regionally; individuals from some populations are monochromatic (blue), others contain 2 dominant colors (blue and orange), whereas others contain a continuum of hues (e.g., reddish blue through greenish blue). To quantify color, we followed the protocol of Robertson JM and Robertson AD (2008) and imported the color-corrected photographs into ImageJ 1.37v (Rasband 2008) for downstream analyses. We selected the entire posterior surface of the leg in ImageJ to create a frequency histogram of the number of pixels of each hue. We then transformed the ImageJ hue data to the standard measure of hue with a range of 0°–360° and divided the color spectrum into 8 equal bins (each spanning 45°) named according to the central hue for each bin. For example, the standard hue definition of pure red is 0; therefore, the red bin spans 22.5° on each side of 0°. Final

hue ranges for the 8 color bins were red (337.6–22.5), 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).

Our measurements of flank color differed from leg color because *A. callidryas* flanks have a series of disruptive, vertical stripes, precluding measurement of the entire flank region. However, flank coloration is nearly monochromatic, thereby justifying subsampling a representative patch of color between stripes at the midline of each frog. For both flank and leg measurements, we quantified the percent pixels in each of 8 hue bins, the average hue in each bin, and saturation of each selected color patch.

To test for population and regional differences in coloration, we used linear discriminant analysis implemented in JMP 7.0 (SAS 2007) to compare each individual with the group multivariate mean. The following color parameters were included in the model: the average hue (leg and flank) for each of 8 bins, the percentage of hue contained in each bin, and percent saturation. To quantify the number of individuals correctly assigned and those misclassified to source populations, we generated a classification matrix and tested the significance of individual assignments using a chi-square test. This method predicts assignment based on multivariate analysis of variance. Accurate assignment indicates that leg and flank coloration are highly diagnostic for the 5 regions examined.

Matrix Regression Analyses

We tested the relative strength of multiple factors contributing to the geographic distribution of genetic and phenotypic diversity at 3 spatial scales: all populations combined, Caribbean populations only, and Pacific populations only. For all analyses, we implemented matrix regression analyses to identify the relative significance of multiple predictor variable, using a forward selection, 1–3 step process (Legendre et al. 1994). In step 1, we used individual pairwise Mantel tests to examine the association between the Y matrix (phylogenetic distance or phenotypic distance) and each X matrix (e.g., geographic distance, barrier, and phylogenetic distance for analyses of phenotype). If all X matrices significantly varied with the Y matrix in pairwise Mantel tests, then variables were included in subsequent partial Mantel tests (step 2). The variable with the highest R^2 was listed first in each partial Mantel test. This examines the effect of the first X matrix while considering the variation due to the second matrix. For analyses that contained 3 variable X matrices, a third step was included, following the same methodology described for steps 1 and 2. At each step, the slope and probability values indicate the relative strength (and significance) of each variable in predicting genetic and phenotypic diversity patterns (Legendre et al. 1994). All tests were implemented in R ver 1.8.5 (R Core Development Team 2007). For each test, the standardized regression coefficients (std b), R^2 , and P values were generated by 9999 permutations.

Table 2. Pairwise ϕ_{ST} for 5 regions, representing 20 populations of *Agalychnis callidryas* in CR and PA

	Northeast CR	Southeast CR	Central PA	Northwest CR	Southwest CR
Northeast CR	4.11				
Southeast CR	0.327*	13.00			
Central PA	0.704*	0.533*	13.01		
Northwest CR	0.863*	0.755*	0.754*	6.80	
Southwest CR	0.806*	0.737*	0.735*	0.781*	20.00

The diagonal elements (bolded) are the average pairwise nucleotide differences between haplotypes within each region.

*Significance $P < 0.0001$.

Distance matrices for phenotype (leg and flank coloration) were derived as the Euclidean pairwise distance among individuals, constructed in R. Calculation of Euclidean distance was based on saturation, average hue, and the percentage of hue for each of 8 bins. For the genetic distance matrix, we calculated pairwise patristic distances from the Bayesian consensus tree using the program TreeEdit 1.0a10 (Rambaut and Charleston 2001). We used 2 measures of geographic distance: The first matrix contained the straight-line distance between all pairs of sites (proximitySTRAIGHT), and the second matrix reflected distances of likely dispersal paths based on our knowledge of the physiology and habitat requirements of the species (proximityAROUND). The second matrix accounted for the inhospitable habitats in the high elevation habitats of the Cordillera de Talamanca and the dry Pacific landscape located between southwestern CR and Central PA (Crawford et al. 2007); both these regions do not contain populations of red-eyed tree frogs. To test for the association between genetic and phenotypic diversity patterns with respect to 5 putative biogeographic regions, we created a “barrier matrix” using binary indicator variables (0,1) to designate whether populations occurred in the same (1) or different (0) regions with respect to each barrier. Three populations included in the mtDNA phylogeny (Universidad de EARTH, Siquirres, Cahuita) were excluded from matrix regression analyses because of insufficient data for coloration (Table 1).

Results

Population Genetic Variation

The mitochondrial fragment used for analyses was 1149 bp in length, including 118 bp of the 16S gene and 1031 bp of NADH1 (Genbank accession numbers: FJ489259–FJ489334). We sequenced this fragment for 125 *A. callidryas* and the outgroup taxon *Agalychnis saltator* and identified

a total of 75 unique haplotypes with 178 variable sites, of which 142 were parsimony informative; no insertions/deletions were detected. Haplotype (h) and nucleotide (π) diversity varied among populations with high h (mean \pm standard deviation [SD] = 0.846 ± 0.145 , range = 50–100%) and relatively low π for most populations (mean \pm SD = 0.006 ± 0.007 , range = 0.0008–0.00345, Table 1). High haplotypic diversity and low nucleotide diversity generally indicate that populations are divergent and geographically subdivided (Grant and Bowen 1998). Our measures of π are similar to estimates for other sympatric anuran taxa sampled at the NADH2 mtDNA gene (Crawford 2003). We detected exceptionally high π for individuals sampled from Uvita (uvi), an order of magnitude greater than any other population of *A. callidryas* (Table 1) or other anuran taxa sampled in this geographic region (Crawford 2003). Pairwise ϕ_{ST} values among regions were high and significant for all comparisons, ranging from 0.327 (northeastern–southeastern CR) to 0.863 (northwestern–northeastern CR, Table 2).

MrModelTest 3.7 selected the model general time reversible + I + Γ with unequal base frequencies (A = 0.3076, C = 0.22190, G = 0.11182, T = 0.35230; pinvar = 0.6311; and α = 2.1712). The Bayesian tree, including all haplotypes, showed an overall pattern of regional differentiation and well-supported regional clades (marginal posterior probabilities ranged from 89–99%); however, haplotypes from none of the regions formed a monophyletic group (Figure 2). The consensus tree showed an early divergence of the clade Pacific A (including only southwestern CR populations) relative to the other 4 regions (Figure 2). Within Pacific A, samples from the Osa Peninsula (Campo [cam]) were genetically distinct from other southwestern CR populations. Haplotypes from the remaining regions fell within 3 clades (Pacific B, Caribbean A, Caribbean B, Central Panama) united at their base by a polytomy (Figure 2). Pacific B included individuals not only from northwestern CR but also 4 from uvi. The other 7 uvi individuals were members of Pacific A (Figure 2). The clade Caribbean A contained individuals from 3 regions (northeastern CR, southeastern CR, northwestern CR), including individuals from both sides of the Cordillera de Talamanca. Caribbean B (sister to Caribbean A) contained individuals from southeastern CR and Central PA. The third major clade included haplotypes exclusively sampled from Central PA (Figure 2).

Phenotypic Variation

Prior to color analyses, we conducted a homogeneity-of-variance test within populations to determine the range of saturation in which hue measurements would be usable. We expected high variance in hue measurements for individuals with “muddy” color patches because hue is determined solely by the colors that most contribute to coloration. For example, a muddy color patch may contain 33.5% red, 31.5% blue, and 35% yellow. In this case, the hue would be described as red/yellow (orange) with low saturation (i.e.,

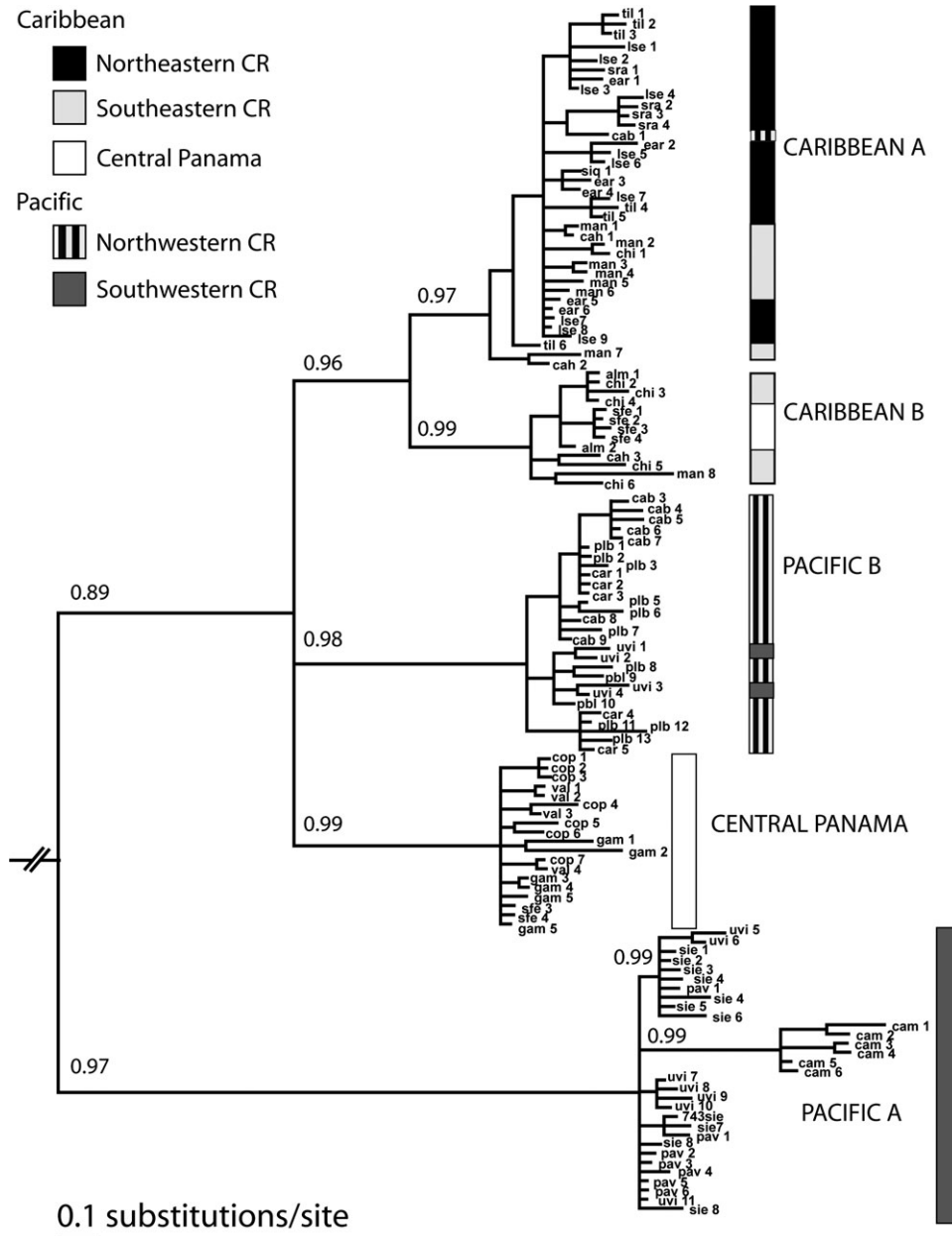


Figure 2. Bayesian consensus phylogram based on 1149 basepairs of the NADH1 mtDNA gene fragment for 125 red-eyed tree frog individuals. Values above branches are marginal posterior probabilities. Phylogram is rooted with the outgroup taxon, *Agalychnis saltator*. Bars are coded according to five geographic regions from Figure 1. Population and regions correspond to those listed in Table 1.

high content of blue, the complement of orange). The color patch of another individual that closely resembles that same phenotype may be 34% red, 35% blue, and 31% yellow. This individual would be designated as red/blue (violet). Thus, slight changes in the relative contribution of any of the 3 colors will significantly skew the hue measurements for low saturation coloration. These values are obviously unreliable and should be excluded from hue-based population studies (McKenna et al. 1999).

We validated this expectation by performing a homogeneity-of-variance test on 3 populations. The other populations did not contain a sufficient number of individuals in both the low- and high-saturation category for statistical analyses. A significant result indicates that variance is not homogenous between high- and low-saturation groups. In 3 populations, the low-saturation group exhibited high variance (Almirante: $F = 13.98$, $P = 0.011$; Pavones: $F = 33.51$, $P < 0.0001$; El Copé: $F = 12.24$, $P = 0.005$), and thus, these individuals were

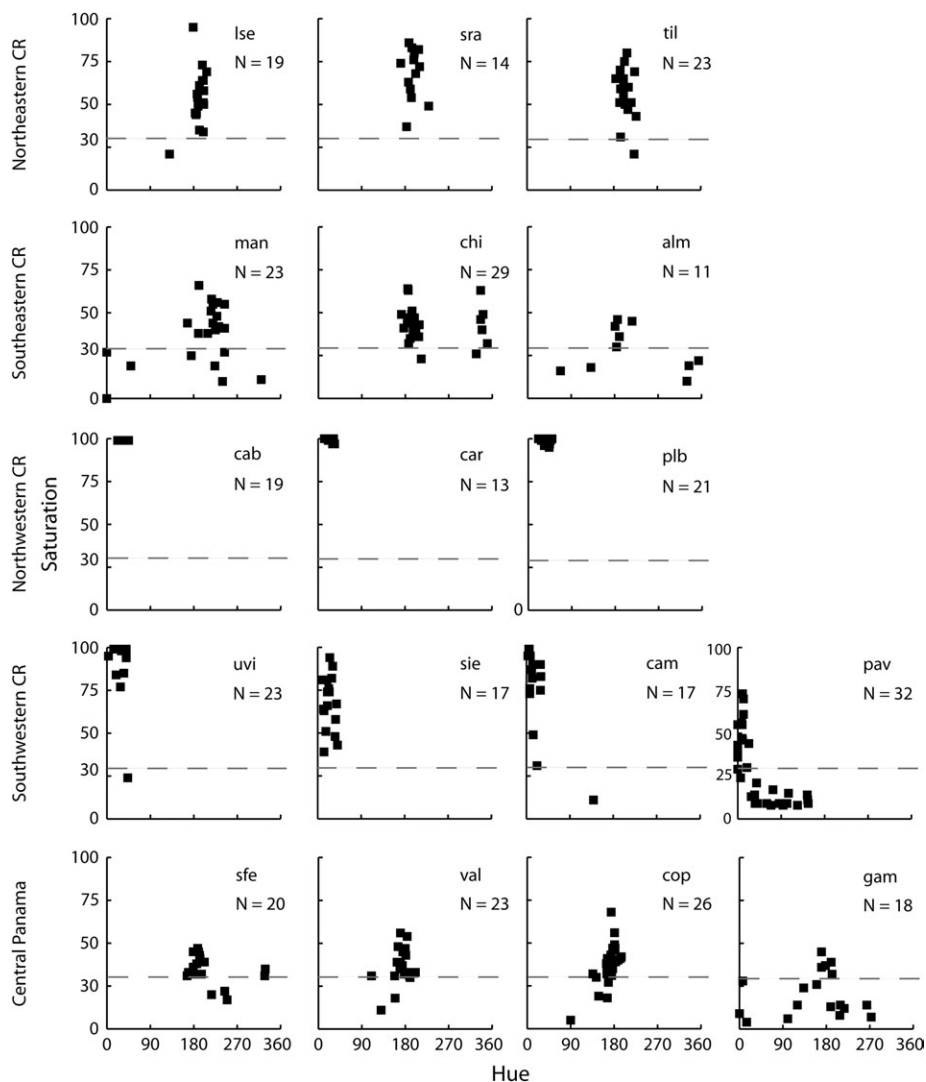


Figure 3. Flank hue and saturation measures for red-eyed tree frog individuals sampled at 17 sites in CR and PA. Saturation ranges from 0–100 % and hue is based on the 0–360 color spectrum. The dotted line at 30 % represents the threshold saturation level for excluding individuals in the study of flank coloration.

excluded from further analyses. Visual inspection of how hue varies with respect to saturation within a population is evident for Gamboa (*gam*; Figure 3): Individuals that exhibit saturation levels less than 30% have hue measures that range from 0–270, whereas the few individuals with higher saturation levels exhibit a small range in hue (170–190). Based on this result, we subsequently measured and analyzed hue only for individuals with saturation levels >30%. The total number of excluded individuals was small (8.2% of all individuals; average number of individuals per region = 3.8). The percent of excluded individuals per population (mean \pm SD = $21 \pm 25\%$, range = 0–72%) varied such that few populations ($n = 3$) contained many excluded individuals, whereas most contained few.

Divergence in flank coloration was most evident between Pacific (orange) and Caribbean (blue) populations.

Pacific populations had mostly orange legs, but a few individuals from the southwestern CR region exhibited some blue and green (Figure 4). In contrast, leg coloration among Caribbean regions varied from blue–violet (northeastern CR) to populations with unequal proportions of blue and orange (Figure 4). The average leg coloration among southeastern CR/PA individuals was approximately 65% orange and 35% blue; in contrast, Central PA individuals had a higher proportion of orange in the legs (ca. 90% orange/10% blue; Figure 5). Overall, these results indicate that leg coloration is more variable than flank coloration. The discriminant function analyses correctly classified 195 of 233 (83.6%) individuals to their region of origin based on leg and flank coloration alone. The number of misclassified individuals per population was very low (mean \pm SD = 0.165 ± 0.659 , range = 0–5). Therefore, these characters are

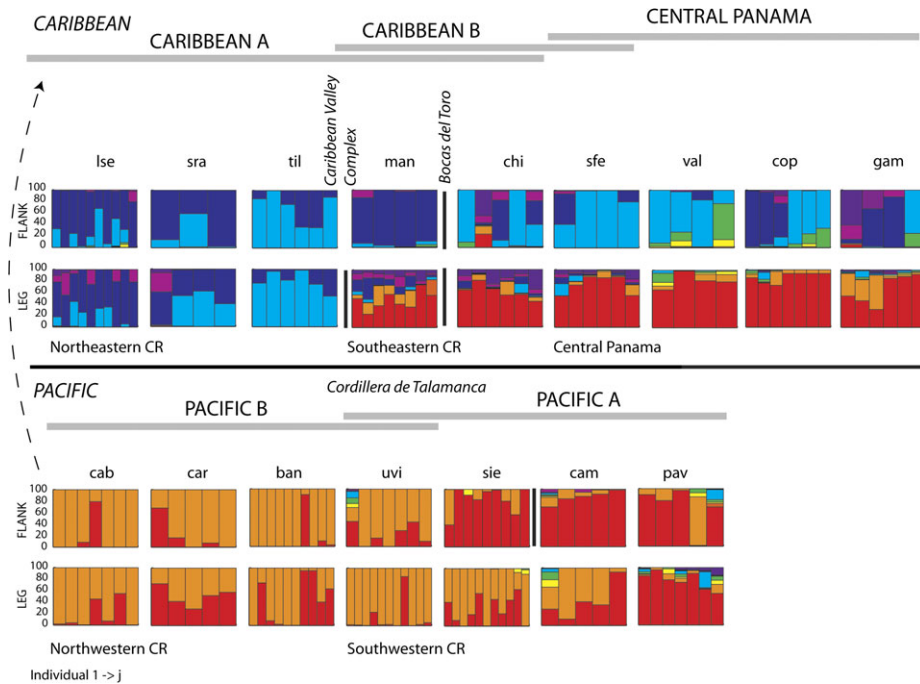


Figure 4. The proportion of the leg and flank (area measured as percent pixels) that falls within eight color bins (red, orange, yellow, green, light blue, dark blue, purple, violet) for 125 red-eyed tree frogs included in the mtDNA analyses. Individuals are represented on the horizontal axes and the proportional leg and flank color for each individual are represented as a vertical histogram. The color frequency graphs underscore the low within-population variation and high regional variation characteristic of this species. The black, bold lines show the biogeographic barriers associated with flank coloration (Bocas del Toro and Osa Peninsula), leg coloration (Caribbean Valley Complex) and both flank and leg coloration (Cordillera de Talamanca). Five phylogenetic clades (light gray bars; Figure 2) show genetic admixture between neighboring regions and reveal the discordance between spatial patterns of genetic and phenotypic divergence. The clade Caribbean A includes one individual sampled from cab (dashed line).

diagnostic for the 5 regions ($\chi^2_{df=16} = 595.45$, $P < 0.0001$). The largest misclassification occurred between northwestern and southwestern CR (52% of all misclassified individuals), reflecting the high similarity in coloration between these 2 regions (Figure 4).

Matrix Regression Analyses

All Sampled Populations

Genetic diversity was largely structured by geographic regional barriers and by geographic distance (Table 3). At the broadest spatial scale, the geographic barrier between Pacific and Caribbean (Cordillera de Talamanca) was the largest determinant of genetic distance (std $b = 0.435$, $P = 0.0001$, Table 3). Accordingly, we also detected an association between genetic and geographic distance around the Cordillera de Talamanca (proximityAROUND) but not across the mountain range (proximitySTRAIGHT, Table 3).

Leg coloration across all populations was strongly associated with a division between Pacific and Caribbean populations (std $b = 0.317$, $P = 0.0001$, Table 3). However, neither geographic nor genetic distance determined leg coloration at this broadest spatial scale (Table 3). Conversely,

the distribution of flank coloration was associated with all factors examined (genetic distance, geographic distance, Cordillera de Talamanca) with the largest effect being the barrier dividing populations into Pacific and Caribbean versants (std $b = 0.598$, $P = 0.0001$, Table 3). At the broadest spatial scale, leg coloration was associated with the distribution of flank coloration (std $b = 0.283$, $P = 0.0001$).

Caribbean Populations Only

Genetic diversification of Caribbean populations was strongly associated with geographic distance and weakly (but significantly) associated with the 2 putative geographic barriers, the Caribbean Valley Complex (between northeastern CR and southeastern CR) and Bocas del Toro (between southeastern CR and PA, Table 3). Leg coloration was associated with geographic distance (std $b = 0.248$, $P = 0.0001$, Table 3) and both barriers but not with genetic distance (std $b = -0.003$, $P = 0.593$, Table 3). In contrast, only the Bocas del Toro barrier significantly structured flank coloration (std $b = -0.125$, $P = 0.0025$, Table 3). Flank and leg coloration were weakly associated across all Caribbean populations (std $b = 0.069$, $P = 0.0143$).

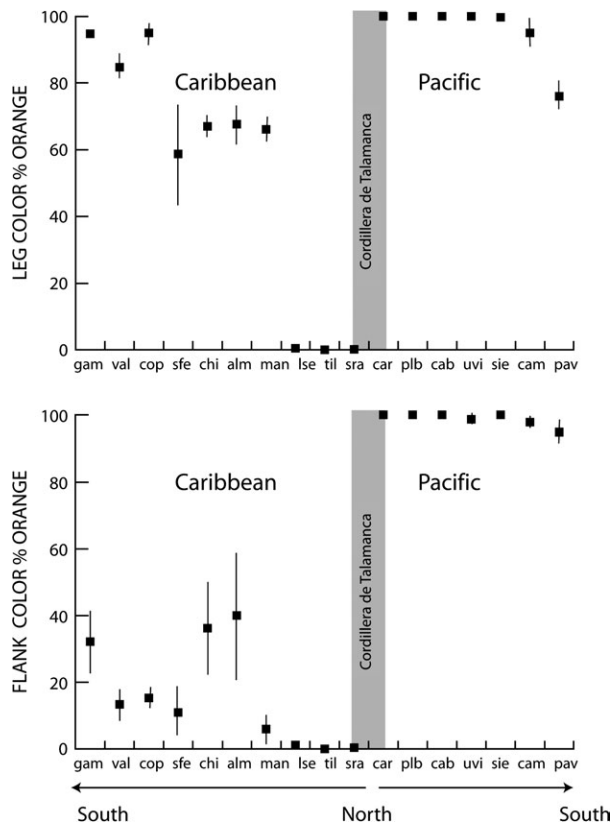


Figure 5. Variation in the average (and standard error) percentage of leg (top) and flank (bottom) coloration measured as ‘orange’ (combined orange and red color bins) for 17 populations of *Agalychnis callidryas*. The greatest divergence in coloration occurred across the Cordillera de Talamanca.

Pacific Populations Only

Geographic factors also structured genetic diversity on the Pacific versant. The barrier Río Parrita (dividing northwestern and southwestern CR) was the strongest predictor (std $b = -0.669$, $P = 0.0001$, Table 3). Leg coloration did not vary with respect to either geographic barrier but was weakly associated with geographic distance (std $b = -0.075$, $P = 0.0065$, Table 3). Flank coloration was strongly associated with whether populations were located on the Osa Peninsula or mainland (std $b = -0.331$, $P = 0.0001$, Table 3), weakly associated with genetic diversity (std $b = -0.0130$, $P = 0.0001$, Table 3), and did not vary with geographic distance (Table 3). Flank and leg coloration were associated across all Pacific populations (std $b = 0.142$, $P = 0.0046$).

Discussion

Our objectives were to quantify genetic and phenotypic diversity in red-eyed tree frogs and examine the extent to which their spatial distribution might be concordant at

multiple geographic scales and across putative biogeographic barriers. Although flank and leg coloration were associated at all spatial scales of this study (albeit only weakly for Caribbean and Pacific populations analyzed separately), leg coloration was more variable than flank coloration. In addition, the geographic and genetic determinants of diversity were not equal for these 2 traits, indicating that different evolutionary mechanisms mediate the spatial distribution of composite body coloration. At some scales, variation in leg and flank coloration was associated with mtDNA diversity, implicating the underlying roles of historical and geological factors in population structure. However, we detected multiple departures from this pattern, demonstrating that limitations to historical connectivity alone cannot fully explain regional diversification in coloration. A direct test for selection on color was not the primary objective of our study; nonetheless, our analyses reveal contexts in which there is opportunity for selection. We discuss the interplay among evolutionary and geographic processes with respect to the 3 general patterns of diversity detected in this study: codistribution of genetic and phenotypic diversity across historical barriers, phenotypic similarity across genetically isolated groups, and phenotypic differentiation in the presence of historical connectivity.

Historical Barriers and Codistributed Patterns of Phenotypic and Genetic Diversity

The codistribution of genetic and phenotypic diversity patterns across geographic barriers indicates that similar processes have shaped aspects of population diversity and that geographic isolation has likely facilitated differentiation. Genetic diversification in *A. callidryas* populations was associated with levels of phenotypic differentiation with respect to 2 biogeographic barriers: the Cordillera de Talamanca and Bocas del Toro.

Matrix regression analyses, pairwise ϕ_{ST} values, and the multivariate discriminant function analyses based on coloration all indicate isolation of Caribbean and Pacific populations with respect to the Cordillera de Talamanca. However, our phylogenetic analyses revealed a single Pacific individual with a haplotype nested within the clade Caribbean A (cab1; Figure 2), indicating either incomplete lineage sorting of mtDNA haplotypes or that historical gene flow connected Pacific and Caribbean populations. The observation that flank-stripe patterns were not clearly distinguishable between northwestern and northeastern CR populations, a trait that otherwise exhibited regional variation, led Savage and Heyer (1967) to hypothesize that mountain passes at these elevations (ca. 900 m) may have historically facilitated migration through dispersal corridors under more lenient climatic and habitat conditions. Three volcanic mountain ranges isolated from the northern edge of the Cordillera de Talamanca (Cordillera de Tilarán, Cordillera Central, and Cordillera Guanacaste) are younger and lower in elevation than the peak height of the Cordillera de Talamanca, possibly facilitating dispersal across the

Table 3. Forward selection matrix multiple regression of factors that predict the spatial variation in genetic diversity (A), leg coloration (B), and flank coloration (C) at 3 spatial scales: all populations, Caribbean populations, Pacific populations

Region	X matrix	Step 1		Step 2		Step 3		Interpretation
		std <i>b</i>	<i>P</i>	std <i>b</i>	<i>P</i>	std <i>b</i>	<i>P</i>	
Determinants of genetic diversity								
All	proximityAROUND	0.398	0.0001	0.168	0.0002			Geographic distance around TAL
	proximitySTRAIGHT	0.142	0.0009	0.019	0.1844			
	Barrier (TAL)	0.533	0.0001	0.435>	0.0001			
Caribbean	KM	0.921	0.0001	0.878>	0.0001	0.757	0.0001	Barrier: Cordillera de Talamanca Geographic distance
	Barrier (CVC)	0.656	0.0001	0.058	0.0001	0.062	0.0001	Barrier: Limon
	Barrier (BDT)	0.762	0.0001	0.165	0.0001	0.167	0.0001	Barrier: Bocas del Toro
Pacific	KM	0.483	0.0001	0.035	0.0247	0.044	0.0087	Geographic distance
	Barrier (RIO)	0.717	0.0001	0.698>	0.0001	0.669	0.0001	Barrier: Rio Parrita
	Barrier (OSA)	0.249	0.0001	0.173	0.0001	0.174	0.0001	Barrier: Osa Peninsula
Determinants of leg color								
All	DNA	0.121	0.0003	-0.053	0.9904			Barrier: Cordillera de Talamanca
	KM	0.139	0.0002	-0.028	0.8231			
	Barrier (TAL)	0.303	0.0001	0.317>	0.0001			
Caribbean	DNA	0.259	0.0001	0.109	0.0001	-0.003	0.5930	Geographic distance
	KM	0.676	0.0001	0.307	0.0001	0.248	0.0001	
	Barrier (CVC)	0.756	0.0001	0.734>	0.0001	0.561	0.0001	
Pacific	Barrier (BDT)	0.515	0.0001	0.215	0.0001	0.072	0.0004	Barrier: Bocas del Toro
	DNA	-0.005	0.4990					Geographic distance
	KM	0.075	0.0065					
	Barrier (RIO)	-0.0110	0.6466					
Barrier (OSA)	-0.0448	0.9725						
Determinants of flank color								
ALL	DNA	0.3500	0.0001	0.029	0.0069	0.026	0.0133	DNA
	KM	0.347	0.0001	0.025	0.0142	0.021	0.0386	Geographic distance
	Barrier (TAL)	0.623	0.0001>	0.607	0.0001	0.598	0.0001	Barrier: Cordillera de Talamanca
Caribbean	DNA	0.099	0.0034	-0.025	0.2135			Barrier: Bocas del Toro
	KM	0.076	0.0091	-0.071	0.9638			
	Barrier (CVC)	-0.018	0.8031					
Pacific	Barrier (BDT)	0.140	0.0006	-0.125>	0.0025			Barrier: Bocas del Toro
	DNA	0.161	0.0010	0.078	0.0130	0.053	0.0560	DNA
	KM	0.089	0.0454	0.075	0.0510	0.050	0.1513	
	Barrier (RIO)	0.105	0.0120	0.068	0.0296	0.880	0.1656	
	Barrier (OSA)	0.346	0.0001>	0.326	0.0001	0.331	0.0001	Barrier: Osa Peninsula

Values reported are the standardized regression coefficients (std *b*) for each Mantel test with *P* values determined by 9999 random permutations. For step 2, > = variable with highest R^2 in step 1. See text for details of forward selection methodology. KM = distance in kilometers, DNA = patristic distance based on consensus Bayesian phylogeny. Barriers examined include Cordillera de Talamanca (TAL), Caribbean Valley Complex (CVC), Bocas del Toro (BDT), Río Parrita (RIO), Osa Peninsula (OSA). In the case where multiple variables contributed to the model, the variable most strongly associated with the Y matrix is in bold face.

Continental Divide, as observed in other anuran taxa (Wang et al. 2008). Our previous analyses of population genetic structure using microsatellite markers revealed that gene flow on more recent time scales was highly restricted across the Continental Divide (Robertson et al. 2009), indicating that phenotypic divergence has likely resulted from divergence in geographic isolation. Increased population sampling could elucidate the genetic nature of a potential contact zone across the Continental Divide.

The greatest divergence in coloration was detected between northwestern and northeastern CR despite close geographic proximity (56 km) and relatively recent time since divergence (Figure 2). Leg and flank color differentiation was greater between these populations than between 2 of the most geographically distant sites in our study, Gamboa “gam” in

Central PA and Pavones (“pav” in Southwest CR), populations that are 800 km apart when considering the distance around the Cordillera de Talamanca (Figure 5). Combined, data from this mtDNA study and prior findings of restricted nuclear gene flow (Robertson et al. 2009) indicate that phenotypic diversity on either side of the Continental Divide is likely due to genetic drift and continued geographic isolation over evolutionary time scales.

Historical limitations to gene flow between populations sampled from southeastern CR and Central PA (either side of Bocas del Toro) contributed to the spatial diversification of flank and leg coloration (Figure 4). However, the effect of this putative barrier was small in matrix regression analyses (compared with the effect of geographic distance) and is consistent with the relatively subtle differences in gene and

color differentiation compared with the divergence observed across the Cordillera de Talamanca. Thus, the Bocas del Toro barrier could be more permeable to historical gene flow, as evidenced by mtDNA admixture of southeastern CR and central PA populations and low to moderate levels of phenotypic differentiation. Multiple colonization events of montane populations in this region have been documented for other taxa (Crawford 2003; Zeh et al. 2003; Weigt et al. 2005), and the permeable nature of this barrier is consistent with findings for other anurans (Crawford et al. 2007). The admixture revealed for *A. callidryas* populations could be due to incomplete lineage sorting reflecting multiple vicariant events in the Bocas del Toro region. Alternatively, the geographic proximity of these admixed populations could arise from secondary contact of previously isolated populations. Recent gene flow estimates, based on nuclear microsatellite loci, revealed substantial gene flow and admixture of genetic demes across this region (Robertson et al. 2009) suggesting that our phylogenetic analyses tracked historical processes of temporal isolation but that more recent gene flow connects populations spanning this region.

Phenotypic Uniformity in Structured Populations

We detected limited phenotypic variation among individuals sampled from genetically differentiated populations along the Pacific versant. The striking similarity among most individuals could be explained by evolutionary stasis (Rutherford 2000; Wellborn and Broughton 2008) or stabilizing selection, although studies are required to differentiate between these mechanisms. The genetic structure of Pacific populations into 2 clades (northwestern and southwestern CR; Figure 2) was consistent with a priori expectations of historical isolation due to plate tectonic activity in CR (Kohlmann et al. 2002). Genetic isolation could be due to these historical geological factors, temporal differences in rainfall and reproductive activity (Grinnell 1914, 1924), and/or dispersal limitations imposed by one of several large riverine barriers that drain from the Cordillera de Talamanca into the Pacific Ocean. Independent of the exact nature of the barrier, the matrix regression analyses and phylogenetic analyses corroborate a geographic division between northern and southern populations ($\phi_{ST} = 0.781$), with a potential contact zone centered between Playa Bandera and uvi. Our estimates of π were exceptionally high for individuals sampled at uvi (Table 1), possibly indicating that this population has a long evolutionary history and experienced secondary contact with divergent lineages (Grant and Bowen 1998). Alternatively, admixed clades in mitochondrial gene genealogies can result from incomplete lineage sorting or historical connectivity (Wright 1937; Slatkin 1985).

A second Pacific barrier indicated population isolation on the Osa Peninsula relative to the mainland: The phylogenetic tree and matrix regression analyses showed that the single population from the Osa Peninsula (cam) forms a deeply divergent monophyletic clade within a larger southwestern CR clade. Genetic isolation of Osa Peninsula populations has also been detected for other vertebrates,

including frogs (Crawford 2003; Crawford et al. 2007) and snakes (Zamudio and Greene 1997) suggesting that Osa Peninsula populations have a history of genetic isolation. Despite this historical isolation, we found little evidence of phenotypic divergence in leg coloration, thus indicating evolutionary stasis for a single phenotype in the Osa Peninsula and adjacent mainland populations or that contemporary gene flow is sufficiently high to result in phenotypic homogeneity of Pacific populations. Estimates of recent gene flow based on nuclear markers show little genetic differentiation between cam and other southwestern CR populations suggesting the later for leg coloration (Robertson et al. 2009). These results favor the hypothesis that more recent gene flow explains uniformity of leg color, but not flank color, between mainland and Osa Peninsula populations.

Phenotypic Divergence in the Presence of Gene Flow

Phenotypic divergence of red-eyed tree frog populations could not be explained by historical isolation of regions that occupy lowland Caribbean forest. Leg coloration varied among populations, ranging from entirely blue legs in the north to mixed blue/orange coloration in the south that did not coincide with genetic diversification of these same populations (Figure 4). Phylogenetic analyses revealed historical cohesion between 2 phenotypically divergent regions, northeastern and southeastern CR (Figure 2), indicating that color differentiation could be mediated by strong localized selection sufficient to counteract the homogenizing effects of historical connectivity. More recent gene flow estimates revealed that northeastern and southeastern CR populations were not isolated but exhibited some genetic admixture (Robertson et al. 2009). Our data thus indicate that a geographic break structures phenotype but not genotype for Caribbean populations. The nature of this break is not well understood for terrestrial taxa, but it does coincide with the northern/southern edges of geographic ranges in other Central American beetle taxa (Kohlmann et al. 2002); delimits color morphs of the lowland frog, *Oophaga* (= *Dendrobates*) *pumilio* (Hagemann and Pröhl 2007); and appears to structure red-eyed tree frog populations, as well. Combined, mitochondrial and nuclear markers (Robertson et al. 2009) provide evidence that color pattern differentiation cannot be solely explained by geographic isolation, indicating the probable role of diversifying selection in the maintenance of color variation. Alternatively, rapid colonization of the Caribbean lowland forest, followed by intermittent geographic barriers to gene flow, could result in a signature of modest gene flow with the accumulation of phenotypic differentiation (Reeves and Bermingham 2006). Under either scenario, differences could be exaggerated by strong selection against maladapted phenotypes (Nosil and Crespi 2004; Nosil et al. 2005; Rosenblum et al. 2007).

Mode of Diversification

Our data indicate that the high degree of phenotypic regionalization in the red-eyed tree frog reflects a number of

evolutionary processes and that these processes vary across spatial scales in this study. The distribution of genetic or phenotypic diversity often correlates with landscape history (Pröhl et al. 2006) and microhabitat differences (Thorpe and Baez 1993; García-París et al. 2000). Although microhabitat features of *A. callidryas* populations have yet to be rigorously quantified, factors that could mediate strong localized selection are not obvious. For example, sampling sites varied in habitat type, elevation, or percent canopy cover within but not among geographic regions (Table 1; Jeanne Robertson, personal observation). Divergent color morphs raised in captivity maintain their respective coloration despite identical feeding regimes (Ivan Gomez-Mestre, personal observation); thus, although regional diet has not been quantified for this species, it is unlikely that differences in prey base among regions determine coloration for this species. The one environmental factor that we know to vary regionally and may contribute to the patterns of genetic and phenotypic distribution is rainfall. Many anurans, including *A. callidryas*, rely on rainfall for reproduction. Because rainfall patterns vary across regions in CR and PA (Holdridge 1947; Kohlmann et al. 2002), these differences might reinforce spatial isolation due to asynchronous reproduction. In addition, subtle regional differences in abiotic features, selection regimes (sexual selection or predator pressures), or both could potentially mediate spatial patterns of phenotypic divergence (Brooks and Endler 2001b; Price 2006; Gosden and Svensson 2008).

Disentangling the selective environment controlling the evolution of color is difficult because several different processes could underlie divergent phenotypic expression, including natural (Hairston 1979; Endler 1980) and sexual selection (Endler 1983; West-Eberhard 1983; Panhuis et al. 2001; Masta and Maddison 2002; Maan et al. 2004). Natural selection for habitat background matching is the most common form of selection documented to date, including for rodents (Hoekstra et al. 2004; Hoekstra et al. 2005), lizards (Thorpe and Baez 1993; Thorpe 2002; Rosenblum 2006; Rosenblum et al. 2007; Stuart-Fox et al. 2007), frogs (Pyburn 1961; Nevo 1973; Stewart 1974; Hoffman and Blouin 2000; Woolbright and Stewart 2008), insects (Kettlewell and Conn 1977; Sandoval and Nosil 2005; Nosil et al. 2006), and snakes (King and Lawson 1995). Background matching is unlikely to mediate leg and flank color divergence in *A. callidryas* because there are no observable sharp environmental gradients that coincide with breaks among regions. In addition, the flank and leg color patches measured in this study are not cryptic. At rest, red-eyed tree frogs lie still with their brightly colored flanks and limbs tucked underneath their body; in this position, only the green dorsum is visible and perfectly matches the leaves they sit on (Schwalm et al. 1977; Emerson et al. 1990). In contrast, whereas active at dusk and throughout the night they sit upright and expose their colorful limbs and flanks. The conspicuous nature of this display suggests that color pattern functions as a visual signal to conspecifics and/or predators at night when this species is active.

Natural selection contributes to the evolution of conspicuous coloration in other anurans (Summers and

Clough 2001; Siddiqi et al. 2004; Reynolds and Fitzpatrick 2007; Rudh et al. 2007). Bright, aposematic coloration is an effective signal to deter predators and is observed for many, usually toxic, dendrobatid species (Summers and Clough 2001; Siddiqi et al. 2004). Natural selection for conspicuous coloration could evolve through predator interactions if the contrasting coloration of *A. callidryas* functions to warn predators of the noxious skin peptides common in this and other phyllomedusine frogs (Sazima 1974; Mignogna et al. 1997; Conlon et al. 2007). Geographic variation in skin peptide composition covaries with differences in coloration in the Australian tree frog, *Litoria rubella* (Steinborner et al. 1996); therefore, color differences could reflect geographic regionalization in skin peptide profiles in *A. callidryas* as well.

Sexual selection can rapidly promote population divergence through female mate choice (West-Eberhard 1983; Masta and Maddison 2002; Maan et al. 2004; Siddiqi et al. 2004; Summers et al. 2004, 2003). Female preferences may vary under different environmental conditions, which in turn will promote population divergence (Maan et al. 2004; Maan, Hopfker et al. 2006; Gray and McKinnon 2007). For the polytypic frog, *Dendrobates* (= *Oophaga*) *pumilio*, mate choice experiments demonstrated that sexual selection mediates intraspecific population divergence in coloration (Summers et al. 1999; Reynolds and Fitzpatrick 2007). More recently, mate preference experiments with *O. pumilio* found that female choice is based on aposematic coloration, indicating the potential for colorful visual signals to evolve through an interaction of natural and sexual selection (Maan and Cummings 2008). If dendrobatid frogs serve as a generalized model of color evolution in aposematic frogs, then we would predict that bright, contrasting coloration evolved as a response to natural predators in *A. callidryas* and that sexual selection through female choice also mediated divergence among populations. For *A. callidryas* populations, divergent hues must be sufficiently perceived and distinguishable when this crepuscular species is active for sexual selection (Hailman and Jaeger 1974; Lythgoe and Patridge 1991; Endler 1992). Visual signaling functions for conspecific communication in many nocturnal anurans, indicating that social cues are transmitted and received, even in low light conditions (McDiarmid and Adler 1974; Buchanan 1994; Haddad and Giaretta 1999; Hartmann et al. 2005; Cummings et al. 2008). Sexual selection provides the most compelling initial hypothesis of selection to examine with future behavioral studies.

The potential combined effects of genetic drift and geographic isolation on differentiation among populations is very high (Slatkin 1985; Orr 1998; Gavrillets 2003; Hoffman et al. 2006). Previous studies have shown that phenotypic variation in behavior (Castellano et al. 2002; Phillips and Johnston 2008), color pattern (Bittner and King 2003; Hoffman et al. 2006; Ohmer et al. 2009), and body size (Knopp et al. 2007) can evolve, in part, through nonselective regimes, and these processes possibly contribute to phenotypic diversification among populations of red-eyed tree frogs. Regional fixation from an ancestral, polymorphic

state, or linkage to other traits evolving through selection, could also account for the patterns we see in *A. callidryas*. However, the high levels of within-population haplotypic diversity (Table 1) and substantial variation at microsatellite loci (Robertson et al. 2009) indicate that genetic drift has not been the primary process shaping divergence among these populations.

Color Evolution in Red-Eyed Tree Frogs

The genetic control of coloration is poorly understood in most anurans. However, our analyses lend support to the hypothesis that regional variation in *A. callidryas* is due to differential fixation of preexisting variation rather than the evolution of novel coloration. Our phylogenetic analyses revealed that Southwestern CR diverged first and is sister to all other regions. This clade contained individuals with predominantly orange legs and orange flanks; however, the 2 southernmost populations in this region were the most variable of all Pacific populations (Figure 5). The presence (albeit limited) of blue coloration in these 2 populations informs us that the full range of coloration occurred deep in the phylogeny and is present across most populations, including populations that are historically isolated from other populations.

Geographic barriers have clearly contributed to isolation of populations or groups of populations in the red-eyed tree frog, and the long-term nature of some of these barriers resulted in genetic and phenotypic divergence. However, not all barriers are absolute, and in some cases, populations will evolve under selection-gene flow equilibrium. Our phylogenetic analyses showed admixture among most neighboring regions, corroborating that both geographic barriers and localized selection contribute to color divergence among *A. callidryas* populations. We found that the relative roles of selection and gene flow likely differ among biogeographic regions: Genetic isolation and divergent coloration appear to be strongest across the Continental Divide, moderate among Caribbean populations, and weakest among Pacific populations. Our study underscores the fact that selection gradients vary across relatively small spatial scales, even in species that occupy a relatively wide range of environments or habitats.

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