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Discordant patterns of evolutionary differentiation in two Neotropical treefrogs

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Abstract

Comparative studies of codistributed taxa test the degree to which historical processes have shaped contemporary population structure. Discordant patterns of lineage divergence among taxa indicate that species differ in their response to common historical processes. The complex geologic landscape of the Isthmus of Central America provides an ideal setting to test the effects of vicariance and other biogeographic factors on population history. We compared divergence patterns between two codistributed Neotropical frogs (*Dendropsophus ebraccatus* and *Agalychnis callidryas*) that exhibit colour pattern polymorphisms among populations, and found significant differences between them in phenotypic and genetic divergence among populations. Colour pattern in *D. ebraccatus* did not vary with genetic or geographic distance, while colour pattern co-varied with patterns of gene flow in *A. callidryas*. In addition, we detected significant species differences in the phylogenetic history of populations, gene flow among them, and the extent to which historical diversification and recent gene flow have been restricted by five biogeographic barriers in Costa Rica and Panama. We inferred that alternate microevolutionary processes explain the unique patterns of diversification in each taxon. Our study underscores how differences in selective regimes and species-typical ecological and life-history traits maintain spatial patterns of diversification.

Keywords: *Agalychnis*, biogeography, colour pattern polymorphisms, *Dendropsophus*, microsatellites, population genetics

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Introduction

Concordant phylogeographic patterns indicate landscape features are predominant determinants of lineage diversification in codistributed taxa (Avise 1986, 1989; Avise *et al*. 1987; Bermingham & Lessios 1993; Knowles 2004; Zeisset & Beebee 2008). The dynamics of natural populations, however, are often more complex and organisms often respond to common historical processes in differing ways (Crawford 2003; Babik *et al*. 2005; Barber *et al*. 2006; Crandall *et al*. 2008). Spatial analyses of phenotypic divergence among populations have been used to quantify the effects of environment and landscape features on morphology and life-history traits (Grinnell 1914, 1924; Wright 1937; McNeilly & Antonovi 1968). More recently, combined analyses of genetic and phenotypic population differentiation have revealed mechanisms leading to concordant or discordant phylogeographic patterns, including the relative roles of localized selection, genetic drift, hybridization, and gene flow (Hoekstra *et al*. 2005; Crews & Hedin 2006; Hoffman *et al*. 2006; Lemmon *et al*. 2007; Rosenblum *et al*. 2007; Wollenberg *et al*. 2008). Comparing evolutionary signals of population differentiation derived from both genetic and phenotypic data, and contrasting these signals among species inhabiting the same landscape (Morgan *et al*. 2001; Rocha 2004; Rosenblum 2006), allows us to distinguish patterns of divergence due to dominant landscape features from those resulting from species-typical or population-specific processes.

Due to its complex and dynamic geologic and climatic history, Central America is an ideal setting for studies

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of biogeographic and ecological processes underlying lineage diversification (Coates & Obando 1996; Campbell 1999; Kohlmann *et al*. 2002; Savage 2002). The history of Costa Rica and Panama, in particular, is dominated by plate tectonics and large-scale landscape changes (Campbell 1999; Kohlmann *et al*. 2002; Savage 2002). Approximately 3 million years ago, uplift formed the Cordillera de Talamanca, a mountain chain extending the length of the lower Central American continental divide. This barrier altered the climate of the Caribbean, Pacific and Central regions (Holdridge 1947; Campbell & Frost 1993; Coates & Obando 1996), resulting in distinct biotic zones that vary from dry, lowland, deciduous forest (north Pacific), to cloud forest (along the divide), to hot and wet lowland rainforest (Caribbean). This diversity likely contributed to the exceptionally high species richness and endemicity characteristic of this area (Holdridge 1947; Savage 2002). Not surprisingly, major geographic features, such as the Cordillera de Talamanca, contribute to concordant diversification patterns across many Central American taxa (Zamudio & Greene 1997; García-París *et al*. 2000; Kohlmann *et al*. 2002; Crawford 2003; Robertson & Robertson 2008).

In this study, we tested whether specific landscape features were reliable predictors of genetic and phenotypic variation in codistributed Central American anuran taxa. If these features effectively isolate groups of populations, they could facilitate lineage diversification through localized selection and genetic drift. To test the generality of these barriers, we quantified gene flow and genetic and phenotypic divergence among populations of two highly polymorphic species, the hourglass treefrog (Dendropsophinii: *Dendropsophus ebraccatus*) and the red-eyed treefrog (Phyllomedusinae: *Agalychnis callidryas*). Our focal taxa are sympatric in Costa Rica and Panama and share many ecological characteristics, including a prolonged reproductive season, leaf-oviposition, aggregate breeding in temporary pools, and colour pattern polymorphism (Savage 2002; Robertson 2008; Robertson & Robertson 2008). Despite these similarities, *D. ebraccatus* and *A. callidryas* show marked differences in phenotypic differentiation across their range. *D. ebraccatus* exhibits multiple dorsal colour pattern types, but all types occur within most populations (Duellman 2001; Ohmer *et al*. in press). In contrast *A. callidryas* exhibits striking, regional differentiation in flank-stripe pattern, and leg and flank coloration ranging from red to blue-violet (Savage & Heyer 1967; Robertson & Robertson 2008). Breeding studies for *D. ebraccatus* (J. Robertson and J. Touchon, unpublished) and captive breeding of *A. callidryas* (I. Gomez-Mestre, personal communication) indicates that colour pattern in both taxa has a strong heritable component. While further studies are necessary to rule out phenotypic plasticity, available evidence indicates that colour pattern is a reliable character for evolutionary studies in both taxa.

Within natural populations, colour pattern polymorphisms are maintained through the combined effects of migration, selection, and genetic drift (Endler 1973, 1980; Hoffman & Blouin 2000; Jordan *et al*. 2005; Hoffman *et al*. 2006; Gray & McKinnon 2007). If populations are linked through high rates of migration and gene flow, then population connectivity likely explains phenotypic similarity (Wright 1937; Ritchie *et al*. 2007; Roberts *et al*. 2007). In contrast, if gene flow is highly restricted, then the maintenance of polymorphisms will reflect population-level processes, and be largely mediated by localized selection and drift (Endler 1982; Lenormand 2002; Saint-Laurent *et al*. 2003; Price 2006; Harper & Pfennig 2008). The form of selection acting on polymorphisms will vary depending on the role that coloration plays in different species (Cott 1940; Gray 1983). Natural selection could favour conspicuous and/or cryptic coloration to deter predators (Cott 1940; Endler 1982, 1990; Hoekstra *et al*. 2005; Rosenblum *et al*. 2007), or sexual selection could favour patterns that augment conspecific recognition or individual fitness (West-Eberhard 1983; Gray & McKinnon 2007). *Agalychnis callidryas* is chemically defended by noxious skin peptides common in phyllomedusine frogs, and its bright coloration may serve as an aposematic warning signal to predators (Sazima 1974; Erspamer *et al*. 1985; Mignogna *et al*. 1997). However, phylogenetic tests for character displacement in Central American phyllomedusine frogs support the hypothesis that colour pattern also serves a species recognition function (Robertson 2008). Thus, a trait that functions in intraspecific communication should be relatively conserved among populations connected through gene flow, but could evolve through relaxed selection among allopatric populations. Therefore, we expect phenotypic differentiation to vary with levels of gene flow in *A. callidryas*. In contrast, phylogenetic tests for the Dendropsophinii, revealed that multiple, syntopic species exhibit similar colour patterns (Robertson 2008), suggesting that those characters maximize survival in a particular visual environment (Grinnell 1924; Warburg 1965; Rand & Williams 1970; Stewart 1974; Endler 1982; Harmon *et al*. 2005). Thus, if colour pattern of *D. ebraccatus* evolved through natural selection, with balancing selection favouring multiple phenotypes, we expect that the frequencies of morphs at any locality will vary independent of the extent of gene flow among populations (Endler 1973; Slatkin 1985; Sandoval 1994; Lenormand 2002).

We compared processes underlying genetic and phenotypic variation in these two common, widespread Neotropical frogs. Our goal was not to directly test the adaptive significance of coloration, but to quantify the geographic distribution of colour pattern in each species and compare them with indices of genetic differentiation. These comparisons were used to infer the mechanisms underlying phenotypic diversification in each species

(Endler 1982; Hoffman *et al*. 2006). We had four specific aims: (i) quantify the geographic distribution of phenotypic diversity within and among regions; (ii) recover historical biogeography and contemporary population genetic structure using mitochondrial DNA (mtDNA) sequencing and nuclear microsatellite genotyping; (iii) examine associations among genetic diversity, colour pattern and geography; and (iv) compare patterns and processes of diversification between our focal taxa. The strength of this study lies in its comparative nature, fine scale sampling, and geographic coverage, allowing for the investigation of the historical and species-typical traits shaping diversity among populations. Our focal taxa offer a unique opportunity to disentangle factors underlying the origin and maintenance of diversity because of their similar ecologies, yet very different geographic patterns of phenotypic diversity.

Materials and methods

We quantified and compared regional phenotypic and genetic diversity in *Dendropsophus ebraccatus* and *Agalychnis callidryas*. For this study, we developed nuclear microsatellite primers for both species and genotyped all individuals for estimates of recent gene flow patterns and population genetic structure. We generated new mtDNA sequences for *D. ebraccatus* and compare the resulting topology to previous phylogenetic analyses of *A. callidryas* (Robertson 2008). We extended previous analyses on the geographic variation of colour pattern by including more populations of *D. ebraccatus* (Ohmer *et al*. in press) and *A. callidryas* (Robertson & Robertson 2008).

Field sampling

We sampled 22 populations representing five regions in Costa Rica (CR) and Panama (PA): northeastern CR, southeastern CR/PA, northwestern CR, southwestern CR and central PA (Kohlmann *et al*. 2002; Savage 2002). Our previous studies showed that distribution patterns of genetic (mtDNA) and phenotypic diversity in *A. callidryas* mostly coincided with these five regions, indicating landscape barriers between them have played a role in historical regional diversification (Robertson 2008). For this study, we gathered a similar data set for 15 populations of *D. ebraccatus* (Fig. 1), 14 of which were also sampled for *A. callidryas* ($n = 21$; Fig. 1b; Table 1). We conducted field surveys during the breeding seasons (May–August) of 2003, 2004 and 2005. At each sampling site, we captured 8–50 adult males and females and collected data on colour pattern through digital photography (Table 1). We sampled all individuals encountered so as to not bias our measures of colour pattern frequencies. Individual tissue samples (toe clips) were collected and stored in 100% ethanol for use in genetic analyses. All individuals were released at their capture site, with the exception of two *D. ebraccatus* and 18 *A. callidryas* individuals that were preserved as voucher specimens and deposited in the Cornell University Museum of Vertebrates (CUMV; Accession nos CU 14029, 14093, 14206–14208, 14210–14213, 14225–14228, 14230–14235) and Museum of Zoology, University of Costa Rica, San José (UCR: Accession nos 19100–19102, 19213). All photographs are archived at the CUMV.

Quantifying phenotypic diversity

The dorsal colour pattern of *D. ebraccatus* is characterized by disruptive yellow, gold and brown blotches and spots over a bright yellow or tan background colour. Following Duellman (1970), we categorized each individual as having an hourglass with extensive spotting (pattern 1), an hourglass without spotting (pattern 2), spotting only (pattern 3), and plain yellow (pattern 4; Fig. 2). Previous range-wide analyses revealed widespread polymorphisms (Duellman 1970) but dorsal pattern frequencies varying among populations at fine spatial scales (Ohmer *et al*. in press). We augmented those previous studies by including two additional populations along the Pacific CR versant (populations 2 and 4; Fig. 1).

Agalychnis callidryas has bright, contrasting white or yellow flank-stripes overlaying a background colour of blue, violet, brown or orange (Savage & Heyer 1967; Duellman 1970; Robertson & Robertson 2008). We reported co-varying regionalization in coloration and flank-stripe patterns in a previous study of *A. callidryas* (Robertson & Robertson 2008). Here we implemented the scoring system designed by Savage & Heyer (1967) and modified by Robertson & Robertson (2008) to assign individuals to seven flank-stripe pattern types: A, AB, B, BC, C, AC, ABC (Fig. 2). We analysed only the left side of the body to avoid bias due to lateral asymmetry (Savage & Heyer 1967; Robertson & Robertson 2008). Here, we expand upon the earlier study of flank-stripe variation by including five additional populations not previously sampled (populations 4, 6, 7, 18, 19; Fig. 1b).

For both species, we conducted contingency analyses to determine whether the frequency distribution of colour pattern morphs varied among populations. Significance of chi-square tests were determined using Fisher's exact test implemented in R version 2.7.0 (R Core Development 2007) and adjusted for the risk of a type I error with Bonferroni corrections.

Population-level phylogenetic analyses

We collected a genetic data set for *D. ebraccatus* that paralleled that available for *A. callidryas* (Robertson 2008). We aimed for similar sample sizes and geographic

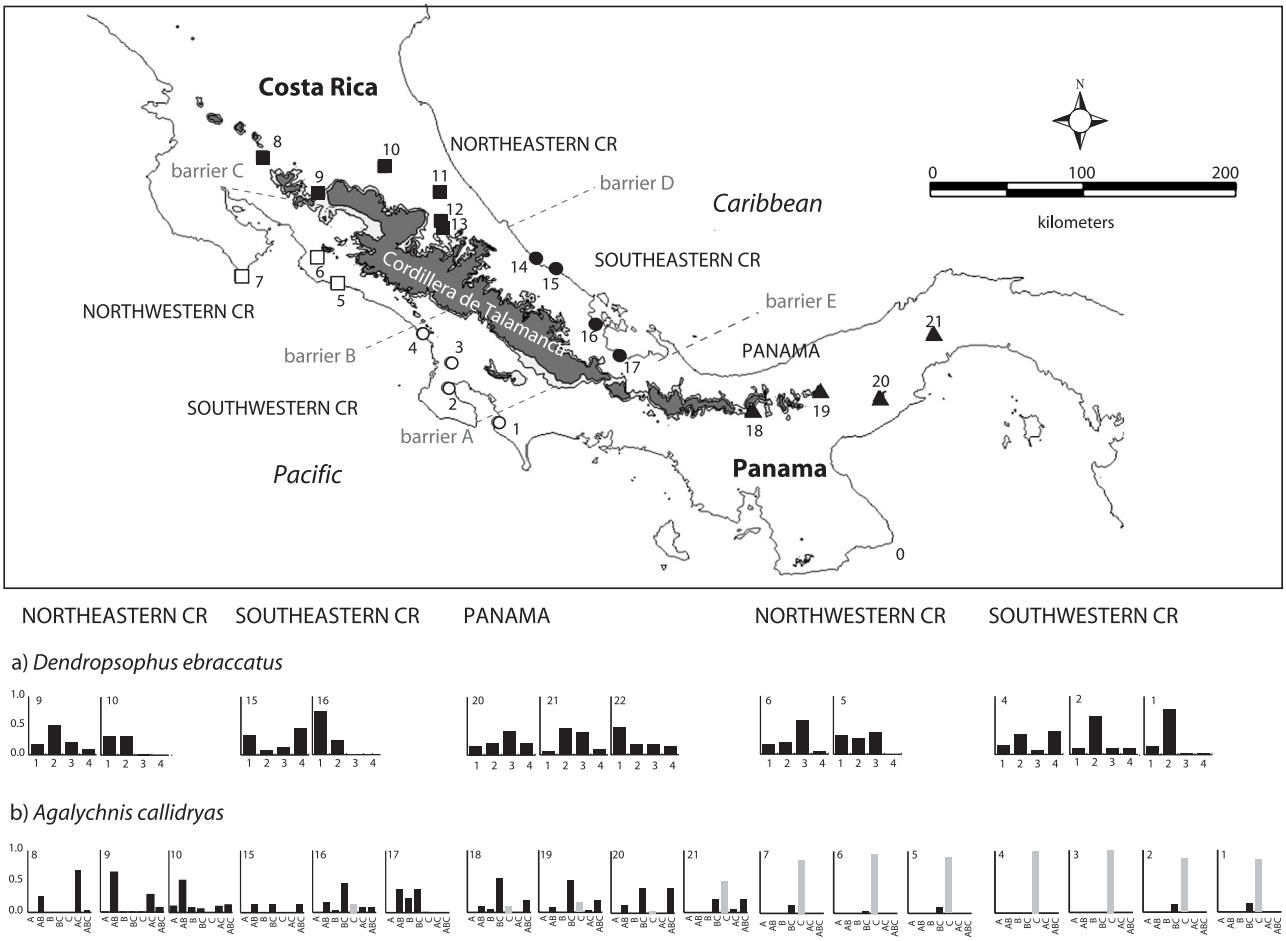

Fig. 1 Population sampling and geographic variation in colour pattern for *Dendropsophus ebraccatus* and *Agalychnis callidryas*. The Cordillera de Talamanca isolates the Pacific and Caribbean versants of Costa Rica and Panama. The shading of topological relief corresponds to elevation: dark grey (> 1300 m above sea level), light grey (300–1299 m), white (0–300 m). Dry forest habitat between southwestern Costa Rica and Central Panama restricts dispersal along the Pacific coast of Panama. Five biogeographic barriers A–E that potentially isolate populations in different regions were examined using matrix correspondence tests. (a) Sampling for 15 populations *Dendropsophus ebraccatus* with the frequency of dorsal patterns in each sampled population (expanded from Ohmer *et al.* in review). (b) Sampling for 21 populations of *Agalychnis callidryas*. The frequency of flank pattern C is shown in grey to illustrate the disjunct phenotypic distribution of Pacific and Caribbean populations. For both taxa, numbers in the upper left hand corner of each frequency histogram refer to population number.

sampling (125 individuals for *A. callidryas* from 20 populations and 79 Individuals for *D. ebraccatus* from 15 populations; Table 1), similar length of mtDNA sequences (1031 bp for *A. callidryas*; 1882 bp for *D. ebraccatus*), and followed the same DNA sequencing procedures and phylogenetic analyses. (*A. callidryas* GenBank Accession nos FJ489259–FJ489334).

We extracted genomic DNA from *D. ebraccatus* and two outgroup taxa (*Pseudohyla puma* and *Dendropsophus microcephalus*) by digesting tissues in standard lysis buffer with Proteinase K, and purifying the extract using the QIAGEN DNeasy Tissue Kit. We amplified a mitochondrial gene fragment that includes partial sequence of the 16S Ribosomal Subunit 1, the leucine and isoleucine transfer RNAs (tRNA) and the complete NADH dehydrogenase subunit 1 gene (ND1) using primers t-met frog, and 16S-frog (Wiens *et al*. 2005). Polymerase chain reactions (PCRs) were performed in a total volume of 25 µL, each containing ~100 ng template DNA, 1× PCR Buffer, 0.75 mM dNTPs, 1.5 mM $MgCl_2$, 1 µM primer, and 0.625 U of *Taq* polymerase. PCR amplification conditions were: 95 °C initial denaturation for 5 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, extension at 72 °C for 1 min, and a final 5-min extension at 72 °C. We used Exonuclease (10 units) and SAP (1 unit) to remove unincorporated oligonucleotides and dNTPs. We used the same sequencing primers in cycle sequencing reactions with Big Dye terminator sequencing kits (Applied Biosystems, Perkin Elmer).

Table 1 Sampling populations of *Dendropsophus ebraccatus* and *Agalychnis callidryas* from five regions in Costa Rica and Panama*

					D. ebraccatus			*A. callidryas*		
No.	Population	Province	Region	GIS (lat., long., el.)	Colour	Micro	Seq	Colour	Micro	SeqΨ
1	Pavones	Puntarenas, CR	Southwestern CR	8.4204, –83.1069, 37	39	30	4	20	26	7
2	Sierpe	Puntarenas, CR		8.8892, –83.477, 17	—	—	—	19	22	11
4	Uvita	Puntarenas, CR		9.123, –83.701, 26	25	28	5	24	32	11
3	Campo	Puntarenas, CR		8.6909, –83.5013, 35	26	25	5	16	19	5
5	Playa Bandera	Puntarenas, CR	Northwestern CR	9.5188, –84.3774, 23	21	28	5	19	27	11
6	Carara	San Jose, CR		9.725, –84.531, 385	24	31	4	25	30	5
7	Cabo Blanco	Guanacaste, CR		9.580, –85.124, 166	—	—	—	18	30	7
8	Tilarán	Guanacaste, CR	Northeastern CR	10.5162, –84.9601, 637	—	—	—	22	23	6
9	San Ramon	Alajuela, CR		10.2335, –84.5287, 638	22	21	5	14	14	4
10	La Selva Biological Station	Heredia, CR		10.4327, –84.0080, 37	50	98	6	20	33	10
11	University of EARTH	Limón, CR		10.2318, –83.5, 44	0	29	6	0	32	6
12	Siquirres	Limón, CR		10.0134, –83.565, 667	0	26	5	0	26	1
13	Guayacan	Limón, CR		10.0134, –83.557, 680	—	—	—	0	7	0
14	Cahuita-Comadre	Limón, CR	Southeastern CR/PA	9.718, –82.814, 16	0	21	9	0	29	3
15	Manzanillo	Limón, CR		9.6332, –82.6556, 2	15	14	5	26	30	8
16	Chiriqui Grande	Bocas del Toro, PA		8.9460, –82.1571, 21	8	11	5	21	24	6
17	Almirante	Bocas del Toro, PA		9. 1980, –82.3445, 13	—	—	—	10	15	2
18	Santa Fé	Veraguas, PA	Central Panama	8.529, –81.139, 714	—	—	—	17	19	6
19	El Cope	Coclé, PA		8.6681, –80.592, 792	—	—	—	22	31	7
20	El Valle	Coclé, PA		8.6299, –80.1159, 866	24	20	5	21	24	4
21	Gamboa	Panamá, PA		9.1231, –79.6930, 51	31	109	5	22	21	6
22	Cerro Azul	Panamá, PA		9.1671, –79.419, 638	27	31	5	—	—	—
				No. of samples	312	522	79	336	514	126
				No. of populations	12	15	15	17	21	20

*Geographic Information System coordinates [GIS: latitude (Lat), longitude (Long), elevation (el., in metres above sea level)]. Sampling sizes for colour pattern (colour), microsatellite genotyping (micro), and mtDNA sequencing (seq) provided. Population number (No.) indicates position on Fig. 1. Species not present at locality (—). Data collected from previous study (Ψ). CR, Costa Rica; PA, Panama.

Cycle sequencing reaction conditions were 25 cycles of 96 °C (30 s), 50 °C (15 s), and 60 °C (4 min). We sequenced the amplicon in both directions to avoid base-calling ambiguities. Products were column-purified to remove non-incorporated terminator dye using Sephadex G-50 and electrophoresed on an ABI 3100 Genetic Analyzer. Electropherograms were checked by eye and contiguous sequences created using Sequencher version 4.1 (GeneCodes).

We partitioned the sequences into two data sets, ND1 and partial 16S + tRNA gene sequences for alignment using ClustalW (Thompson *et al*. 1994) in the MegAlign version 6.1.2 program of the Lasergene sequence analysis software (DNASTAR, Inc.). The initial guide tree was aligned using gap length penalty = 6.66; gap extension penalty = 0.05; delay divergence sequences = 30%; and transitions = 0.5. For subsequent alignments, we kept all parameters constant but varied gap costs (4, 8, 10, 15) using the 'slow/accurate' alignment option to identify regions of ambiguous homology (Gatesy *et al*. 1993); positions that varied in alignment were excluded as characters in phylogenetic analyses.

We conducted phylogenetic analyses using maximum-likelihood (ML) in PAUP* version 4.0 b10 (Swafford 2001) and Bayesian inference in MrBayes 3.0b4. For both analyses, we first used ModelTest version 3.04 (Posada & Crandall 1998) and hierarchical likelihood ratio tests to determine the model of DNA substitution and parameter estimates that best fit our data. The GTR + I + Γ model ($\mu = 2.61\%$; $\sigma = 1.65\%$) was selected as the preferred model. We used unequal base frequencies (A = 0.3076, C = 0.22190, G = 0.11182), pinvar of 0.340837 and an alpha shape parameter of the gamma distribution of 0.676678 in a heuristic maximum-likelihood search. Bayesian analyses used four chains (one cold and three heated) sampled every 1000 generations for 10 million generations with the first 1000 trees discarded as burn-in. We applied default prior distributions for all model parameters in MrBayes with the exception of the alpha shape parameter (exponential, mean = 1.0) and branch lengths (exponential, mean = 0.1). We calculated standard measures of haplotype and nucleotide diversities (Nei 1987) within each population, implemented an analysis of molecular variance (AMOVA) to determine the genetic variation among and within groups, and estimated pairwise F_{ST} between all populations, in Arlequin version 3.01 (Schneider *et al*. 2000).

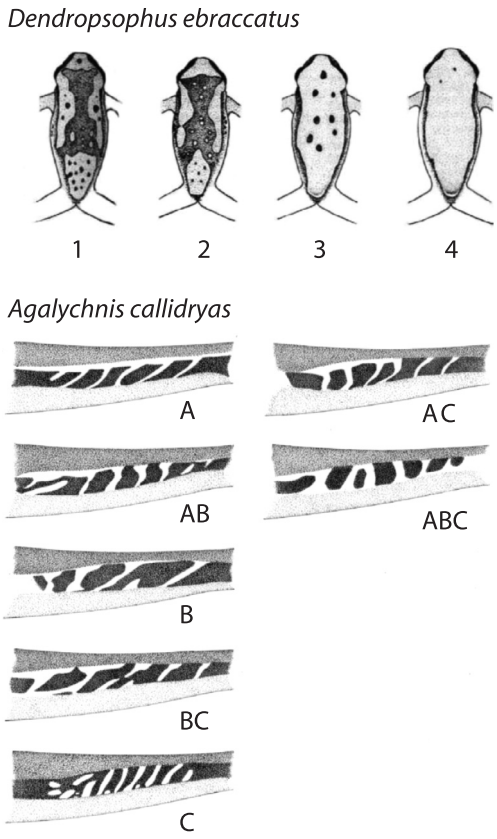

Fig. 2 Colour pattern polymorphisms for *Dendropsophus ebraccatus* and *Agalychnis callidryas*. We recognize four dorsal colour patterns in *D. ebraccatus*: (1) hourglass with spots (2) hourglass shape with no spots (3) spots, (4) and plain (Duellman 2001). We categorized flank stripe patterns of *A. callidryas* into seven groups: A, AB, B, BC, C, AC, ABC (Savage & Heyer 1967; Robertson & Robertson 2008). Pattern A individuals 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, AC, ABC.

Microsatellite characterization and genotyping

We constructed an enriched partial genomic library to isolate and characterize microsatellite loci for both *D. ebraccatus* and *A. callidryas* (Hamilton *et al*. 1999). For both taxa, we extracted genomic DNA from three individuals using a QIAGEN DNeasy kit and pooled the extracts for library development. DNA was digested with two restriction enzymes, *Alu*I and *Hae*III, and ligated to a double-stranded SNX linker using T4 DNA ligase. We probed DNA fragments with di-, tri-, and tetra- biotinylated oligonucleotides and captured them with streptavidin-coated magnetic beads, followed by PCR amplification using the SNX primer and Vent exo-polymerase for 35 cycles under the following conditions: 95 °C for 50 s; 60 °C for 60 s; 72 °C for 90 s. The PCR products were cut with *Nhe*I and the product was electrophoresed on a 1% agarose gel for size selection. We excised a product 300–900 bp in size, followed by purification using QIAquick Gel Extraction kit (QIAGEN). The digested and gel-purified PCR products were ligated to pUC19 cloning vector plasmids for transformation in *Escherichia coli* XL1-Blue MRF′ supercompetent cells. We plated cells on Luria-Burtani agar/ampicillin plates and transferred colonies to nylon membranes hybridized to ^{32}P radiolabelled probes. Positive clones were cultured overnight in Luria broth and DNA was extracted with a QIAprep Spin Miniprep Kit (QIAGEN). Colonies containing microsatellites were sequenced with M13 forward and reverse primers using Big-Dye Terminator Cycle Sequencing Kit (Applied Biosystems) on an ABI 3100 Genetic Analyzer. We characterized and designed primers for 54 microsatellite loci for *D. ebraccatus* and 83 loci for *A. callidryas*; of these six were polymorphic and amplified reliably across all populations in the study (Appendix I). Cloned sequences containing microsatellite motifs were accessioned in GenBank (*D. ebraccatus*: FJ546203–FJ546208; *A. callidryas*: FJ546197–FJ546202).

We digested tissues (approximately 1 mm^3) in 150 µL of a 5% Chelex solution (Chelex-100, Bio-Rad) incubated with 19 µg proteinase K for 120 min at 55 °C followed by denaturation at 90 °C for 10 min; the supernatant was used directly in PCR amplifications using a Hybaid Gradient Thermocycler. PCR conditions consisted of an initial 90 °C denaturation for 2 min, followed by 35 amplification cycles of denaturation at 94 °C for 50 s, locus-specific annealing temperature (ranging from 55–67.6 °C; Appendix I) for 60 s, extension at 72 °C for 60 s, and a final 5-min extension at 72 °C. We performed PCRs in 10-µL reaction volumes containing: 1× Roche reaction buffer without $MgCl_2$, 1 mM $MgCl_2$, 0.2 µM of each PCR primer, 0.2 µM dNTPs, 2.5 U *Taq* polymerase and template DNA. Each forward primer was 5′-end labelled with a fluorescent dye (NED, 6-FAM, PET, VIC; Appendix I); amplicons were multiplexed in two groups and electrophoresed on an Applied Biosystems 3730xl DNA Analyzer. We assigned fragment sizes by comparison with a LIZ 500 bp ladder and binned alleles into discrete size categories according to microsatellite repeat motif using GeneMapper version 3.5 software (Applied Biosystems).

Estimating gene flow and population genetic analyses

For both species, we estimated allelic diversity and observed and expected heterozygosities for each locus in FSTAT version 2.1. We used FSTAT to test for significant deviation from Hardy–Weinberg equilibrium (HWE) accounting for unequal sample sizes with 2520 permutations of the data across loci. Likewise, we tested for linkage disequilibrium (LD) based on 300 permutations. Significance for both HWE and LD were determined at

$\alpha = 0.05$ after Bonferroni correction. Pairwise F_{ST} estimates among all populations were estimated in FSTAT and significance obtained after 2100 permutations for *D. ebraccatus* and 4200 permutations for *A. callidryas*, adjusted for multiple comparison (Weir & Cockerham 1984). For both species, we conducted an analysis of molecular variance (AMOVA) implemented in Arlequin version 2.0 (Schneider *et al*. 2000). Significance for this test was based on 1023 permutations for *D. ebraccatus* and 2020 permutations for *A. callidryas*.

We used Bayesian assignment tests (Pritchard *et al*. 2000) to estimate the number of genetic demes and to evaluate the degree of admixture among them. The program Structure version 2 (Pritchard *et al*. 2000) uses a Markov chain Monte Carlo algorithm to find the posterior probability that individuals belong to each of K clusters assuming linkage equilibrium and HWE across multiple, unlinked loci. We applied an admixture model with correlated allele frequencies and alpha max = 10.0. Each run included 3 million generations, following a burn-in of 1 million iterations. The average maximum-likelihood values, for each of 25 runs at each number of genetic demes ($K = 1$ to $K = 15$) were examined to determine the plateau in likelihood scores. We also calculated ΔK to identify the greatest rate of change between each subsequent K (Evanno *et al*. 2005). Assignment profiles for 25 runs at the best value of K were coalesced in CLUMPP (Jakobsson & Rosenberg 2007) for the final deme assignment graph for each species.

Isolation-by-distance: comparison between species and Caribbean and Pacific populations

Using the microsatellite data set for each species, we tested for a pattern of genetic isolation by distance (IBD) by comparing the relationship among geographic distance (natural logarithm) and genetic distance $[F_{ST}/(1 - F_{ST})]$ Rousset 1997). We computed a standardized measure of genetic differentiation because estimates of genetic divergence based on allele frequencies are a function of absolute heterozygosity of the genetic markers (Meirmans 2006). Therefore, we transformed data sets in GenoDive version 2.0b9 (Meirmans 2006) to assign each population a unique set of alleles to calculate the maximum population pairwise genetic differentiation (φ_{STmax}). Standardized measures of genetic differentiation (φ'_{ST}) were computed by dividing the true pairwise values of φ_{ST} by φ_{STmax}. We graphically examined the relationship between geographic distance and the standardized values of $[\varphi'_{ST}/(1-\varphi'_{ST})]$ and performed 10 000 permutations of the Mantel test implemented in R version 2.7.0 to determine whether the slope of each regression was significantly greater than zero. Because of unequal variances in our estimates of migration between species and between Caribbean and Pacific populations, we could not directly compare correlation coefficients. Therefore, these graphs were used only to uncover qualitative differences in IBD between taxa and/or regions.

Matrix correspondence tests: associations of gene flow, phenotype, and geography

We conducted matrix correspondence tests (MCT) and partial MCT (pMCT) to examine the associations among genetic distance, geographic factors, and colour pattern, a standard methodology that tests for the correlation of multiple parameters (Thorpe & Richard 2001; Harmon & Gibson 2006; Rosenblum 2006). MCT use repeated randomization and permutation to test for the correlation between two distance matrices by comparing the individual pairwise distance for each parameter (Manly 1986). Randomized values provide a null distribution to test the hypothesis of no association. We conducted pMCT when more than one independent variable was significant in individual MCT ($P < 0.05$). Partial MCT measure the association between two matrices, while controlling for the variation in a third. As with many partial regression analyses, pMCT can be biased when dependent variables are correlated (Raufaste & Rousset 2001; Castellano & Balletto 2002; Raufaste *et al*. 2005). We compared the pairwise regression and partial regression analyses and interpreted our results in light of this possible limitation. Significance values were determined by comparing the observed and expected Z-statistic, generated by 10 000 permutations in the program R version 2.7.0.

We constructed pairwise dissimilarity matrices and conducted MCT and pMCT in R version 2.7.0. For colour pattern, we constructed pairwise Euclidian distance matrices based on the frequency of each colour pattern within populations. We estimated two measures of genetic differentiation, based on the individual pairwise patristic distance from mtDNA sequences and estimated pairwise F_{ST} from multilocus genotypes (*D. ebraccatus*: Appendix II; *A. callidryas*: Appendix III). Geographic distance was the minimum linear distance between populations based on UTM coordinates. Because our focal species are low to mid-elevation frogs, and thus cannot disperse over high elevation mountain passes in this region, we calculated the shortest distance around the Cordillera de Talamanca, instead of across the mountain range, thus better representing the 'effective dispersal' required to maintain connectivity among populations.

We compared the isolating effect of five barriers on gene flow and colour pattern: the Golfo Dulce (barrier A), Rio Tárcoles (barrier B), Cordillera de Talamanca (barrier C), Caribbean Valley Complex (barrier D), and the Bocas del Toro region (barrier E; Fig. 1a, b). A matrix for each barrier was constructed using a binary assignment with 0 for populations on the same side and 1 for populations located

on opposite sides of the barrier. For each test, we only included populations from the two regions adjacent to the putative barrier.

The effectiveness of the Cordillera de Talamanca in structuring Caribbean and Pacific populations is discussed above. The other four putative biogeographic barriers examined in this study were known to structure populations in other Central American taxa. Specifically, the Golfo Dulce (barrier A) of Costa Rica, juxtaposes the Osa Peninsula and lowland Pacific dry forest in southwestern Costa Rica (Fig. 1a, b). The Osa Peninsula is well known for its high endemicity 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 Costa Rica approximately 2 million years ago (Kohlmann *et al*. 2002). Our analyses examined the water barrier situated between population 1 (Pavones) and the Osa Peninsula. The Río Tárcoles (barrier B) drains from the Cordillera de Talamanca into the Pacific Ocean and coincides with plate tectonic and microplate tectonic activity (Kohlmann *et al*. 2002), separating populations into northern and southern bioregions. Genetic isolation could be due to the effects of these historical geological factors and/or dispersal limitations imposed by a riverine barrier. The Caribbean Valley complex (barrier D), a series of floodplain valleys of the south Caribbean region, includes Valle de la Estrella, Valle de Talamanca and Llanura de Santa Clara (Kohlmann *et al*. 2002; Figs 1 and 2). Although the nature of this barrier is not well understood, it coincides with the northern/southern edges of geographic ranges in other Central American taxa (Kohlmann *et al*. 2002; Savage 2002), delimits colour morphs of the poison arrow frog, *Dendrobates pumilio* (Hagemann & Prohl 2007) and leg coloration in red-eyed treefrog populations (Robertson & Robertson 2008). Finally, prior to the formation of the Cordillera de Talamanca, the region of Bocas del Toro (barrier E) experienced a short-lived uplift, approximately 5–7 million years ago. Multiple colonization events (Zeh *et al*. 2003; Weigt *et al*. 2005) consistent with the uplift have been documented for other anuran taxa (Crawford 2003; Crawford *et al*. 2007).

Results

Quantifying phenotypic diversity

Our phenotype data set for *Dendropsophus ebraccatus* included 12 of 15 sampled populations (Table 1). We sampled 8–50 individuals (average ± SD = 26 ± 10.72) per population for a total of 312 individuals. The frequency distributions of dorsal pattern differed among all populations ($P = 0.001$) and among populations within all regions except for northwestern CR ($P = 0.2882$) and southeastern CR ($P = 0.013$; not significant after Bonferroni correction). We detected differences among populations for three regions (northeastern CR: $P < 0.001$; Panama: $P = 0.003$; and southwestern CR: $P = 0.002$; Fig. 1a). Overall, two hourglass patterns (patterns 1 and 2; Fig. 1a) accounted for 68% of the total variation of all individuals while the plain type was least common (11.5%). Not all populations contained all pattern types: we did not observe the plain pattern in three populations (5, 10 and 16) or the spotted pattern in one population (16; Fig. 1a).

Our phenotype data set for *Agalychnis callidryas* included data from 17 of 21 populations (Table 1). We sampled 13–48 individuals (average 26 ± SD = 19.7 ± 4.0) per population for a total of 336 individuals (Table 1). The frequency distribution of flank stripe pattern differed among all populations ($P < 0.001$) and among populations within two Caribbean regions (northeastern CR: $P = 0.0005$; southeastern CR: $P = 0.002$) but did not differ among Pacific populations (northwestern CR: $P = 0.554$; southwestern CR: $P = 0.059$) or Panama ($P = 0.040$; not significant after Bonferroni correction). Caribbean populations were more variable than Pacific populations, and although Caribbean populations contained all pattern types, pattern A was observed in only one population (10; Fig. 1b). In contrast, Pacific populations were nearly monomorphic for flank pattern C (92%), with only a few individuals exhibiting pattern BC.

Population-level phylogenetic analyses

We amplified the ND1/16S mtDNA fragment from 79 *D. ebraccatus* and two outgroup taxa, resulting in 37 unique sequences, 1882 nucleotides in length (Fig. 3; GenBank Accession nos FJ542147–FJ542198). The sequences contained 685 variable sites, 217 parsimony-informative sites, and no insertions or deletions. Haplotypic (h) and nucleotide (π) diversity varied among populations with high h and relatively low π for most populations (Table 2). Significant genetic structure was evident both within and among five regions based on mtDNA: the AMOVA estimated that 19.52% of genetic variance was partitioned among populations and 18.40% among regions. Pairwise gene flow estimates (F_{ST}) revealed high average differentiation among regions (0.65 ± 0.28; 0.18–0.97; Appendix II).

The Bayesian consensus topology for *D. ebraccatus* showed high regional differentiation; all biogeographic regions formed highly supported, reciprocally monophyletic clades (Fig. 3). Populations from Panama diverged earliest from all other regions. Following that, the consensus phylogeny revealed two major clades (i) southwestern CR clade and (ii) southeastern–northeastern–northwestern clades. The southwestern CR clade diverged from the more widespread sister clade, indicating the species likely had an ancestral widespread distribution and that southwestern CR populations became isolated from the remaining isthmus populations, either due to vicariance or dispersal.

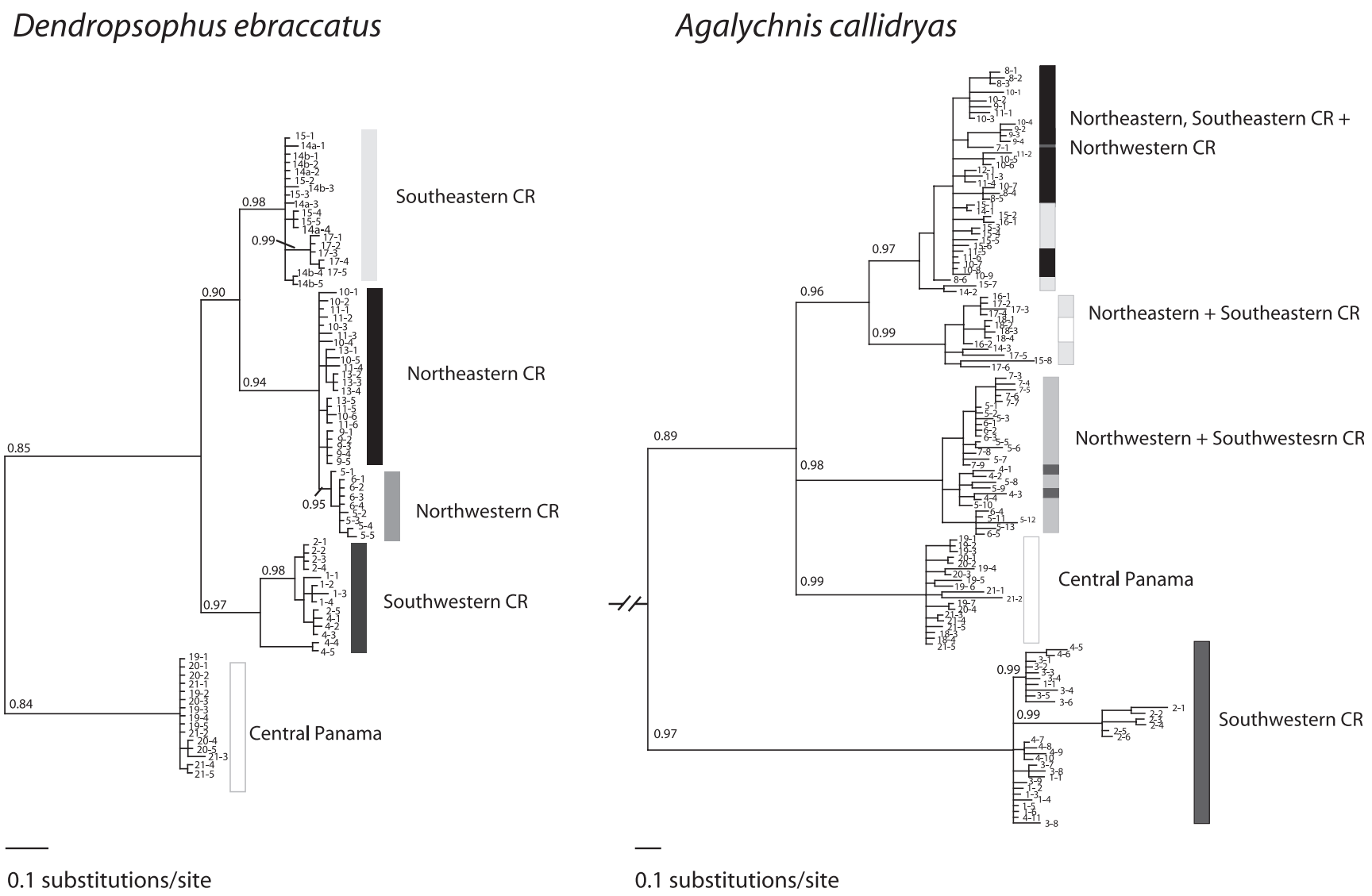

Fig. 3 Left panel. Bayesian consensus phylogram for *Dendropsophus ebraccatus* based on 1882 base pairs of the 16S ribosomal subunit + NADH1 mitochondrial DNA gene fragment. Phylogram is rooted with the outgroup taxa, *D. microcephalus* and *P. puma* (not shown). Right panel. Bayesian consensus phylogram for *Agalychnis callidryas* based on 1149 base pairs of the NADH1 mitochondrial DNA gene fragment (modified from Robertson 2008). Phylogram is rooted with the outgroup taxon, *Agalychnis saltator*. In both topologies bars identify groups of populations or clades belonging to five sampled geographic regions and values above branches are posterior probabilities for each clade.

Table 2 Summary of within-population diversity at NADH1 and 16S mtDNA gene sequences for *Dendropsophus ebraccatus**

Region	Population	N	*H*	Po	Π
Southwest CR	1	4	0.83	7	0.0018 (0.0014)
	2	5	0.40	3	0.0006 (0.0005)
	4	5	0.40	1	0.0002 (0.0027)
Northwest CR	5	5	0.0	0	0.0000 (0.0000)
	6	4	0	0	0
Northeast CR	9	5	0.00	0	0.0000 (0.0000)
	10	6	0.33	1	0.0001 (0.0002)
	11	6	0.80	5	0.0010 (0.0007)
	12	5	0.00	0	0.0000 (0.0000)
Southeast CR/PA	14	9	1.00	4	0.0010 (0.0008)
	15	5	0.60	1	0.0003 (0.0003)
	16	5	0.80	2	0.0005 (0.0004)
Central PA	20	5	0.00	0	0.0000 (0.0000)
	21	5	0.48	1	0.0003 (0.0003)
	22	5	0.80	5	0.0011 (0.0009)

*the number of individuals sampled per population (N), haplotypic diversity (*H*), number of polymorphic sites (Po), nucleotide diversity (π) with standard error. Summary statistics for *A. callidryas* available in Robertson (2008). CR, Costa Rica; PA, Panama.

The widespread clade contained both Caribbean and Pacific populations, but with no evidence of admixture among regions; the southeastern CR clade is sister to a clade containing populations on either side of the continental divide (northwestern CR and northeastern CR; Fig. 3), indicating populations at the northern edge of the Cordillera de Talamanca share a more recent common ancestry than do the two CR Caribbean regions. We detected no evidence of historical connectivity between southwestern CR and Panama: our inferences based on both the tree topology and high pairwise F_{ST} estimates between these two regions indicate that migration along the Panamanian pacific versant has been highly restricted for hourglass treefrogs.

The Bayesian consensus topology for *A. callidryas* indicated a pattern of regional differentiation among five clades, but with admixture among most neighbouring populations (Robertson 2008). We detected an early divergence of southwestern CR relative to the other four regions (Fig. 3). Populations from remaining regions belong to three admixed clades united at their base by a polytomy (Fig. 3). We detected no evidence of historical connectivity between southwestern CR and Panama: our inferences based on both the tree topology and high pairwise F_{ST} estimates between these two regions indicated that migration along the Panamanian Pacific versant has been highly restricted for red-eyed treefrogs (Appendix III).

The greatest distinction between tree topologies of our study taxa is the monophyly of regional clades for the hourglass treefrog and significant admixture among the same regional clades for the red-eyed treefrog. For *D. ebraccatus*, per cent sequence divergence among five monophyletic clades ranged from 0.76 to 2.48% for the 16S gene region and from 0.49 to 7.63% for ND1 (Table 3). Similarly, for *A. callidryas*, per cent sequence divergence among all five regions ranged from 1.25 to 6.65% for ND1 and from 1.25 to 6.14% among admixed lineages (Table 3). Thus, despite differences in tree topology and degree of monophyly, overall sequence divergence estimates were similar between taxa.

Gene flow and population genetic structure

Hourglass treefrogs exhibited significant population genetic structure at microsatellite loci both within and among five regions, corroborating that gene flow is highly restricted among populations. The number of alleles per locus ranged from 24 to 51 and averaged 30.3 across all loci. Mean observed heterozygosity was 0.898, ranging from 0.815 to 0.944 at individual loci (Appendix I). Overall, we detected no consistent deviation in HWE or LD among populations. We detected significance in 92 of 105 population pairwise F_{ST} comparisons, indicating restricted gene flow even among populations within regions (range: 0.025–0.348; Appendix II). Likewise, AMOVA showed 13% of the genetic variation could be explained by divergence among populations and 11% by divergence among regions.

Bayesian assignment analyses for *D. ebraccatus* identified six demes (four Caribbean and two Pacific demes) with no detectable admixture between Pacific and Caribbean populations (Fig. 4). Among Caribbean populations, northeast populations were comprised of two demes, one containing a single isolated population (population 9; Fig. 4) and the other deme containing all other northeastern populations. Two other Caribbean demes corresponded to southeastern CR and Panamanian populations, with limited admixture detected at the geographic borders of

Table 3 Per cent sequence divergence among mtDNA lineages for *Dendroposphus ebraccatus* and *Agalychnis callidryas*. Pairwise divergence shown among unique NADH1 sequences (below the diagonal) and 16S sequence (above the diagonal)*

A. *Dendropsophus ebraccatus*

	Northeast CR	Southeast CR	Northwest CR	Southwest CR	Panama
Northeast CR	.	0.76	0.02	1.03	1.89
Southeast CR	2.11	.	0.764	1.32	2.34
Northwest CR	0.49	2.21	.	0.94	1.86
Southwest CR	3.44	3.05	3.46	.	2.48
Panama	7.62	6.65	7.63	6.66	.

B. *Agalychnis callidryas*

	Northeast CR	Southeast CR	Northwest CR	Southwest CR	Panama
Northeast CR	.	–	–	–	–
Southeast CR	1.25	.	–	–	–
Northwest CR	4.05	4.01	.	–	–
Southwest CR	6.65	6.55	6.14	.	–
Panama	2.93	2.96	4.03	6.25	.

*Shaded boxes indicate admixed regions for *A. callidryas*. CR, Costa Rica; PA, Panama.

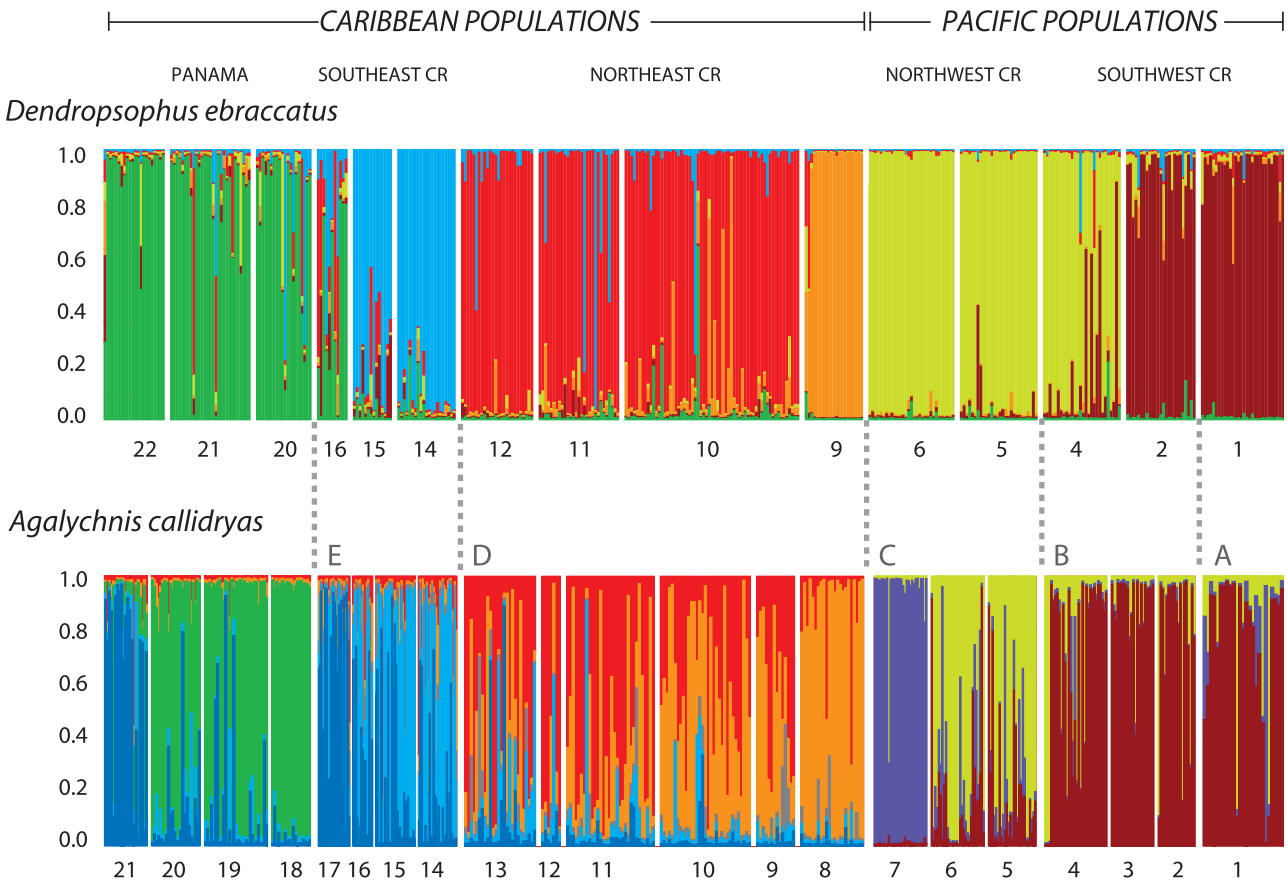

Fig. 4 Bayesian assignment probabilities of *Dendropsophus ebraccatus* (above) and *Agalychnis callidryas* populations (below) inferred in the program Structure (Pritchard *et al*. 2000). For *D. ebraccatus*, analyses revealed two Pacific demes and four Caribbean demes with limited admixture among neighbouring biogeographic regions. *A. callidryas* populations were comprised of eight genetic demes (three Pacific and five Caribbean demes) with significant admixture among neighbouring demes. Each vertical line corresponds to a single individual and colours represent the proportional membership coefficient of that individual to each of six genetic clusters. Dashed grey lines, labelled A–E, indicate the position of putative geographic barriers that lie between biogeographic regions.

these regions. Structure analyses of Pacific populations identified two genetic demes (Fig. 4) with some interdeme admixture: one deme comprised northwestern CR populations plus one southwestern CR population (population 4) and the other deme contained the two southernmost populations from southwestern CR (Fig. 4). Despite limited admixture among demes, geographic breaks delimiting demes were coincident with only two of five putative geographic barriers (barriers C and D; Fig. 4).

Red-eyed treefrogs also exhibited population genetic structure at microsatellite loci both within and among five regions. The number of alleles per locus ranged from 7 to 53 and averaged 30.3 across all loci (Robertson 2008). Mean heterozygosity was 0.487, ranging from 0.194 to 0.672 at specific loci (Appendix I). Overall, we detected no consistent deviation in HWE or LD among populations. We detected significance in 204 of 210 population pairwise F_{ST} comparisons, underscoring the significant restriction in gene flow among populations, even within regions (range: 0.00–0.019; Appendix III). In contrast to our findings for the hourglass treefrogs, more genetic variation was partitioned among regions (22%) than among populations (8%) in the AMOVA.

In Bayesian assignment analyses, red-eyed treefrog populations were more admixed, relative to the hourglass treefrog (Fig. 4). We identified eight demes: the northernmost Caribbean population (population 8) included individuals almost exclusively assigned to a single deme, whereas neighbouring populations (populations 9 and 10) contained individuals of mixed genetic ancestry (Fig. 4). Southeastern CR and Panamanian populations also exhibited mixed ancestry between two primary demes. Among three Pacific demes, a generalized divide was evident between northwestern and southwestern populations, with significant admixture at the centrally located population. The single population sampled from the Nicoya Peninsula (population 7) was genetically isolated from all other populations. The geographic borders of these admixed demes were coincident with geographic barriers delimiting only one of five regions (barrier C).

Our two focal taxa exhibited a nearly equal number of demes after adjusting for the number of sampled Caribbean populations (red-eyed treefrog = 0.35; hourglass treefrog = 0.40) and Pacific populations (red-eyed treefrog = 0.42; hourglass treefrog = 0.40). However, differences in the relative isolation of populations 9 and 21 and admixture of populations 4 and 5 (Fig. 4) indicate that geographic factors underlying connectivity are unequal between taxa.

Isolation-by-distance: comparison between Caribbean and Pacific populations

We calculated φ'_{ST} to compare patterns of IBD in *D. ebraccatus* and *A. callidryas*. Mantel tests revealed IBD for both species among Caribbean (*D. ebraccatus* $r = 0.331$, $P = 0.026$; *A. callidryas* $r = 0.623$, $P = 0.001$) and Pacific populations (*D. ebraccatus* $r = 0.784$, $P = 0.037$; *A. callidryas* $r = 0.872$, $P = 0.0011$). A scatterplot of genetic differentiation $[\varphi'_{ST}/(1-\varphi'_{ST})]$ against geographic distance showed higher variance in differentiation at larger geographic distances for both species along the Caribbean (Fig. 5). In contrast, geographic distance was a stronger predictor of genetic differentiation for Pacific populations for both species (Fig. 5). For *D. ebraccatus*, our interpretation is tempered by small sample size.

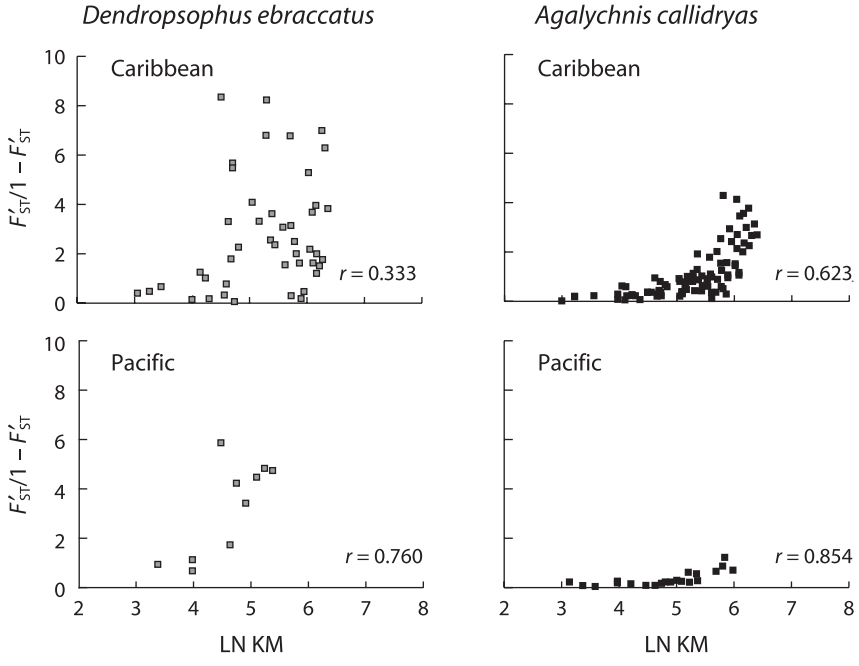

Fig. 5 Genetic isolation by distance (IBD) among *Dendropsophus ebraccatus* (grey squares) and *Agalychnis callidryas* (black squares) populations expressed as the relationship between $(F'_{ST}/1-F'_{ST})$ and natural log of among site distance (km). F'_{ST} represents φ'_{ST}. For each species, we tested for IBD among populations on the Caribbean (top) and Pacific (bottom) versants of Costa Rica and Panama. Correlation coefficients were inferred from Mantel tests and based on 10 000 permutations.

Table 4 Matrix and partial matrix correspondence tests of the associations between genetic and geographic distance for *Dendropsophus ebraccatus* and *Agalychnis callidryas* populations within regions and across biogeographic barriers for mitochondrial and nuclear DNA*

Dendropsophus ebraccatus

Independent Variable	Dependent Variable	WITHIN REGIONS SW	NW	NE	SE	PA
mtDNA	KM	**0.2816 (0.014)**	–0.0556 (0.672)	**0.2819 (0.001)**	**0.9158 (0.001)**	–0.504 (0.639)
ncDNA	KM	0.3538 (0.676)	na	0.3188 (0.298)	0.9732 (0.135)	–0.180 (0.6657)
		BETWEEN REGIONS				
		barrier A	barrier B	barrier C	barrier D	barrier E
mtDNA	KM	**0.8221 (0.001)**	**0.8221 (0.001)**	**0.8245 (0.001)**	**0.789 (0.001)**	**0.9552 (0.001)**
	barrier	0.0387 (0.188)	**0.9668 (0.001)**	0.0110 (0.366)	**0.5451 (0.001)**	**0.9982 (0.001)**
	KM (barrier)		***0.2818 (0.002)***		***0.7049 (0.001)***	*0.069 (0.060)*
	barrier (KM)		***0.9026 (0.001)***		***0.1633 (0.003)***	***0.9789 (0.001)***
ncDNA	KM	0.3538 (0.676)	0.8817 (0.049)	**0.4814 (0.0001)**	0.0852 (0.359)	**0.8696 (0.005)**
	barrier	0.00 (0.79)	0.7471 (0.112)	**0.1922 (0.032)**	0.1729 (0.121)	**0.8351 (0.0293)**
	KM (barrier)			***0.447 (0.0001)***		*0.4417 (0.074)*
	barrier (KM)			*–0.225 (0.5715)*		*0.033 (0.499)*

Agalychnis callidryas

Independent Variable	Dependent Variable	WITHIN REGIONS SW	NW	NE	SE	PA
ncDNA	KM	**0.612 (0.0001)**	**0.996 (0.0001)**	**0.470 (0.0001)**	**0.851 (0.0001)**	**0.778 (0.0001)**
		BETWEEN REGIONS				
ncDNA	KM	**0.724 (0.0001)**	**0.938 (0.0001)**	**0.931 (0.0001)**	**0.940 (0.0001)**	**0.984 (0.001)**
	barrier	**0.598 (0.0001)**	0.08 (0.133)	**0.903 (0.0001)**	**0.866 (0.0001)**	–0.642 (1.00)
	KM (barrier)	***0.597 (0.0001)***		***0.835 (0.0001)***	***0.881 (0.0001)***	
	barrier (KM)	*–0.363 (1.0)*		***0.763 (0.0001)***	*–0.722 (1.000)*	

*five biogeographic barriers (A–E) across biogeographic regions: southwestern CR (SW), northwestern CR (NW), northeastern CR (NE), southeastern CR/PA (SE), and Central Panama (PA). Measures of genetic distance include mitochondrial DNA pairwise patristic distance (mtDNA) and pairwise F_{ST} inferred from microsatellite loci (ncDNA). Geographic distance (km). Partial MCT are indicated in italics. The correlation coefficient and *P* value for MCT and pMCT provided for each test. Significance values were determined by comparing the observed and expected Z-statistic, generated by 10 000 permutations. *P* values that were significant following Bonferroni correction for multiple tests are shown in bold. Tests with an insufficient number of populations (≤ 2) were not conducted (na). Mitochondrial DNA for *Agalychnis callidryas* reported in Robertson (2008).

Matrix correspondence tests: associations of gene flow, phenotype, and geography

We examined the associations among genetic distance, geographic factors, and colour pattern, using both historical genetic distance estimated from mtDNA patristic distances and more recent gene flow estimates (pairwise F'_{ST}) derived from microsatellite genotypes. For *D. ebraccatus*, we found a positive, relationship between historical genetic distance (mtDNA) and geographic distance within three of five regions (southwestern CR, northeastern CR, southeastern CR; Table 4) but no association between geographic distance and gene flow (ncDNA) within any region (Table 4). We detected an effect of three barriers on historical genetic divergence (barriers B, D, E; Table 4). While restrictions in nuclear gene flow were detected across two barriers in MCT (C and E), the partial MCT showed that this variance was largely driven by geographic distance (Table 4). Colour pattern for *D. ebraccatus* at the population level was random with respect to nuclear gene flow patterns ($r = 0.026$, $P = 0.454$) and geographic distance ($r = 0.0009$, $P = 0.47$).

For *A. callidryas*, we detected a positive relationship between mtDNA divergence and geographic distance within all regions (Robertson 2008). Restrictions to mtDNA gene flow were evident across four barriers (barriers A, B, C, E; Table 5). However, nuclear gene flow estimates were significantly associated with only one of these barriers,

Table 5 Comparison of the effectiveness of putative biogeographic barriers in shaping historical divergence (mtDNA) and recent patterns of gene flow (ncDNA) of *Dendropsophus ebraccatus* and *Agalychnis callidryas* populations. Ψ indicates data from Robertson (2008)

	D. ebraccatus		*A. callidryas*		
Barrier	mtDNA	ncDNA	mtDNAΨ	ncDNA	Studies showing population isolation across barrier
A Golfo Dulce			√		(Crawford *et al*. 2007)
B Rio Tárcoles	√		√		(Kohlmann *et al*. 2002)
C Cordillera de Talamanca			√	√	(Demastes *et al*. 1996; Zamudio & Greene 1997; García-París *et al*. 2000; Crawford 2003)
D Caribbean Valley Complex	√				(Kohlmann *et al*. 2002; Hagemann & Pröhl 2007)
E Bocas del Toro	√		√		(Crawford 2003; Crawford *et al*. 2007; Weigt *et al*. 2005; Zeh *et al*. 2003; Hagemann & Pröhl 2007)

barrier C (Robertson 2008; Table 4). We detected an association between flank stripe patterns and nuclear gene flow ($r = 0.668$, $P < 0.001$).

Discussion

Our analyses of genetic and phenotypic differentiation in codistributed species provide abundant evidence that historical mechanisms have resulted in very different geographic patterns of diversification. This discordance between taxa highlights the multiple processes underlying population structure, including landscape features, gene flow, localized selection and drift. We focus on four aspects of diversification. First, examining patterns at a deeper temporal scale, we address species differences in the colonization history of isthmus populations. Next, we evaluate IBD patterns between species. Third, we draw attention to the isolating effects of biogeographic barriers at two temporal scales. Finally, we discuss how different modes of selection acting on colour pattern polymorphisms have most likely shaped the evolution of populations.

Colonization history of the Central American Isthmus

Phylogenetic inference based on mtDNA comparisons revealed both similarities and differences between focal taxa in the colonization history of the Central American Isthmus. For *Dendropsophus ebraccatus*, we detected five, deeply divergent, reciprocally monophyletic regional mtDNA clades with no admixture among lineages (Fig. 3). Our topology showed that the two earliest divergences occurred within Panamanian and southwestern CR populations. Thus, for *D. ebraccatus*, as for other species in this area (Crawford 2003), a simple vicariance model associated with the uplift of the Cordillera de Talamanca does not explain the complex relationship between Caribbean and Pacific populations. For *Agalychnis callidryas*, we detected five regional mtDNA clades with admixture among neighbouring lineages and no regional monophyly. The phylogenetic topology indicated an early divergence of southwestern CR, sister to all other clades (Robertson 2008). Combined, phylogenetic analyses for both species underscore the early isolation of more southerly populations (SW Costa Rica for *A. callidryas*; Central Panama and SW Costa Rica for *D. ebraccatus*). Both taxa showed more recent divergences throughout remaining parts of the range, either due to dispersal, or sequential vicariance of populations in isolated regions. The Cordillera de Talamanca has effectively isolated South Pacific and Caribbean populations, a pattern observed in other frogs with similar geographic distributions (Crawford *et al*. 2007). This is most likely a result of the inhospitable, dry habitat of the Pacific versant in western Panama (Kohlmann *et al*. 2002; Savage 2002; Crawford *et al*. 2007).

Overall, the per cent sequence divergence between clades at ND1 did not differ significantly between taxa (Table 3), indicating a role of natural history and ecology in explaining topological differences between the two focal taxa. However, differences in specific regional pairwise estimates demonstrate that vicariance and historical processes have also likely resulted in unique taxon phylogenetic topologies. For example, the average pairwise sequence divergence between northwest CR and northeast CR was 0.49% and 4.05% for *D. ebraccatus* and *A. callidryas*, respectively. Yet despite the large sequence divergence for *A. callidryas*, we detected admixture among those lineages, but not for *D. ebraccatus* (Table 3). A comparison between northwest and southwest CR revealed divergence estimates of 3.46% (*D. ebraccatus*) and 6.14% (*A. callidryas*), but with admixture among *A. callidryas* lineages, only. Additionally, we detected admixture among neighbouring southeast and northeast CR lineages of *A. callidryas* (but not *D. ebraccatus*) despite similar estimates of sequence divergence (Table 3). Thus, our two taxa differed significantly in their response to major landscape features, both in terms of colonization history of Central America and the degree of regional admixture, particularly among more recently

derived clades. Combined, these analyses indicate that both unique ecological and natural history traits and vicariance/historical scenarios could explain differences in the phylogenetic histories of our focal taxa.

Genetic IBD across lower Central America

The overall magnitude of gene flow differed between *A. callidryas* and *D. ebraccatus* populations. Hourglass treefrog populations exhibited very restricted gene flow, reduced admixture among Caribbean demes (Fig. 4), and no IBD within regions (Table 4). In contrast, red-eyed treefrog populations exhibited higher levels of gene flow and concordantly greater admixture among demes (Robertson 2008). Reduced gene flow among *D. ebraccatus* populations could result from unique habitat or environmental barriers or species-specific traits such as limited dispersal capability, small body size, lower tolerance to desiccation, and higher philopatry. Similar levels of population differentiation were observed for other small-bodied, specialized frogs (Crawford 2003). *Agalychnis callidryas* is significantly larger than *D. ebraccatus* (up to fourfold; Savage 2002), and broader physiological tolerance afforded by larger body size could account, at least in part, for differences in dispersal capacity.

Both hourglass and red-eyed treefrogs exhibited higher genetic diversity and larger variance in gene flow among Caribbean populations, relative to Pacific populations (Figs 4 and 5). The diversification of Caribbean populations could be due to climatic and topographic differences between Pacific and Caribbean versants of the Cordillera de Talamanca (Kohlmann *et al*. 2002; Savage 2002) that impacts population connectivity. We also sampled populations over a broader elevational range in the Caribbean (Table 1). Both elevation and montane ridges act as gene flow barriers in other frogs (Funk *et al*. 2005a; Funk *et al*. 2005b). Therefore, a combination of landscape and altitudinal complexity could explain broad-scale differences in population structure (Fig. 4).

Biogeographic barriers vary between species and across temporal scales

Combined analyses derived from mitochondrial and nuclear markers permit quantification of gene flow at both deeper and more recent timescales (Zeisset & Beebee 2008). At deeper timescales, two biogeographic barriers (B and E) effectively isolated populations for both species. Southwestern CR, delimited by barrier B, is characterized by exceptional endemism due to early isolation from northwestern CR and Caribbean lowland forests following the uplift of the Cordillera de Talamanca (Holdridge 1947; Savage 2002), as well as isolation of the Osa Peninsula, an offshore island that drifted into the mainland of Costa Rica approximately 2 million years ago (Kohlmann *et al*. 2002). Similar genetic isolation of Osa Peninsula populations has been documented in frogs (Crawford 2003; Crawford *et al*. 2007; Robertson 2008) and snakes (Zamudio & Greene 1997) suggesting that Osa Peninsula populations have retained a signature of isolation, despite reconnection to the mainland. Our data demonstrate that this vicariance resulted in intraspecific genetic divergence early in the history of both species. The second common barrier (E), located within the Caribbean region of Bocas del Toro (Figs 1 and 2), is a significant biogeographic feature for other animal and plant taxa, including frogs (Crawford 2003; Weigt *et al*. 2005; Crawford *et al*. 2007; Hagemann & Prohl 2007). This region has a complex history, marked by habitat instability and repeated isolation and formation of gene flow corridors over the last 5 million years (Coates & Obando 1996; Kohlmann *et al*. 2002). However, despite signatures of historical isolation across barriers B and E, microsatellite data revealed that gene flow was not impeded by either barrier (Table 4, Fig. 5). The discordance between mtDNA and ncDNA gene flow estimates could reflect population connectivity on recent timescales, and/or other factors related to differences in the inheritance and mutation rate of these two molecular markers, including incomplete lineage sorting, differences in effective population sizes, and male-biased dispersal (Avise *et al*. 1987; Avise 1989).

The isolating effects of the three other barriers (A, C, D) were unequal for the two taxa. The Cordillera de Talamanca (barrier C) delimits Caribbean and Pacific populations and is known to structure the distribution of many terrestrial organisms in Costa Rica (Table 5). Yet, surprisingly, for *D. ebraccatus*, MCT analyses did not reveal an isolating effect of this barrier. In contrast, for *A. callidryas*, estimates of genetic divergence from mitochondrial and nuclear markers showed the highest degree of isolation across barrier C. This barrier was also associated with high phenotypic divergence in *A. callidryas* coloration (Robertson 2008) and flank-stripe pattern (this study), providing substantial evidence that colour pattern diversification is driven by genetic isolation. It is highly unlikely that individual *D. ebraccatus* are migrating across the northern edge of the Cordillera de Talamanca. Rather, the relative distribution of genetic diversity among populations relative to regions (based on AMOVA) may explain our inability to detect isolation across this barrier in hourglass treefrogs. We detected higher variation among populations (13%) than among regions (11%), indicating that genetic variance is partitioned across the landscape at small spatial scales, reducing the measurable effect of large-scale barriers. In contrast, most of the genetic variation in *A. callidryas* could be explained among regions, rather than among populations, thus positively biasing correlation tests such as MCTs to identify barriers that fall along these regional breaks. This discordance underscores the importance of sampling

scale in studies of population genetic differentiation (Irwin 2002) and the utility of comparing markers with different spatial resolution.

Historical genetic isolation was detected across the Caribbean Valley Complex (barrier D), but for *D. ebraccatus* only. This region is characterized by two valleys at the foothills of the Cordillera de Talamanca (Valle de Talamanca and Valle de Estrella) and coincident with geographic range limits of several plant and animal taxa (Kohlmann *et al*. 2002) and has recently been shown to delimit colour morphs and mtDNA lineages of the strawberry poison frog, *Dendrobates pumilio* (Hagemann & Prohl 2007). Despite historical limitations to migration, our estimates of nuclear gene flow in *Dendropsophus ebraccatus* revealed no obvious restrictions in gene flow due to this landscape feature (Fig. 4), nor did colour pattern vary across this barrier. For *A. callidryas*, gene flow was unimpeded by barrier D at both temporal scales.

Barrier A lies within the Golfo Dulce region of Costa Rica, a region known for disjunct plant and animal distribution patterns (Savage 2002; Crawford *et al*. 2007). This barrier did not impose a significant barrier to gene flow for *D. ebraccatus* at either temporal scale (Table 5; Fig. 4), but did significantly impact historical genetic differentiation for *A. callidryas* (Robertson 2008).

Modes of selection on colour pattern and the evolution of populations

Our previous phylogenetic analyses indicated that mechanisms for colour pattern diversification differ between our focal taxa: flank and leg coloration in *A. callidryas* co-varied with phylogenetic history (Robertson 2008) but dorsal pattern in *D. ebraccatus* did not (Ohmer *et al*. in press). To further examine these mechanisms, we used estimates of recent gene flow with colour pattern variation in this study. Our data clearly showed that dorsal colour pattern of *D. ebraccatus* varied randomly with respect to gene flow and geographic distance. In contrast, flank stripe pattern of *A. callidryas* was associated with patterns of gene flow among populations. Thus, independent of the adaptive significance of colour pattern or the strength of selection acting in each species, we showed that gene flow and geographic factors play a more important overall role in structuring phenotype for red-eyed treefrog populations than hourglass treefrog populations.

Adaptive explanations for the evolution of colour pattern must consider the mode of selection as well as the strength of local selection, gene flow and drift (Endler 1980, 1983; King & Lawson 1995; Hoffman & Blouin 2000; Hoffman *et al*. 2006; Gray & McKinnon 2007). Although all *D. ebraccatus* morphs occurred in all five regions, the frequency distribution of colour patterns varied among most populations. Combined with reduced gene flow among populations, our findings underscore the role of within-population processes for the maintenance of polymorphism. Mechanisms such as frequency-dependent or directional selection, non-assortative mating, or natural selection underlie within-population polymorphisms in many anurans (Hoffman & Blouin 2000). For example, substantial discordance between mtDNA and 'display' colour pattern of the dendrobatid frog, *Dendrobates tinctorius*, indicated a role of selection in colour pattern differentiation among populations (Wollenberg *et al*. 2008). Dorsal blotches and spots are a common form of disruptive coloration in frogs, making individuals cryptic against a heterogeneous background (Cott 1940; Endler *et al*. 2005) and have a presumed cryptic function in hourglass treefrogs (Duellman 2001). Predator pressures could underlie the maintenance of polymorphisms if visual predators more readily identify common morphs as potential prey items, thus allowing rarer forms to persist (Cott 1940). However, the apparent loss of one colour morph in three disjunct populations across the study region implicates genetic drift as an additional factor (Fig. 1b). The large sample size ($n = 50$) for population 10 (Fig. 1) corroborates that these losses were due to stochastic population-level processes rather than insufficient sampling. Combined, the discordant distribution of genetic and phenotypic diversity in *Dendropsophus ebraccatus* suggests that stochastic and frequency-dependent processes most likely interact to maintain colour pattern polymorphisms. We do not fully understand mechanisms of mate choice in this species, nor are there sufficient microhabitat data to measure co-variation of environmental features. Therefore, while we acknowledge the potential for directional selection in determining population differences in morph frequency, we lack sufficient data to examine this hypothesis. The hourglass treefrog, with its variable colour pattern and strong population genetic structuring, is an ideal candidate for future behavioural studies on the adaptive significance of colour pattern within populations.

In contrast, our analyses of *A. callidryas* indicate that genetic isolation (albeit incomplete) among regions has played a larger role in shaping the distribution of phenotypic differentiation. Flank stripe pattern was associated with patterns of gene flow and geographic barriers. The sharpest distinction in flank pattern occurred between Caribbean and Pacific populations (barrier C), corroborating the strong barrier to gene flow observed on both historical and recent timescales. Pacific populations of *A. callidryas* were nearly monomorphic for flank pattern C, concordant higher levels of gene flow among these populations (Robertson 2008). In contrast, we observed overall higher flank pattern diversity, as well changes in flank stripe distribution with geographic distance along Caribbean populations. Higher phenotypic diversity among Caribbean populations is also consistent with observed variation in

flank and leg coloration from prior studies (Robertson & Robertson 2008; Robertson 2008).

Evolved colour pattern differences among Caribbean and Pacific populations of *A. callidryas* may also be reinforced by sexual selection (Summers *et al*. 2003; Siddiqi *et al*. 2004; Boul *et al*. 2007; Rudh *et al*. 2007). *Agalychnis callidryas* possesses the visual pigments to detect and discriminate flank pattern, as well as the full range of hues exhibited across its range (Robertson 2008), a prerequisite for colour pattern evolution through sexual selection. We lack direct evidence of sexual selection on colour pattern in this species, but the combined evidence favours the hypothesis that flank stripes, in combination with flank and leg coloration, contributes to species recognition and mate choice in *A. callidryas* (Robertson 2008). Future behavioural studies of choice and assortative mating will help define the relative importance of sexual selection and migration–drift equilibrium for the distribution of colour and pattern in this species.

Our comparative study revealed differences in the biogeographic history, population genetic structure, and dispersal biology of *A. callidryas* and *D. ebraccatus* populations inhabiting five biogeographic regions. We found that different processes underlie spatial patterns in coloration among populations and that both historical contingencies and current selective pressures contribute to the maintenance of phenotypic diversity. More generally, our study shows that species-typical characteristics can alter the direction and magnitude of genetic and phenotypic divergence, resulting in different evolutionary outcomes even for codistributed taxa that experience common histories.

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This research forms part of J. Robertson's PhD thesis on geographic variation and the evolutionary mechanisms that underlie genetic and phenotypic diversity in Neotropical frogs. M. C. Duryea is a graduate student at Dartmouth College where she is exploring questions related to genetic and behavioural mechanisms that generate diversity. Research in the Zamudio laboratory focuses on population differentiation and mating system evolution in reptiles and amphibians. We are particularly interested in the distribution of population genetic variation in complex landscapes.

Appendix I

Polymorphic di- and tetranucleotide microsatellite loci characterized for *Dendropsophus ebraccatus* and *Agalychnis callidryas**

M	Locus	Primer sequences 5′– 3′	F 5′ label	Repeat structures	T_a (°C)	A	bp	H_O	H_E	F_{IT}	F_{IS}
Dendropsophus ebraccatus											
1	HEB 165	F: GTGGGTAGCCATGTATTCAGAGAT	NED	$(GT)_8AT(GT)_4G(GT)_3$	55.6	48	114–392	0.463	0.815	0.410	0.293
		R: ACACCCCAACACCGCTCACA									
1	HEB 231	F: GCACTGCCCGGGAATAAAG	6-FAM	$(TG)_{32}$	58.6	51	134–286	0.727	0.944	0.343	0.285
		R: AATGAGGAGAGGGTTGGGGAAAAA									
1	HEB 310	F: TCCCCTGCATAAAGTAAGAGTGAG	PET	$(TG)_{15}(N)_8(TG)_6(N)_8(TG)_6$	55.0	30	201–299	0.539	0.868	0.347	0.292
		R: ACCCCTCTGTCCCCTTCAGAC									
2	HEB 161	F: TCACATGACGTCCRGAGCCAATC	PET	$(TG)_{12}(N)_{25}(TG)_6$	67.6	24	207–257	0.550	0.900	0.414	0.313
		R: CAGCCACCCATGAGCACTAA									
2	HEB 337	F: GCACTGCTACGCATATACATGTG	NED	$(CA)_{14}$	55.0	27	143–213	0.734	0.921	0.172	0.059
		R: GAGTGCTGGGTTCTTTCTATGC									
2	HEB 226	F: TGGGATGGTCACGTTTTGA	6-FAM	$(TG)_{23}$	55.0	30	217–305	0.604	0.937	0.449	0.407
		R: ATTCGCACACTTATTTGTGAAAAT									
	Mean				—	35	—	0.603	0.898	0.353	0.241
Agalychnis callidryas											
1	ACA126	F: GGGCCCCTGAAATGT	NED	$(TG)_{16}$	64–56	53	105–305	0.518	0.948	0.435	0.384
		R: TACACAAAGCATACATAGATACAA									
1	ACA36	F: CCACCCCTGCTAAAACACTACATCCTA	6-FAM	$(TG)_{12}$	64–56	10	384–402	0.372	0.526	0.303	0.163
		R: CCACCTTGCACCACAGACTATCCA									
1	ACA7	F: AATAAAGTGGCAGAACCGTGATC	PET	$(TG)_{16}$	64–56	32	268–348	0.623	0.923	0.338	0.194
		R: TGTCTCTGCTGGCACTTGTTG									
2	ACA148	F: CGGGAGGTTTCGCCCACCCTTCT	PET	$(TG)_4(N)_{23}(TG)_2(N)_{11}(TG)_3$	64–56	7	224–244	0.194	0.671	0.760	0.141
		R: TCTTTATCCCCACTCTACTCCCATACGCACACT		$(N)_{10}(TG)_3(N)_{24}(TG)_4$							
2	ACA127	F: ACCGGTGCACCCCTTCCTA	VIC	$(TGTC)_{13}$	65–54	31	172–260	0.672	0.912	0.260	0.197
		R: CCGGCTCCTGCAAAAACTT									
2	ACA29	F: GTCAATTACAGGCCTCTTATCTTTTTA	PET	$(TG)_{26}$	65–54	49	100–216	0.543	0.963	0.440	0.375
		R: GATTCGCTTTCTCATTTTGTCCCTCATA									
	Mean				—	30	—	0.487	0.824	0.417	0.270

*annealing temperature [T_a (°C)] used in PCRs. The number of alleles (A), product range size (bp), proportion of observed (H_O) and expected (H_E) heterozygosities, are listed for each locus averaged across all populations sampled. Estimates of Weir and Cockerham F_{IT} and F_{IS} were calculated in FSTAT.

Appendix II

Pairwise F_{ST} estimates based on mtDNA (below diagonal) and six microsatellite loci (above diagonal) for 15 populations of *Dendropsophus ebraccatus**

		Southwest CR			Northwest CR		Northeast CR				Southeast CR			Panama		
		1	2	4	5	6	9	10	11	12	14	15	16	20	21	22
Southwest CR	1		**0.13**	**0.19**	**0.20**	**0.19**	**0.31**	**0.18**	**0.17**	**0.21**	**0.21**	**0.16**	**0.18**	**0.25**	**0.21**	**0.14**
	2	0.21		**0.18**	**0.20**	**0.20**	**0.34**	**0.20**	**0.20**	**0.23**	**0.24**	**0.19**	**0.22**	**0.27**	**0.22**	**0.18**
	4	0.45	**0.5**		0.22	0.20	0.31	0.18	0.18	0.22	0.13	0.10	0.12	0.26	**0.21**	0.13
Northwest CR	5	**0.80**	**0.82**	**0.51**		**0.08**	**0.25**	**0.08**	**0.07**	**0.10**	**0.20**	**0.16**	**0.17**	**0.18**	**0.14**	**0.13**
	6	**0.90**	**0.92**	0.46	0.33		**0.21**	**0.07**	**0.06**	**0.11**	**0.17**	**0.12**	**0.15**	0.07	**0.04**	**0.07**
Northeast CR	9	**0.26**	**0.30**	0.42	**0.07**	0		**0.13**	**0.16**	**0.24**	**0.30**	**0.24**	**0.29**	**0.29**	**0.24**	**0.21**
	10	0.03	0.07	**0.20**	–0.01	–0.06	–0.1		**0.01**	**0.08**	**0.16**	**0.13**	**0.14**	**0.13**	**0.10**	**0.09**
	11	0.03	**0.07**	0.20	–0.01	–0.07	–0.1	–0.2		**0.05**	**0.16**	**0.12**	**0.14**	**0.14**	**0.10**	**0.08**
	12	**0.92**	**0.93**	**0.51**	**0.62**	**0.96**	**0.21**	0.04	0.03		**0.22**	**0.18**	**0.20**	**0.19**	**0.15**	**0.14**
Southeast CR/PA	14	**0.89**	**0.9**	**0.51**	**0.81**	**0.97**	**0.24**	0.04	0.04	**0.97**		**0.07**	–0.01	**0.20**	**0.14**	**0.06**
	15	**0.83**	**0.84**	**0.51**	**0.75**	**0.88**	**0.22**	0.04	0.04	**0.92**			**0.05**	**0.19**	**0.14**	**0.02**
	16	**0.90**	**0.91**	**0.51**	**0.84**	**0.98**	**0.28**	**0.06**	**0.05**	**0.97**	**0.84**	**0.63**		**0.19**	**0.13**	**0.04**
Panama	20	**0.95**	**0.96**	**0.52**	**0.93**	**1.00**	**0.50**	**0.18**	**0.18**	**0.99**	**0.99**	**0.96**	**0.99**		**0.02**	**0.09**
	21	**0.95**	**0.96**	**0.52**	**0.93**	**1.00**	**0.50**	**0.18**	**0.18**	**0.99**	**0.99**	**0.96**	**0.99**	0.13		**0.07**
	22	**0.93**	**0.94**	**0.52**	**0.92**	**0.98**	**0.50**	**0.18**	**0.18**	**0.97**	**0.97**	**0.94**	**0.97**	–0.03	0.02	

*F_{ST} significance corrected for multiple comparisons at $\alpha = 0.05$ in bold. Negative F_{ST} values are not different from zero. Population numbers correspond to Fig. 1. Shaded boxes indicate within region population sampling.

Appendix III

Pairwise F_{ST} estimates based on mtDNA (below diagonal) and six microsatellite loci (above diagonal) for 21 populations of *Agalychnis callidryas**

		Southwest CR				Northwest CR			Northeast CR						Southeast CR				Panama			
		1	2	3	4	5	6	7	8	9	10	11	13	12	14	15	17	16	18	19	20	21
Southwest CR	1		**0.06**	**0.10**	0.04	**0.08**	**0.09**	**0.23**	**0.32**	**0.33**	**0.29**	**0.28**	0.28	**0.26**	**0.25**	**0.25**	**0.28**	**0.30**	**0.41**	**0.34**	**0.34**	**0.30**
	2	0.09		**0.08**	0.02	**0.08**	**0.09**	**0.26**	**0.30**	**0.32**	**0.26**	**0.27**	0.26	**0.24**	**0.23**	**0.25**	**0.26**	**0.28**	**0.39**	**0.31**	**0.32**	**0.29**
	3	**0.21**	0.27		**0.07**	**0.08**	**0.08**	**0.30**	**0.30**	**0.32**	**0.27**	**0.26**	0.26	**0.25**	**0.23**	**0.24**	**0.26**	**0.28**	**0.39**	**0.32**	**0.33**	**0.28**
	4	**0.86**	**0.84**	0.33		**0.03**	**0.05**	**0.20**	**0.26**	**0.26**	**0.23**	**0.23**	0.20	**0.20**	**0.19**	**0.20**	**0.21**	**0.24**	**0.34**	**0.27**	**0.28**	**0.25**
Northwest CR	5	**0.96**	**0.96**	**0.55**	**0.95**		0.03	**0.19**	**0.29**	**0.29**	**0.26**	**0.25**	**0.24**	**0.23**	**0.20**	**0.22**	**0.24**	**0.26**	**0.36**	**0.30**	**0.31**	**0.27**
	6	**0.97**	**0.97**	**0.47**	**0.96**	–0.07		**0.19**	**0.24**	**0.23**	**0.21**	**0.21**	0.15	**0.17**	**0.15**	**0.16**	**0.16**	**0.20**	**0.29**	**0.22**	**0.24**	**0.18**
	7	**0.91**	**0.92**	**0.47**	**0.90**	**0.13**	0.05		**0.40**	**0.43**	**0.37**	**0.37**	0.40	**0.35**	**0.33**	**0.34**	**0.37**	**0.40**	**0.50**	**0.42**	**0.44**	**0.41**
Northeast CR	8	**0.97**	**0.96**	**0.57**	**0.95**	**0.90**	**0.92**	**0.78**		**0.13**	**0.04**	**0.05**	**0.07**	**0.06**	**0.12**	**0.12**	**0.14**	**0.16**	**0.24**	**0.22**	**0.22**	**0.21**
	9	**0.97**	**0.97**	**0.53**	**0.96**	**0.90**	**0.93**	**0.76**	**0.42**		**0.06**	**0.07**	**0.09**	**0.09**	**0.16**	**0.14**	**0.16**	**0.21**	**0.28**	**0.25**	**0.28**	**0.25**
	10	**0.96**	**0.96**	**0.62**	**0.95**	**0.90**	**0.91**	**0.80**	0.08	**0.25**		**0.02**	**0.05**	**0.05**	**0.11**	**0.10**	**0.12**	**0.15**	**0.23**	**0.22**	**0.21**	**0.20**
	11	**0.97**	**0.96**	**0.57**	**0.95**	**0.90**	**0.92**	**0.78**	0.13	**0.37**	–0.04		**0.04**	**0.04**	**0.12**	**0.10**	**0.12**	**0.16**	**0.27**	**0.23**	**0.23**	**0.21**
	13													–0.01	0.05	0.04	0.06	**0.11**	0.24	0.15	0.16	0.13
	12	0.98	0.97	0.33	0.96	0.89	0.93	0.69	–0.01	0.33	–0.26	0.21			**0.06**	**0.06**	**0.07**	**0.10**	**0.19**	**0.15**	**0.16**	**0.14**
Southeast CR	14	**0.93**	**0.94**	**0.44**	**0.90**	**0.84**	**0.82**	**0.66**	0.24	0.19	**0.24**	**0.61**		–0.58		0.00	0.01	**0.04**	**0.12**	**0.08**	**0.10**	**0.06**
	15	**0.93**	**0.93**	**0.56**	**0.91**	**0.85**	**0.84**	**0.73**	**0.09**	**0.15**	0.03	0.85		0.39	–0.05		**0.01**	**0.06**	**0.18**	**0.12**	**0.13**	**0.09**
	17	**0.92**	**0.93**	**0.52**	**0.90**	**0.84**	**0.82**	**0.70**	**0.62**	**0.59**	**0.64**	**0.52**		0.95	0.17	**0.45**		0.04	**0.15**	**0.09**	**0.11**	**0.07**
	16	**0.98**	**0.97**	**0.46**	0.97	**0.90**	0.94	0.75	**0.85**	0.88	**0.83**	**0.87**		0.18	0.37	**0.62**	–0.25		**0.12**	**0.10**	**0.08**	**0.03**
Panama	18	**0.87**	**0.89**	**0.47**	**0.85**	**0.77**	**0.72**	**0.62**	**0.53**	**0.48**	**0.57**	**0.90**		0.87	0.16	**0.41**	0.07	–0.05		**0.14**	**0.16**	**0.14**
	19	**0.96**	**0.96**	**0.57**	**0.95**	**0.90**	**0.91**	**0.80**	**0.88**	**0.88**	**0.87**	**0.86**		0.93	**0.77**	**0.79**	**0.78**	**0.89**	**0.56**		**0.08**	**0.09**
	20	**0.98**	**0.97**	**0.52**	**0.97**	**0.90**	**0.94**	**0.79**	**0.90**	**0.91**	**0.88**	0.21		0.83	**0.76**	**0.79**	**0.77**	**0.94**	**0.51**	0.01		**0.06**
	21	**0.96**	**0.96**	**0.55**	**0.95**	**0.89**	**0.90**	**0.78**	**0.86**	**0.86**	**0.85**	0.01		–0.63	**0.74**	**0.74**	**0.76**	**0.87**	0.53	**0.07**	0.06	

*F_{ST} significance corrected for multiple comparisons at $\alpha = 0.05$ in bold. Negative F_{ST} values are not different from zero. Population numbers correspond to Fig. 2. Population 13 not sampled at mtDNA. Shaded boxes indicate within region population-sampling.