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Genetic isolation by distance underlies colour pattern divergence in red-eyed treefrogs (*Agalychnis callidryas*)

Meaghan I. Clark[1,2,3] | Gideon S. Bradburd[2] | Maria Akopyan[1,4] | Andres Vega[5] | Erica Bree Rosenblum[6,7] | Jeanne M. Robertson[1,8]

[1]Department of Biology, California State University Northridge, Northridge, California, USA

[2]Department of Integrative Biology, Ecology, Evolution and Behavior Program, Michigan State University, East Lansing, Michigan, USA

[3]W.K. Kellogg Biological Station, Michigan State University, Hickory Corners, Michigan, USA

[4]Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, New York, USA

[5]AMBICOR, Tibas, Costa Rica

[6]Department of Environmental Science, Policy, and Management, University of California Berkeley, Berkeley, California, USA

[7]Museum of Vertebrate Zoology, University of California Berkeley, Berkeley, California, USA

[8]Department of Herpetology, Natural History Museum of Los Angeles County, Los Angeles, California, USA

Correspondence
Meaghan I. Clark, Department of Integrative Biology, Ecology, Evolution and Behavior Program, Michigan State University, East Lansing, MI, USA.
Email: clarkm89@msu.edu

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Abstract

Investigating the spatial distribution of genetic and phenotypic variation can provide insights into the evolutionary processes that shape diversity in natural systems. We characterized patterns of genetic and phenotypic diversity to learn about drivers of colour-pattern diversification in red-eyed treefrogs (*Agalychnis callidryas*) in Costa Rica. Along the Pacific coast, red-eyed treefrogs have conspicuous leg colour patterning that transitions from orange in the north to purple in the south. We measured phenotypic variation of frogs, with increased sampling at sites where the orange-to-purple transition occurs. At the transition zone, we discovered the co-occurrence of multiple colour-pattern morphs. To explore possible causes of this variation, we generated a single nucleotide polymorphism data set to analyse population genetic structure, measure genetic diversity and infer the processes that mediate genotype–phenotype dynamics. We investigated how patterns of genetic relatedness correspond to individual measures of colour pattern along the coast, including testing for the role of hybridization in geographic regions where orange and purple phenotypic groups co-occur. We found no evidence that colour-pattern polymorphism in the transition zone arose through recent hybridization. Instead, a strong pattern of genetic isolation by distance indicates that colour-pattern variation was either retained through other processes such as ancestral colour polymorphisms or ancient secondary contact, or else it was generated by novel mutations. We found that phenotype changes along the Pacific coast more than would be expected based on genetic divergence and geographic distance alone. Combined, our results suggest the possibility of selective pressures acting on colour pattern at a small geographic scale.

KEYWORDS
colour-pattern polymorphism, Costa Rica, hybridization, population genetics

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1 | INTRODUCTION

A central goal of evolutionary biology is to understand how phenotypic variation is generated, distributed and maintained in natural systems (Mayr, 1963; Mitchell-Olds et al., 2007). Colour pattern is a phenotype well suited for evolutionary study because it is easily quantifiable and often has adaptive value (Cott, 1940; Orteu & Jiggins, 2020). Many compelling examples of natural selection in the wild have come from studying colour-pattern variation across diverse empirical systems (e.g., Corl et al., 2010; Hoekstra et al., 2004; Lowry et al., 2012; Maan et al., 2008; Nachman et al., 2003; Pfeifer et al., 2018; Rosenblum et al., 2004; Streisfeld & Kohn, 2005; Supple et al., 2015; Twomey et al., 2015). In animals, colour and colour pattern can play important ecological roles, serving as, for example, a deterrent to predation (crypsis, aposematism), as a signal to conspecifics (mate choice, territoriality) or both (Cummings & Crothers, 2013; Rojas, 2016; Selz et al., 2016; Stevens & Merilaita, 2009). Accordingly, colour and colour pattern are often shaped by selection, and variation in colour traits can provide insight into the evolutionary dynamics that shape diversity. When present, colour variation within a species can be distributed at a regional level with little variation within sites (polytypic), found at a single locality (polymorphic) or both (e.g., Wang & Summers, 2010). The distribution of colour variation within species can provide particular insight into the spatial scale over which evolutionary dynamics act (Svensson, 2017).

A variety of evolutionary scenarios could explain phenotypic polytypism, polymorphism, and uniformity. For example, polytypic variation can arise and persist due to genetic drift, limited gene flow among populations due to environmental, geographic or reproductive barriers, or selection against maladapted migrants (Endler, 1973; Lehtonen et al., 2009; Rosenblum et al., 2004, 2010). Polymorphism often occurs at transition zones between distinct morphs (Planes & Doherty, 1997) and can arise through novel mutations, contemporary gene flow, the retention of ancestral polymorphism and/or ancient secondary contact (Lim et al., 2010; Roland et al., 2017). In such transition zones, hybridization could increase phenotypic variation within a population via the co-occurrence of distinct phenotypes unique to each parental population, or the generation of novel phenotypes in hybrids (Akopyan et al., 2020b; Anderson & Stebbins, 1954). Uniformity in colour pattern is expected when there is substantial gene flow among morphs, or when colour is under stabilizing selection (Duftner et al., 2006; Slatkin, 1985). Studying polytypism and polymorphism requires both georeferenced colour-pattern data to quantify spatial patterns of phenotypic variation, and genotypes for those sampled individuals to understand the evolutionary processes generating those patterns of variation.

The striking colour-pattern variation of red-eyed treefrogs, *Agalychnis callidryas* Cope 1862, provides an excellent system in which to study the mechanisms of extreme phenotypic variability. Red-eyed treefrogs exhibit colour variation across their range from central Mexico to northern Colombia (Campbell, 1999; Savage & Heyer, 1967), with hypervariability in flank and leg colour-pattern across populations in Costa Rica and Panama (Robertson & Robertson, 2008; Robertson & Zamudio, 2009). Flank and leg colour are not sexually dimorphic, do not change within individuals in response to light intensity (Schliwa & Euteneuer, 1983), and are known to be heritable based on breeding studies that found intrapopulation crosses produce offspring with parental phenotypes (J. M. Robertson, unpublished data). Four distinct flank and leg colour morphs exist in Costa Rica and Panama: blue, red/blue, orange, and purple (Robertson & Robertson, 2008). Across this part of the range, red-eyed treefrogs are polytypic, but generally not polymorphic (Robertson & Robertson, 2008; Robertson & Vega, 2011), except in a contact zone on the Caribbean slope of Costa Rica (Akopyan et al., 2020b). Flank and leg colour-pattern probably evolve through both sexual selection due to preference for local morphs during mate choice (Akopyan et al., 2018; Jacobs et al., 2016; Kaiser et al., 2018) and natural selection for colour pattern to potentially act as a warning signal of unpalatability to predators (Clark, 2019; Davis et al., 2016; Robertson & Greene, 2017). Thus, the evolutionary dynamics and impacts of selection have probably shaped the polytypic and polymorphic distributions of colour pattern in red-eyed treefrogs.

We focused on populations of red-eyed treefrogs from mainland and peninsular populations of the Pacific slope of Costa Rica, where colour variation is characterized by a north–south gradient. In the north, frogs generally have orange flanks and legs (orange morph), while in the south, they display purple flanks and legs (purple morph, Figure 1). Recent field sampling efforts identified polymorphic sites where colour pattern transitions from the orange to the purple morph (Central 2, Figure 1). Previous research revealed a complex relationship between geography, colour pattern, and mitochondrial and nuclear markers (Robertson et al., 2009). Within the Pacific slope, northern and southern sites do not share mitochondrial haplotypes, except at a single centrally located site (Robertson & Zamudio, 2009; Figure 1, Site 4; Table 1). We aim to understand the evolutionary processes underlying both polytypic and polymorphic colour-pattern distributions on the Pacific coast.

In this study, we document the distribution of polytypic and polymorphic populations across the orange–purple colour-pattern gradient and combine those data with a genomic data set generated using restriction-site associated DNA sequencing (RAD-seq) to understand the evolutionary mechanisms that generate and maintain patterns of diversity. We expand sampling from previous studies to examine genomic ancestry and phenotypic patterns across and within orange, purple and polymorphic sites. We first address two hypotheses regarding polytypic patterns across the entire study area: (1A) purple and orange morphs represent distinct genetic demes, consistent with selection for reproductive isolation among these morphs (Akopyan et al., 2018), or alternatively, (1B) samples along the coast are characterized by a pattern of genetic isolation by distance (IBD) that is not correlated with phenotypic variation, which could indicate local selection acting to maintain colour morphs (here we refer to IBD as a pattern of increasing genetic divergence with geographic distance). We investigate the relationships between phenotype and geographic distance, latitude, and genetic diversity using Mantel tests and Bayesian models.

Focusing on the transition zone, we then combine analyses of colour pattern, genetic variation, and relatedness to investigate the phenotypic and genomic profile of polymorphic sites. First, we characterize the distribution of colour phenotypes at polymorphic sites using principal components analysis (PCA) and discriminate function analysis to ask if (2A) only orange and purple morphs are present, or (2B) unique colour phenotypes are present alongside orange and purple morphs. We then use genomic clustering analyses to examine the genomic makeup of phenotypically polymorphic populations compared to other (nonpolymorphic) populations. (3A) If there is little or no gene flow between orange and purple morphs, clustering analyses will show distinct parental groups at polymorphic sites. (3B) Alternatively, if there is gene flow between morphs, clustering analyses will show individuals at polymorphic sites assigned to the same genetic cluster, despite distinct phenotypes. Next, we use the program ENTROPY (Gompert et al., 2014) to determine if (4A) hybridization underlies polymorphism, or if (4B) polymorphism has been generated through another mechanism (i.e., retained from an ancestral population or ancient secondary contact, and/or novel morphs have been generated by new mutations). By integrating analyses of variation at both large (transect from north to south, including polytypic sites and transition zone) and small spatial scales (polymorphic sites in transition zone), we can test hypotheses about how colour pattern evolves at microgeographic scales.

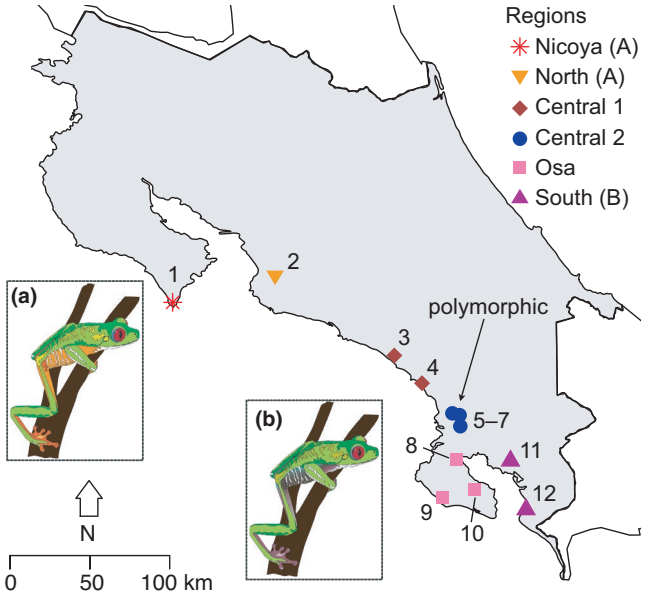

FIGURE 1 Map indicating the 12 sampling sites for red-eyed treefrogs (*Agalychnis callidryas*) along the Pacific coast of Costa Rica. Each site is assigned to one of six biogeographic regions: Nicoya, North, Central 1, Central 2, Osa and South. Arrow indicates polymorphic sites. Insets A and B: red-eyed treefrog colour morphs, drawn from photographs, illustrating colour patterns typical of northern orange (A, Nicoya, North) and southern purple (B, South) regions (illustrations: Cynthia J. Hitchcock)

2 | METHODS

2.1 | Field sampling and study sites

Our study focused on 12 sites along the Pacific coast of Costa Rica, west of the continental divide (Figure 1). Pacific red-eyed treefrogs are isolated from Caribbean populations by the Cordillera de Talamanca (Robertson & Zamudio, 2009). The Pacific slope is geographically and environmentally complex: two peninsulas, the Nicoya and Osa, are potentially isolated from mainland sites through geographic barriers and inhospitable habitat (Robertson et al., 2009). Previous studies characterized red-eyed treefrogs at

TABLE 1 Location, geographic coordinates, sample size, mtDNA clade (Robertson & Zamudio, 2009) genetic diversity, and number of private alleles for 12 sampling sites of red-eyed treefrogs (*Agalychnis callidryas*) along the Pacific coast of Costa Rica

No.	Site	Province	Region	Latitude (N)	Longitude (W)	*n*	mtDNA clade	π_p	θ_w	Private alleles
1	Cabo Blanco	Guanacaste	Nicoya	9.5805	−85.1246	9	B	0.088	0.071	907
2	Bijagual	San Jose	North	9.7256	−84.5313	9	B	0.154	0.131	213
3	Firestone	Puntarenas	Central 1	9.274927	−83.8589	3	--	0.167	0.126	0
4	Uvita	Puntarenas	Central 1	9.1235	−83.7011	5	A, B	0.165	0.149	3
5	Cortes	Puntarenas	Central 2	8.95517	−83.52177	12	--	0.169	0.152	66
6	Palmar Sur	Puntarenas	Central 2	8.94488	−83.48444	20	--	0.168	0.144	104
7	Sierpe	Puntarenas	Central 2	8.8892	−83.477	17	A	0.174	0.163	34
8	El Campo	Puntarenas	Osa	8.6909	−83.5013	17	A	0.163	0.144	83
9	Sirena	Puntarenas	Osa	8.480261	−83.59012	19	--	0.165	0.145	80
10	El Tigre	Puntarenas	Osa	8.52678	−83.40053	19	--	0.165	0.143	52
11	Gamba	Puntarenas	South	8.69333	−83.19522	19	--	0.164	0.143	172
12	Pavones	Puntarenas	South	8.4204	−83.1069	25	A	0.161	0.138	173

Note: mtDNA haplotype was not identified (−−) for some sites. Estimates of nucleotide diversity (π) and Wu and Watterson's theta (θ_w) were based on the RADseq data set. See Figure 1 for region designations.

northern sites (Sites 1–8) as orange and at a southern site as purple (Site 12) (Robertson & Vega, 2011). Here, we expanded previous geographic sampling (Robertson et al., 2009) by adding six sites to assess fine-scale variation in phenotype and genotype, including: the central coast (Site 3), the Osa Peninsula (Sites 9 and 10), mainland adjacent to Osa Peninsula (Sites 5 and 6) and Gulfo Dulce (Site 11). We included 9–25 individuals from previously sampled sites, and 3–20 individuals from new sites (Table 1). We bred frogs from Site 2 with frogs from Site 12 in captivity at California State University, Northridge (IACUC 1819–005) to produce F_1 hybrids to serve as controls in ancestry analyses (see genomic analyses below). Unfortunately, these frogs did not survive until adulthood, so we were unable to assess their phenotype.

We grouped sites together into six regions based on potential geographic barriers and divisions between known phenotypic and genetic groups (Figure 1). The Nicoya Peninsula (Site 1) is geographically isolated from all mainland populations by dry forest, which is an unsuitable habitat for red-eyed treefrogs. The Central 1 region (Sites 3 and 4) is separated from the North (Site 2) based on distinct mitochondrial DNA (mtDNA) haplotypes (Robertson & Zamudio, 2009). The Central 2 region (Sites 5–7) contains both orange and purple frogs whereas frogs in the South (Sites 11 and 12) only have purple legs. The Terraba and Sierpe Rivers separate the Osa Peninsula (Sites 8–10) from the mainland (Kohlmann et al., 2002; Robertson & Vega, 2011).

At each site, we captured frogs by hand and took digital photographs with a black–white–grey standard (QPcard 101 or 102, Adorama Camera Inc.) of the posterior aspect of their legs (Table S1; for samples collected before 2015, see Robertson & Robertson, 2008, Robertson & Vega, 2011; for samples collected in 2015, Akopyan et al., 2020b). We obtained a toe clip for genomic analyses, which was stored in 100% ethanol. Frogs were released within 1 day at the site of capture. We also took toe clips from eight laboratory-bred F_1 hybrids.

2.2 | Quantifying colour pattern

Photos were colour corrected in PHOTOSHOP CC version 18.0.0 (Adobe) using the QPcard 101 and QP102 grey standard (same subset of standards in both cards). To quantify colour pattern, we focused on the posterior leg because leg and flank colour-pattern are strongly correlated (Robertson & Robertson, 2008). The coloured region of the frogs' legs can be either uniform in colour or contain a grey/blue patch on the inner leg (Figure S1). In all cases, we first excluded the patch and selected the coloured region of one leg and used the "average" function in PHOTOSHOP to measure hue, saturation and brightness (Adobe). Because orange and purple have similar hue values, the saturation and brightness values were useful to distinguish colour pattern within and among populations (Robertson & Robertson, 2008). We then used IMAGEJ (Abràmoff et al., 2004) to quantify the percentage of pixels on the leg that were covered by the grey patch, which was easily distinguished from the dominant portion of the leg using saturation values.

We used PCA in R version 3.5.3 (R Core Team 2016) based on hue, saturation, brightness and percentage grey of the hind legs to visualize the distribution of colour-pattern variation among red-eyed treefrog populations within and among regions. Because it is likely that different loci underly colour and pattern in red-eyed treefrogs, we also used PCA to visualize the distribution of colour measurements separate from pattern. Photographs were taken with different cameras (Canon PowerShot SX160 and Nikon Coolpix 5700), but we do not see evidence of batch effects corresponding to camera type (Figures S2 and S3). We conducted a linear discriminant function analysis (DFA) using the "lda" function in the MASS version 7.3-51.4 package in R version 3.5.3. to test whether individuals were correctly assigned to their site of origin based on either hue, saturation, brightness, and percentage grey. Individuals were classified to the site with the highest posterior probability. We conducted this analysis with all measurements and each measurement separately.

2.3 | Molecular methods

DNA was extracted from frog toe clips using a DNeasy Blood and Tissue kit (Qiagen) as per the manufacturer's protocol, except as described below. We eluted DNA in 50 μl Buffer AE to increase concentration. After extraction, 4 μl of 1 mg ml^{-1} RNAse A was added to each sample to remove RNA contamination. DNA concentration was measured using a Qubit 2.0 Fluorometer (ThermoFisher). RAD-seq libraries (Ali et al., 2016; Etter et al., 2011) containing samples from 12 sites and eight laboratory-bred F_1 hybrids were prepared with restriction enzyme *Sbf*1. We included four to 20 individuals per site (Table 1). Libraries were sequenced on one lane of a HiSeq4000 (PE 150; Illumina).

2.4 | Quality filtering and SNP calling

We processed RAD-seq data as in Akopyan et al. (2020b). Briefly, raw reads with the cut site on the reverse read were rescued using a custom Perl script. The *clone_filer* program in STACKS version 1.34 (Catchen et al., 2013) was used to remove PCR (polymerase chain reaction) clones. Sequence data were demultiplexed using the *process_radtags* function in STACKS. Reads containing base pairs with a phred score less than 10 were removed. We used *ustacks* in STACKS for *de novo* assembly, requiring a minimum of three reads to create a stack, and a maximum of 4-bp differences between stacks. A pseudoreference genome was created using a custom Perl script. Over half of the individuals had to have a stack for it to be used in the pseudoreference genome. Reads were aligned to the pseudoreference genome using "aln" and "samse" in the program BWA version 0.7.15-r1142 (Li & Durbin, 2009). We set the maximum edit distance to four, seed to 20, maximum edit distance within the seed to two,

and the read trimming parameter to 10. SAMTOOLS version 1.9 (Li et al., 2009) was used to index, sort and merge alignments. We used the BCFTOOLS version 1.3.1 "call" function to call variants and create a VCF file (Li, 2011). We filtered the data to remove low-coverage loci (<2× per individual) and to retain a single nucleotide polymorphism (SNP) per contig. Loci with mean allele frequencies <0.5% and individuals with >70% missing data were not included in subsequent analyses. Pipeline and scripts are available online (Akopyan et al., 2020a).

2.5 | Population genomic analyses

We calculated two measures of relative genetic variability, pi at polymorphic sites (π_p), and Wu and Watterson's theta (θ_w, Watterson, 1975) at polymorphic sites, in R using custom scripts. We defined S as the total number of segregating sites in the global data set, and s_i as the number of those sites genotyped in the ith individual. To calculate π_p, we first calculated heterozygosity at polymorphic sites for each individual; in the ith individual, this is the number of sites at which that individual was called heterozygous divided by s_i. To calculate π_p for a sampling site, we then averaged the heterozygosities of all individuals within that site (Charlesworth & Charlesworth, 2010). Wu and Watterson's theta for a population was calculated as the number of polymorphic sites in that population divided by the product of S and a_k, where a_k is Watterson's correction factor for the number of individuals in that population (Charlesworth & Charlesworth, 2010; Watterson, 1975). We calculated the number of private alleles in each population in R. We used a Bayesian clustering method implemented in the program ENTROPY (Gompert et al., 2014) to estimate admixture proportions and interpopulation ancestry. We excluded sites 1 and 8–10 from the ENTROPY analysis to limit the data set to putative parental and hybrid groups based on admixture proportions. To assess the possibility of hybridization across sites, we plotted interpopulation ancestry (Q_{12}) against admixture proportion (q) (Gompert & Buerkle, 2016). Interpopulation ancestry, the proportion of an individual's genome where each allele at a locus is from a different putative parental group, separates out F_1 hybrids (Q_{12} near 1), multigenerational hybrids (intermediate Q_{12}) and individuals with late-stage hybrid genomes (Q_{12} near 0). The laboratory-reared F_1 hybrids served as a useful control for what we expect for F_1 hybrids. Multigenerational hybrids were expected to show elevated interpopulation ancestry, but below that of F_1 hybrids.

We ran Mantel tests in the R package ADE4 version 1.7–13 (10,000 replicates) to test for correlations between geographic distance and genetic distance; geographic distance and phenotypic distance; and genetic and phenotypic distance (Chessel et al., 2004). We assumed frogs would avoid crossing bodies of saltwater, so we calculated geographic distances among populations as over-land distances. We calculated pairwise F_{ST} between sites in the R package BEDASSLE version 1.5 (Bradburd et al., 2013; Weir & Hill, 2002). We estimated phenotypic distance between sites by calculating the pairwise Euclidean distance of average hue, saturation, brightness and percentage grey for each site. We also tested for relationships between each phenotypic measurement and geographic/genetic distance individually. A Q_{ST}–F_{ST} or P_{ST}–F_{ST} approach is not justified for this study because we did not measure phenotype in a common garden, and the genetic basis of colour pattern in red-eyed treefrogs is unknown (Pujol et al., 2008).

Because we observed a strong pattern of genetic IBD (see Results), we used the R package CONSTRUCT version 1.0.4 to assess evidence for discrete genetic clusters while accounting for continuous differentiation resulting from geographic distance (Bradburd et al., 2018). The spatial model in CONSTRUCT estimates admixture proportions for each sample while simultaneously accounting for the decay in genetic relatedness with distance within each discrete group. We ran four chains for each spatial and nonspatial model specifying a number of discrete groups (K) between 1 and 6. We selected the chain with the highest mean posterior probability and assessed the biological importance of each added K-value by (i) comparing the contribution of each layer to overall covariance, (ii) cross-validation analysis that compares the predictive accuracy of models using eight replicates and 1000 iterations, and (iii) assessing the biological realism of the model given previous knowledge of the system. To explore genetic clustering in restricted spatial scales, we repeated CONSTRUCT analyses for three subsets of the data: without Site 1 ("Without Nicoya"), northern sites (Sites 1 and 2) and southern sites (Sites 5–12).

To evaluate phenotypic patterns while accounting for genetic relatedness, we fitted three sets of Bayesian linear models implemented in RSTAN version 2.12.2 (Stan Development Team, 2018): (i) we modelled individual phenotype measurements (hue, saturation, brightness and colour-pattern PC1) each as a function of latitude, (ii) we modelled the average phenotype for each site as a function of latitude, and (iii) we modelled phenotypic variation at a site as a function of latitude and, separately, of θ_w. All models included a covariance structure that was a function of the sample covariance of allele frequencies between individuals and were run four times, for 2000 iterations each. These models allowed us to detect variation in phenotype above and beyond that explained by covariance at putatively neutral genetic markers.

3 | RESULTS

3.1 | Phenotypic variation

Phenotypic patterns along the Pacific coast matched previous studies: frogs had orange legs and flanks in the northern regions and purple flanks and legs in the south. We confirmed that the three sites in the Central 2 region were polymorphic. The first two principal components in our colour-pattern PCA explained 75.6% of the total variance in measured leg colour-pattern metrics (Figure 2; Figure S4). Sites in the Nicoya, North and Central 1 regions (orange legs) formed one cluster in phenotypic space, and sites in the South (purple legs) formed another. Sites in the Central 2 region were generally intermediate, but closer to the orange cluster. Osa sites were

variable: Site 10 clustered with the purple/south sites, Site 8 clustered with the orange/northern sites and Site 9 was intermediate. Colour pattern PCs 2 and 3 are visualized in Figure S5. The first two principal components in the colour-only PCA explained 84.3% of the total variance in the leg coloration metrics. The relative location of sites in the colour-only PCA was similar to the colour-pattern PCA, but there was less overlap between sites (Figure S6). The percentage of the hind leg taken up by the inner grey patch increased from 0% in the Nicoya and North regions to an average of 33.3% in the South region (Figure S7).

The DFA run with all phenotypic measurements had an overall predictive accuracy of 69.0%. Individuals from the Nicoya region had 100% correct assignment (Table 2). The majority of frogs were correctly assigned to their region of origin, with the exception of orange frogs, which were frequently assigned to phenotypically similar sites in different regions (Table 2). Frogs from polymorphic sites in the Central 2 region had high misclassification probabilities (Table 2). Most (72.7%) purple individuals from the South were assigned to the correct region. Over half of the individuals from the Osa peninsula were assigned to the correct site; misclassified individuals were most often assigned to sites with colour patterns similar to their respective Osa site (e.g., 26% of purple morph frogs from Site 10 were misassigned to Site 11, another purple morph site). Overall predictive accuracy decreased in DFA performed using single phenotypic measurements (hue: 31.0%, saturation: 30.5%, brightness: 23.0%, grey patch percentage: 19.5%; Tables S2–S5).

3.2 | Genetic variation

After filtering, we retained 71,746 SNPs in 174 individuals. Mean coverage per SNP was 9.3× with a standard deviation of 2.97.

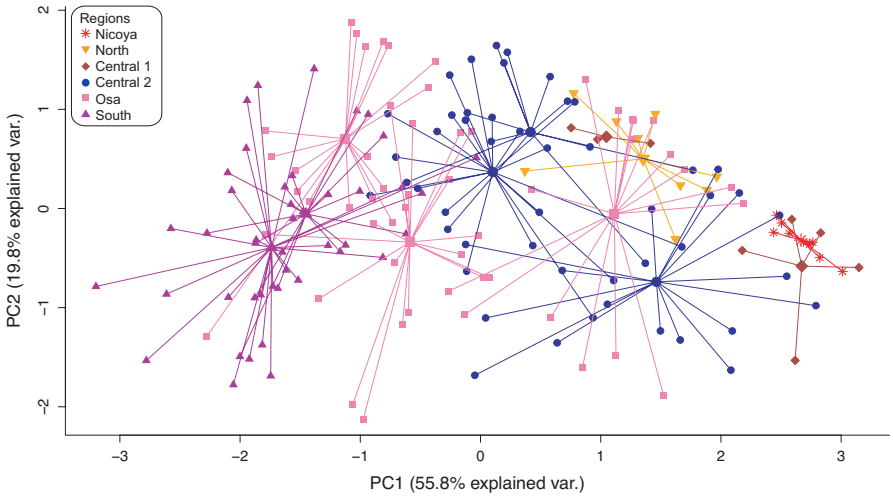

FIGURE 2 Principal component analysis of leg phenotype for red-eyed treefrogs (*Agalychnis callidryas*) sampled from 12 sites along the Pacific coast of Costa Rica. Lines connect individuals (small symbol) with the mean phenotype of each sampling site (large symbol). Individuals and means are colour coded by region

TABLE 2 Discriminant function analysis of leg colour pattern for 12 sampling sites of red-eyed treefrogs (*Agalychnis callidryas*) along the Pacific coast of Costa Rica. Each individual was classified based on its leg colour pattern and assigned to a sampling site

		Region of origin											
		Nicoya	North	Central 1		Central 2			Osa			South	
		1	2	3	4	5	6	7	8	9	10	11	12
Classification	1	100%	0	0	0	0	0	24%	6%	0	0	0	0
	2	0	22%	67%	0	8%	0	6%	6	0	0	0	0
	3	0	0	0	0	0	0	0	0	0	0	0	0
	4	0	0	0	100%	0	0	6%	0	0	0	0	0
	5	0	22%	33%	0	69%	15%	0	0	0	0	0	0
	6	0	0	0	0	15%	65%	6%	12%	0	0	0	0
	7	0	11%	0	0	8%	15%	41%	18%	0	0	0	0
	8	0	33%	0	0	0	0	12%	59%	0	0	0	0
	9	0	11%	0	0	0	0	6%	0	95%	0	0	20%
	10	0	0	0	0	0	5%	0	0	0	74%	25%	0
	11	0	0	0	0	0	0	0	0	0	26%	75%	4%
	12	0	0	0	0	0	0	0	0	5%	0	0	76%

Descriptive statistics indicated similarity in genetic diversity among sites, except for Site 1, which had lower genetic diversity (Table 1). Site 1 also had the highest number of private alleles (907, Table 1). The highest genetic diversity was found at Site 7 in the Central 2 region. Pairwise F_{ST} values ranged from 0.025 to 0.422, with the highest value between Sites 1 and 3 (Table 3). The genetic PCA revealed genetic clustering corresponding to geographic regions (Figure 3; Figure S8). Combined, the first two principal components explained 12.6% of the variation. The Nicoya, North, Central 1, Osa and laboratory-generated hybrids were genetically distinct, while South and Central 2 regions clustered together. Genetic PCs 2 and 3 are visualized in Figure S9.

The comparison of admixture proportion (q) and interpopulation ancestry (Q_{12}) from ENTROPY showed no evidence of recent hybridization when parental populations were assigned as North and South. Laboratory-generated hybrids had intermediate admixture proportions and high interpopulation ancestry scores, as expected. No wild frogs had elevated interpopulation ancestry scores (Figure 4).

3.3 | Genotypic and phenotypic patterns

Mantel tests revealed a strong positive relationship between geographic and genetic distance ($r = .965$, $p < .001$) and weak positive relationships between geographic and phenotypic distance (Mantel test, $r = .336$, $p = .0085$) and genetic and phenotypic distance (Mantel test, $r = .219$, $p = .106$; Figure 5). Results between composite phenotypic distance and individual phenotypic measurements were consistent, and results for individual phenotypic measurement tests are presented in the Figure S10.

We used the R package CONSTRUCT to quantify genetic clustering while accounting for geographic distance. We ran CONSTRUCT on

TABLE 3 Pairwise F_{ST} (below diagonal) and geographic distance in kilometres (above diagonal) for 12 sampling sites along the Pacific coast of Costa Rica. Lines denote regional boundaries

	Nicoya	North	Central 1		Central 2			Osa			South	
	1	2	3	4	5	6	7	8	9	10	11	12
1		212.2	299.4	323.5	350.6	354.4	359.1	385.4	410.7	412.8	396.7	439.3
2	0.286		89.3	113.2	139.9	143.9	148.5	168.8	193.6	189.1	186.1	215.8
3	0.422	0.163		24.2	51.4	55.2	60.1	80.2	104.9	100.5	97.6	127.2
4	0.419	0.206	0.075		27.2	31.0	35.9	56.1	80.8	76.4	73.4	103.1
5	0.394	0.231	0.099	0.058		4.3	8.8	29.9	54.6	50.2	46.3	76.0
6	0.393	0.250	0.126	0.088	0.069		6.3	28.2	52.9	48.5	42.4	72.3
7	0.382	0.235	0.107	0.070	0.054	0.033		22.1	46.9	42.5	37.9	67.3
8	0.415	0.272	0.168	0.137	0.126	0.115	0.090		25.4	21.4	38.4	66.9
9	0.408	0.270	0.168	0.139	0.129	0.119	0.092	0.036		21.5	60.4	89.5
10	0.412	0.274	0.172	0.143	0.134	0.124	0.097	0.031	0.025		55.0	84.1
11	0.411	0.273	0.156	0.116	0.095	0.075	0.059	0.122	0.127	0.131		32.5
12	0.412	0.279	0.166	0.125	0.104	0.082	0.066	0.130	0.135	0.139	0.039	

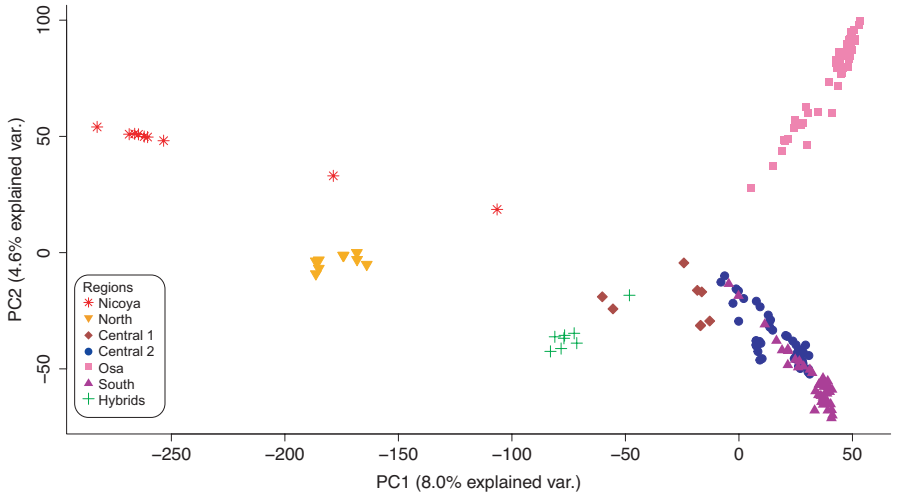

FIGURE 3 Principal component analysis of genomic SNPs for red-eyed treefrogs (*Agalychnis callidryas*) sampled from 12 sites along the Pacific coast of Costa Rica. Symbols represent individuals. Both type of symbol and colour represent region. Green plus symbols are laboratory-generated hybrids between Site 2 and Site 12

four sets of individuals: (i) the full data set, (ii) without the Nicoya Peninsula (Sites 2–12), (iii) northern sites (Sites 1 and 2) and (iv) southern sites (Sites 5–12). Cross-validation analyses showed that spatial models have better predictive accuracy than nonspatial models for all data subsets and *K*-values (Figure S11).

CONSTRUCT results from the full data set (i) show that nonspatial models inferred more discrete genetic structure compared to spatial models (Figure 6). The nonspatial model at $K = 2$ shows a separation between the Nicoya Peninsula and other sites, and at $K = 3$ shows a separation between the Osa Peninsula and other sites. Although each additional layer from $K = 1$ to 6 increased the predictive accuracy of the model (Figure S11), layer contributions for the spatial model decreased dramatically after $K = 1$ (Figure S12). The spatial model at $K = 3$ assigned individuals on the Nicoya and Osa Peninsulas similar admixture proportions. There is no obvious biological explanation for why the populations at these sites would be assigned to the same discrete cluster. It is likely that runs at $K \geq 3$ are describing the genetic dissimilarity between frogs on the Nicoya and Osa Peninsulas with the IBD component of the model within a single layer. This may not reflect recent shared ancestry, and instead may be an artefact of the large geographic distance between these locations combined with gaps in our sampling. Therefore, we interpret $K = 1$ as the model with the most biological significance, and subset the data set to investigate genetic trends within restricted geographic areas.

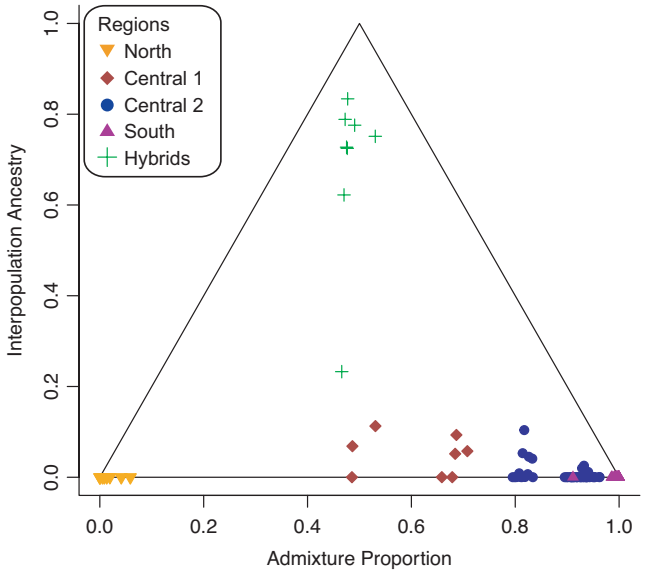

FIGURE 4 Scatterplot of admixture proportion (*q*) and interpopulation ancestry (Q_{12}), the proportion of an individual's genome where one allele is assigned to each parental group, for red-eyed treefrogs (*Agalychnis callidryas*) sampled from five sites along the Pacific coast of Costa Rica. Symbols represent individuals and are colour coded by region. Only laboratory-generated hybrids (green) show high interpopulation ancestry indicative of hybrid origin

We ran CONSTRUCT on a subset of the data that excluded the Nicoya Peninsula (ii). Results show very little assignment to the second layer (Figure 7), and layer contributions are minimal after $K = 1$ (Figure S12). For genetic clustering with just northern sites (iii), there was a difference in assignment between sites, and layer contributions indicate that the most biological relevant *K*-value is $K = 2$ (Figure 7; Figure S12). When considering the southern sites (iv), individuals on the Osa Peninsula and mainland are partially assigned to different layers, but layer contributions indicate that the most biologically relevant *K*-value is $K = 1$ (Figure 7; Figure S12). Admixture proportions for additional *K*-values and nonspatial models are available in Figures S13–S17. Partitioning the data allowed us to investigate discrete processes within restricted geographic ranges that might not be inferred in the full analysis.

We used Bayesian linear models to evaluate relationships between phenotypic measurements (colour-pattern PC1, hue, saturation and brightness) and latitude while accounting for genetic covariance. Using 95% credible intervals, with a Dunn–Bonferroni correction for multiple tests, we found that individual leg saturation, hue and colour-pattern PC1, but not brightness, vary significantly with latitude while accounting for genetic covariance among

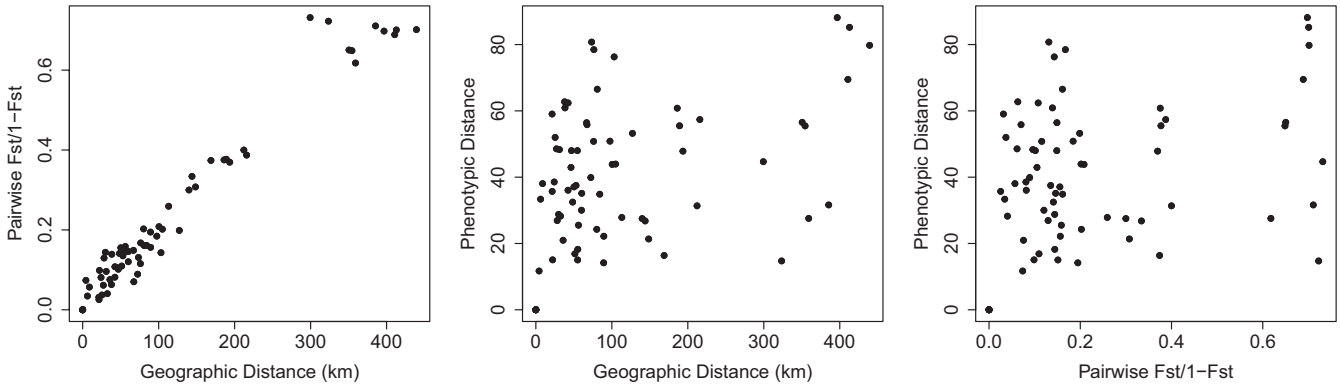

FIGURE 5 Relationship between over-land geographic distance and genetic distance for 12 sites of red-eyed treefrogs (*Agalychnis callidryas*) along the Pacific coast of Costa Rica. There is a strong positive relationship between geographic and genetic distance, suggesting a pattern of isolation by distance ($r = .965$, $p < .001$); the relationships between phenotypic and geographic distance ($r = .336$, $p = .0085$) and genetic and phenotypic distance ($r = .219$, $p = .106$) were considerably weaker

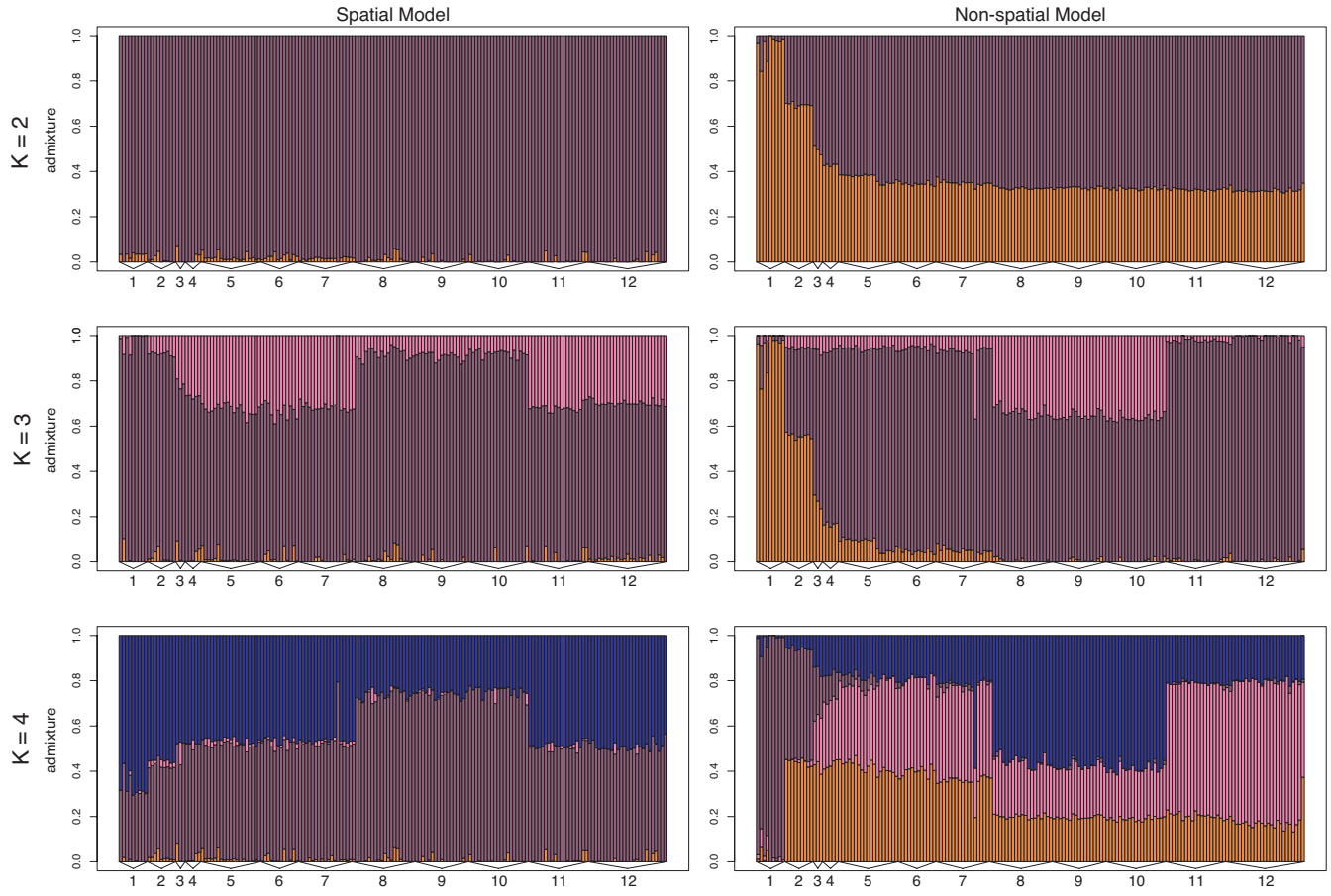

FIGURE 6 Barplots of admixture proportions for red-eyed treefrogs (*Agalychnis callidryas*) sampled from 12 sites along the Pacific coast of Costa Rica. Admixture proportions shown were generated using CONSTRUCT for $K = 2$–4 for both nonspatial and spatial models. Each bar represents an individual. The colour of the bar shows the proportion of the individual's genome that is assigned to each of K layers

sampled individuals (Figure 8). These correlations are not significant when using phenotypic averages for each site. We did not find significant relationships between variation in phenotypic measures and θ_w, or latitude (Figures S18 and S19).

4 | DISCUSSION

The distribution of colour pattern in natural systems is complex and can reveal underlying evolutionary dynamics when compared to genetic patterns. Red-eyed treefrogs display dramatic variation in colour pattern, yet the two primary morphs found on the Pacific coast of Costa Rica are not restricted to distinct genetic demes (Hypotheses 1B and 3B). Rather, we identified a transition from orange to purple legs and polymorphic sites that contain orange, purple and novel morphs (Hypothesis 2B) that cannot be explained by recent hybridization between discrete ancestral genetic groups (Hypothesis 4B). Our analyses of the complex relationship between genetic and colour-pattern variation along a narrow stretch (~215 km) of the Pacific coast of Costa Rica suggest that selective forces plus patterns of IBD influence colour-pattern distribution in the region.

4.1 | Polytypic patterns: Pattern of IBD found across polytypic regions

We found that red-eyed treefrogs at some sites had relatively uniform leg colour-pattern, such as orange frogs on the Nicoya Peninsula and purple frogs in the South region, whereas other sites, such as those in the C2 region, were variable (Table 2). Across the Pacific coast, most of the genetic variation among red-eyed treefrog populations is explained by over-land distance among sites; orange and purple morphs do not represent separate genetic demes (Figures 5 and 6). Red-eyed treefrogs are probably semicontinuously distributed, yet, as with most population genetic studies, we sampled at discrete sampling locations; this risks over-estimating the number of discrete genetic groups inferred using clustering models (Bradburd et al., 2018; Frantz et al., 2009; Meirmans, 2012). Therefore, we used CONSTRUCT to compare models of population structure with and without spatial information (Bradburd et al., 2018). The nonspatial CONSTRUCT models infer more genetic clustering than the spatial models, indicating that clustering in nonspatial models is due to the geographic distance between sites (Figure 6). Our results highlight the importance of considering patterns of IBD as they relate to genetic relatedness

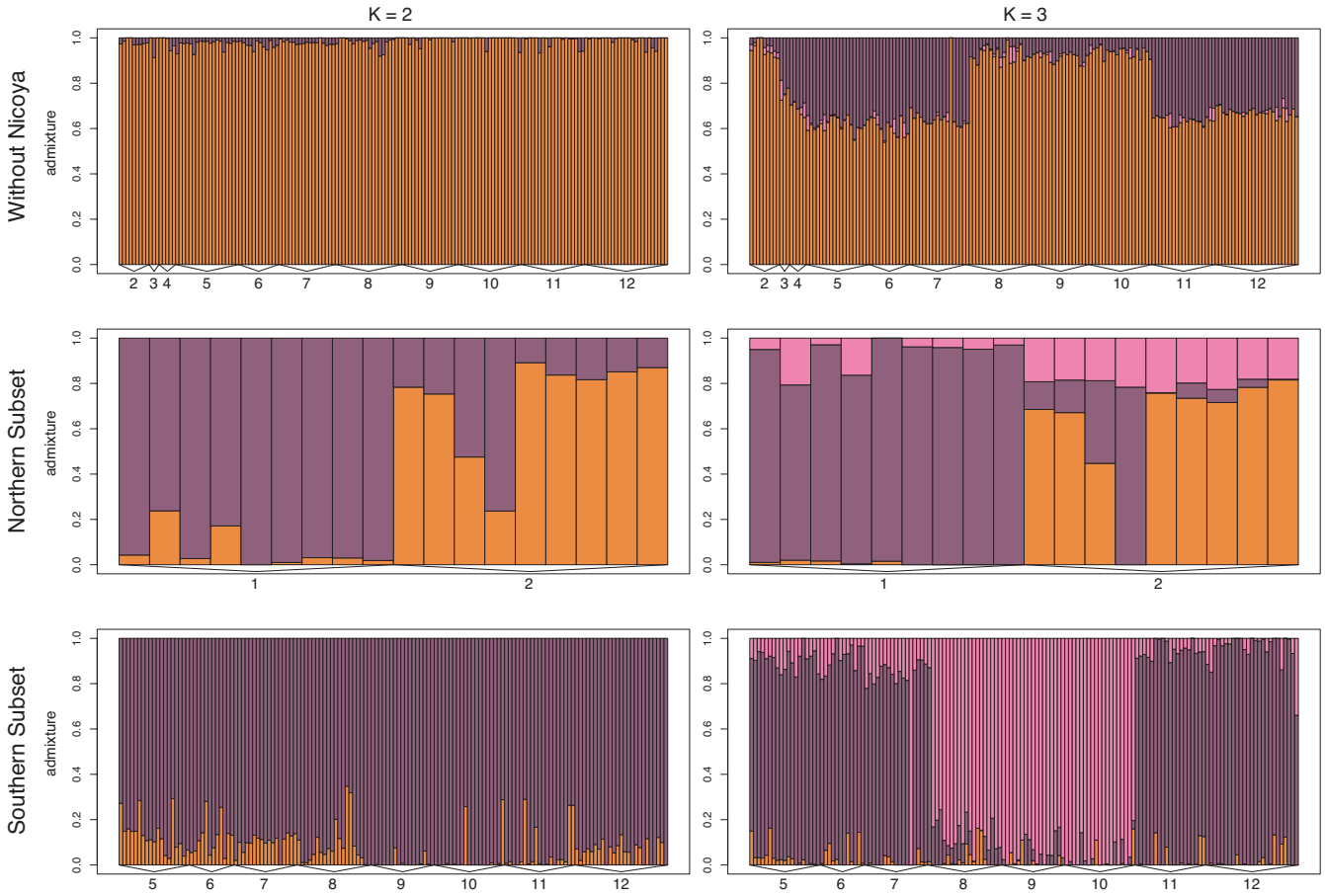

FIGURE 7 Barplots of admixture proportions for red-eyed treefrogs (*Agalychnis callidryas*) sampled from three data subsets: without Nicoya (Sites 2–12), northern sites (Sites 1 and 2) and southern sites (5–12). Admixture proportions shown were generated using CONSTRUCT for $K = 2$–3 for spatial models. Each bar represents an individual. The colour of the bar shows the proportion of the individual's genome that is assigned to each of K layers

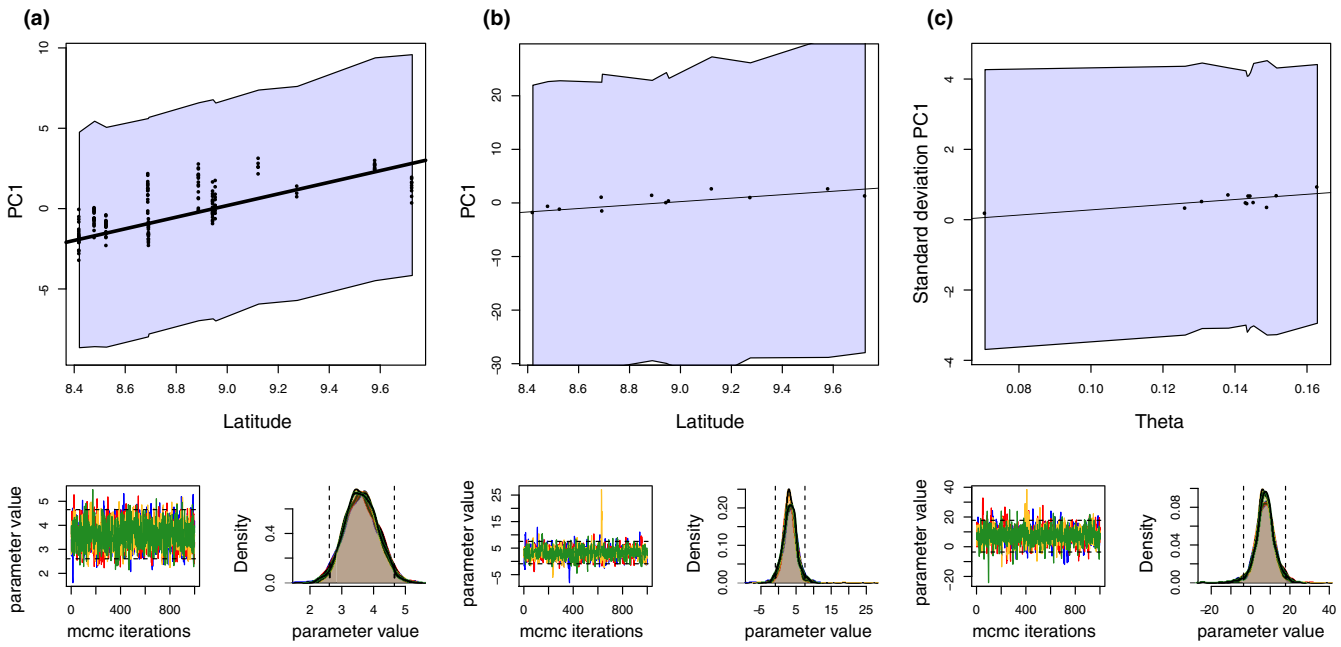

FIGURE 8 Results of Bayesian models with Dunn–Bonferroni-corrected credible intervals (CI) showing the relationships between (a) latitude and colour-pattern PC1, (b) latitude and average PC1 value per site, and (c) Wu and Watterson's theta and variation in PC1 at each site. Each model contains genetic covariance as a covariate. Bold line indicates that the 95% CI did not overlap with zero. Insets below model fit show estimated parameter values for 5,000 Markov chain Monte Carlo iterations

among discrete sampling sites: we would have both over-estimated the number of genetic demes and come to strikingly different conclusions about how diversity is distributed had we relied solely on nonspatial clustering models.

Spatial CONSTRUCT models show a division between the mainland and the two peninsular regions (Nicoya and Osa), but we assert that $K = 1$ best describes the data because there is no *a priori* reason to expect that sites on the Nicoya and Osa Peninsulas exchange migrants or share a more recent common ancestor with each other than with neighbouring mainland sites. In fact, pairwise F_{ST} values between the Nicoya site and Osa sites are among the highest found in the data set (Table 3, 0.408–0.415). Furthermore, the nonspatial model did not support shared ancestry between Nicoya and Osa.

On both the Nicoya and Osa Peninsulas, we expected red-eyed treefrogs to be isolated from neighbouring sites on the mainland: Site 1 at the tip of the Nicoya Peninsula is one of the only known populations of red-eyed treefrogs in the region, as dry forest unsuitable for red-eyed treefrogs covers most of far northwestern Costa Rica, including the Nicoya Peninsula (Kohlmann et al., 2002; Savage, 2002). The Osa Peninsula (Sites 8–10) is genetically isolated from mainland sites for many other taxa, including snakes and other frogs (Crawford, 2003; Crawford et al., 2007; Zamudio & Greene, 1997), and we thought this might also be true for red-eyed treefrogs.

Using the full data set, we did not detect more genetic divergence between mainland and peninsular sites than would be expected given their geographic distance and the rate at which genetic divergence accrues at smaller spatial scales. However, when we restricted analyses to peninsular and nearby mainland sites, we see divergence between the Nicoya Peninsula and mainland, but not between the mainland and the Osa Peninsula (Figure 7; Figure S12). This suggests that the total divergence between red-eyed treefrogs on the Nicoya Peninsula and mainland is explained by both IBD and barriers to gene flow. As dry forest on the Nicoya Peninsula probably acts as a barrier to gene flow, it is possible that populations along the coast (e.g., Gonzalez, 2013) provide a path for gene flow between the peninsula and mainland. Other studies that have found divergence between the Osa Peninsula and mainland populations have not explicitly incorporated IBD into their analyses, which may explain the discrepancies between our conclusions.

4.2 | Polymorphic patterns: Hybridization does not underlie colour pattern polymorphism

We tested whether colour-pattern polymorphism at a particular site arose through recent hybridization of divergent parent populations, the retention of ancestral polymorphism, and/or ancient secondary contact. We did not find evidence of discrete parental groups, nor recent hybridization in the Central 2 region, despite the colour-pattern polymorphism in this region (Table 2; Figure 2). Rather, our findings support that either colour-pattern polymorphism was retained from an ancestral population, there has been ancient secondary contact with extended gene flow spreading alleles through the system, and/or that novel morphs have been generated by new mutations. Laboratory-bred hybrids provided examples of admixed ancestry (F_1 stage) and were the result of crossing orange (Site 2) and purple (Site 12) morph parents from geographically distant locations. Thus, these parents were more genetically differentiated than the wild-caught orange and purple morph individuals living in the transition zone. Orange and purple frogs to the north, south and within the transition zone were assigned to similar genetic groups in both our spatial and nonspatial CONSTRUCT analyses, eliminating the possibility of genetic hybrids. Strong signals of IBD (Figure 5) suggest that gene flow among sites is uninhibited by geographic barriers or other forms of premating reproductive isolation.

While the distribution of colour-pattern variation on the Pacific slope of Costa Rica seemingly mirrors patterns on the Caribbean slope, the underlying processes differ. Red-eyed treefrogs on both the Pacific and Caribbean slopes exhibit colour-pattern polymorphism located within a "transition zone" between two distinct morphs. However, polymorphism in the Caribbean is the result of hybridization between two distinct genetic groups of different colour morphs (Akopyan et al., 2020b). There, red and blue morph frogs meet in a contact zone and produce frogs with either a novel purple colour pattern or a red/blue phenotype distinct from either parental population. Our contrasting findings for the Pacific slope emphasize that diverse evolutionary forces can produce similar patterns of phenotypic variation even within intraspecific lineages. Whereas there is direct evidence that polymorphism on the Caribbean coast is the result of hybridization, the lack of recent hybridization along the Pacific slope suggests that variation in leg phenotype could have arisen due to the retention of ancestral colour-pattern polymorphism in the region (as in Corl et al., 2010), or ancient secondary contact (as in Lim et al., 2010). Additionally, polymorphism could be generated by novel mutations (as in Rosenblum et al., 2004, 2010).

4.3 | Spatial patterns of phenotype and genotype explained by multiple processes

Patterns of phenotypic and genetic variation permit us to test hypotheses about the evolutionary forces that influence lineage divergence. We found that genetic relatedness predicts phenotypic similarity in some regions of the Pacific coast, but not in others.

Evolution via drift is a common pattern for insular populations (e.g., Knopp et al., 2007; Lehtonen et al., 2009). In our study system, frogs on the Nicoya Peninsula had the highest pairwise F_{ST} values to sampled populations at other locations (Table 3), the lowest nucleotide diversity and highest number of private alleles (Table 1). Individuals from the Nicoya Peninsula have a bright orange phenotype that is distinct from mainland orange frogs (Table 2). Although we cannot rule out local adaptation driving both phenotypic and genetic divergence, red-eyed treefrogs on the Nicoya Peninsula are isolated from the mainland by both geographic distance and tropical

dry forests (see above), suggesting that genetic and phenotypic differentiation in this region could have resulted from genetic drift.

In other instances along the Pacific coast, we found that phenotypic similarity is not well predicted by neutral genetic relatedness at our genotyped loci, supporting the hypothesis that colour pattern evolves through selection in this region (see below for a discussion of selection). Orange and purple morph frogs along the mainland coast and in the polymorphic Central 2 region were not part of separate genetic demes, and instead conformed well to the coast-wide pattern of genetic IBD (Figures 6 and 7), despite different phenotypes (Table 2; Figure 2). We found additional incongruent patterns of variation on the Osa Peninsula, where the three sampled sites are genetically similar (Figures 3, 6 and 7), but phenotypically distinct from one another (Table 2). These incongruences between genetic patterns and phenotype suggest that phenotypic differences are maintained by selection in the presence of gene flow. Whether colour-pattern differentiation in these regions was generated by novel mutations, ancestral polymorphisms and/or the result of genetic drift in these relativity isolated and small populations remains untested. Phenotypic divergence, despite genetic homogeneity, is a common pattern in nature, and usually has been attributed to sexual selection or adaptation to environmental variation. Some examples include *Epipedobates* poison frogs in Peru and Colombia (Tarvin et al., 2017), mimic poison frogs in Peru (Twomey et al., 2013), and redpoll finches in the Arctic circle (Mason & Taylor, 2015). The phenotypic variation on the Osa Peninsula is particularly surprising given the small geographic scale (distance among sites ranges from 20 to 33 km).

As reported above, the transition in phenotype from orange legs to purple legs is not the result of recent hybridization between two genetic demes meeting at a contact zone. It is possible that it was facilitated by ancient secondary contact between ancestral demes, one with orange flanks and legs and the other with purple flanks and legs, and that colour polymorphism is the result of continued gene flow (Endler, 1977). Frogs from the southern sites (7–12) and the Osa Peninsula are part of a different mtDNA clade (clade A; Table 1) from the north (clade B; Table 1; Robertson & Zamudio, 2009). This biogeographic discordance between mtDNA and nuclear loci suggests past secondary contact (as in Toews & Brelsford, 2012). Along the mainland Pacific slope, frogs belong to the same genetic cluster, but have different mitochondrial haplotypes and distinct colour patterns. This suggests that ancestral mitochondrial groups and colour-pattern divergence have been maintained over time, whereas nuclear structure has been lost as a result of gene flow. However, it is also possible that the geographic structure of mtDNA haplotypes is the result of IBD alone (Irwin, 2002). Differentiating between these scenarios would require additional genomic data from these regions. These results highlight the complexity and nuance of this natural system, where multiple processes potentially shape diversity at a fine spatial scale, including genetic drift, selection and ancient evolutionary history.

Because there is spatial autocorrelation in colour pattern along the Pacific coast, it is difficult to decouple phenotypic changes from patterns of IBD to explore possible drivers of colour-pattern variation. We therefore used Bayesian linear models to test whether colour-pattern phenotype differences are greater than expected given genetic relatedness, and whether those differences are correlated with latitude or genetic diversity. Specifically, we were interested in whether ecological changes correlated with latitude might play a role in colour-pattern variation in red-eyed treefrogs, and whether sites with more genetic variation might have more phenotypic variation. We found a significant effect of latitude on phenotype for colour-pattern PC1 and leg saturation and hue, but not for brightness measures (Figure 8; Figures S18 and S19). The relationship between phenotype and latitude disappeared at the site level, possibly due to reduced sample size. Future work could investigate the relationship between phenotype and latitude to explore the potential for localized ecological selection on colour pattern.

4.4 | Role and mechanisms of selection

A complex set of selective pressures probably underlies the genetic and phenotypic patterns observed between and within red-eyed treefrog populations. Although hidden during the day when the frogs are at rest, the flanks and legs of red-eyed treefrogs are visible during activity at night, including during mating aggregations (Pyburn, 1970). Colour pattern serves as a species and population-recognition cue in mate choice (Kaiser et al., 2018; Robertson & Greene, 2017). Mate preference for local colour pattern has been demonstrated in this species: females from Site 2 (orange) and Site 12 (purple) have a preference for males with their local colour pattern in mate-choice trials, suggesting a role for sexual selection on visual signals (Akopyan et al., 2018; Jacobs et al., 2016). Frogs from these sites also have distinguishable male calls and exhibit differences in female courtship behaviours (Akopyan et al., 2018), suggesting that preference for male traits could maintain local colour-pattern differences even in the face of gene flow.

Other forms of selection could also shape geographic variation in colour pattern. Red-eyed treefrogs secrete noxious host-defence polypeptides from their skin (Conlon et al., 2007; Davis et al., 2016; Mignogna et al., 1997; Sazima, 1974), presenting the possibility that flank- and leg-colour patterns act as aposematic signals to visual predators (Robertson & Greene, 2017). Geographic variation in host-defence polypeptides is correlated with colour pattern in red-eyed treefrogs, indicating a role for ecological selection (Clark, 2019; Davis et al., 2016). Aposematic coloration is common among other brightly coloured anurans (e.g., many species within Dendrobatidae and Mantellidae), and has been shown to vary among and within populations (Klonoski et al., 2019; Maan & Cummings, 2008; Rojas et al., 2014; Roland et al., 2017; Summers & Clough, 2001; Tarvin et al., 2017). Variation in the colour pattern of aposematic species is often attributed to the interplay between ecological and sexual selection (Maan & Cummings, 2008; Nokelainen et al., 2012; Rojas & Endler, 2013). Future studies of red-eyed treefrogs that test the role of colour pattern in predator avoidance would inform how

ecological and sexual selective pressures interact to shape colour-pattern distribution.

5 | CONCLUSIONS

Our results highlight the complex evolutionary dynamics that shape colour pattern in natural systems. We found that, despite a dramatic and discrete phenotypic change from orange to purple morphs, genetic variation in our study region follows a continuous pattern of IBD. Phenotypic polymorphism at sites located where the orange morph transitions to purple cannot be explained by recent hybridization as the orange and purple morphs are not genetically differentiated groups. Comparison of colour pattern and genetic relatedness indicates that selective forces have probably maintained colour-pattern divergence in some regions, while genetic drift may play a role in others. Understanding the relative strength of sexual and ecological selection on colour pattern is a logical next step for this system.

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CONFLICT OF INTEREST

The authors have no conflicts of interest.

AUTHOR CONTRIBUTIONS

M.I.C., J.M.R., M.A., E.B.R. and A.V. designed this study. J.M.R., M.A. and A.V. carried out field sampling. M.I.C. performed phenotypic analyses, library preparation and bioinformatics. G.S.B. and M.I.C. carried out statistical and computational modelling. Funding support was received for J.M.R., G.S.B., E.B.R. and M.I.C. M.I.C. drafted the manuscript, and all authors contributed to and approved the final version.

DATA AVAILABILITY STATEMENT

Raw data, input files, and analysis scripts are available on figshare at https://doi.org/10.6084/m9.figshare.14622744.v2. Bioinformatic scripts are available at https://doi.org/10.6084/m9.figshare.11923017.v2.

ORCID

Meaghan I. Clark https://orcid.org/0000-0003-3297-8372
Gideon S. Bradburd https://orcid.org/0000-0001-8009-0154
Maria Akopyan https://orcid.org/0000-0001-7956-3196
Erica Bree Rosenblum https://orcid.org/0000-0002-7384-8032
Jeanne M. Robertson https://orcid.org/0000-0003-2642-6280

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