GENETIC AND PHENOTYPIC DIVERSITY PATTERNS IN TWO POLYMORPHIC, NEOTROPICAL ANURANS: BIOGEOGRAPHY, GENE FLOW AND SELECTION

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by
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GENETIC AND PHENOTYPIC DIVERSITY PATTERNS IN TWO POLYMORPHIC, NEOTROPICAL ANURANS: BIOGEOGRAPHY, GENE FLOW AND SELECTION

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Geographic patterns of phenotypic and genetic differentiation among populations provide critical insight for understanding the processes that underlie the origin and maintenance of biological diversity. Fully concordant phylogeographic patterns among co-distributed taxa are an indication that landscape features promote lineage diversification. The dynamics of natural populations, however, are often more complex and organisms respond to common historical processes in different ways. I quantified variation in two co-distributed and wide-ranging Neotropical frogs, Agalychnis callidryas and Dendropsophus ebraccatus that share many ecological traits, yet differ in the geographic distribution of color pattern polymorphisms. Specifically, I compared divergence patterns across multiple populations of each species to determine whether: 1) spatial patterns of phenotypic and genetic diversity were congruent; 2) the complex biogeographic history of the Central American Isthmus has resulted in a similar evolutionary history of vicariance and dispersal; 3) landscape features limited gene flow; 4) gene flow patterns explained the geographic distribution of phenotypic diversity. I compared historical (mitochondrial DNA) and contemporary (nuclear DNA) gene flow to patterns of phenotypic differentiation. I determined cases where gene flow processes alone could not explain the patterns of phenotypic diversity, implying that selection (sexual and/or natural selection) has played a role in diversification. My results indicated that Agalychnis callidryas and D. ebraccatus have differences in their biogeographic history, population genetic structure and dispersal biology; therefore color pattern and genetic differentiation have evolved due to independent mechanisms in each taxon.
BIOGRAPHICAL SKETCH

Jeanne Marie Robertson was born to Anne Leone and Arthur D. Robertson in Cupertino, California on July 20, 1970. Along with her older brother, Stephen, and younger sister, Diana, the Robertson kids grew up playing in the foothills of the Santa Cruz Mountains. They explored the redwood forest, fruit orchards and small creeks that characterized a place that would later become home to Apple Computer. Summer trips to upstate New York were at the heart of Jeanne’s childhood fascination and admiration of frogs.

Each family member contributed significantly to Jeanne’s emotional, intellectual, athletic and artistic growth. Her father, a modern day da Vinci and the original, walking ‘wikipedia’ (although more accurate than wiki), often conducted experiments in the kitchen sink, garage and backyard, demonstrating the principles and laws of physics and chemistry. It was no surprise to any Robertson that Arthur would continue to mentor Jeanne throughout her baccalaureate education and become a valued collaborator during her later doctoral training including co-authorship of a paper. Her mom, Anne, was an artist, comedian, social worker and teacher, as well as a forward thinker who strongly believed that any child or adult can and should achieve their human potential. She continues to have a profound influence on the way that Jeanne experiences the world, interacts with others, and enjoys red wine.

Jeanne pursued an undergraduate degree in human physiology and spent most of her free time playing varsity soccer and volunteering at the U.C. Davis Medical Center. She worked with Dr. Tissa Kappagoda for over two years in the Division of Cardiovascular Medicine before taking a 6 week leave to pursue a “nagging” interest that developed from years of surfing the chilly Pacific ocean of the Northern California coast – kelp ecology and systematics.
Jeanne took her first Field and Marine Ecology class at Bodega Marine Laboratory in 1996 and remained there to work as a field assistant and research technician. She was fortunate to work closely with highly influential and wonderful mentors, including: Alex Reich, John Maron, Pam Kittelson, Susan Harrison, Dick Root, and Peter Alpert. The salmon genetics lab, headed by Dennis Hedgecock, hired her to work closely with Kate Bucklin and Michael Banks on the population genetics of coho salmon. The experiences at Bodega Marine Lab and the people Jeanne worked with changed her future.

Tropical frogs consumed the next decade of her academic life. It began with an eye-opening experience working on the evolution of parental care in microhylid frogs in Papua New Guinea. Her job as a field assistant to David Bickford, then graduate student of Jay Savage, initiated her training in tropical biology, natural history, and herpetology.

Jeanne had the opportunity to conduct her Masters research with Karen R. Lips at Southern Illinois University, Carbondale. Karen and Jeanne became fast friends during the summer months spent in El Cope, catching herps in one of the most beautiful national parks in Central America. She prepared her to be an independent field researcher, and imparted the skills and wisdom needed for conducting research alone in Central America. Jeanne’s Master’s research on gene flow and individual movement patterns of a stream-breeding glassfrog solidified her academic interests and inspired her to pursue a Ph.D.

Kelly R. Zamudio accepted Jeanne into her lab at Cornell in 2001. A dynamic, invigorating friendship and professional relationship developed quickly and matured beautifully over time. Several memorable experiences marked her graduate student career, including field-work in Brazil with Lauren and Kelly, participation in a field course at Archbold Biological Station and one through the Organization for Tropical
Studies. Jeanne’s field experiences over five summers in Costa Rica and Panama and one month in Atlantic Forest in Brazil have had profound effects on the way she thinks about organisms and evolution.

Jeanne will begin a postdoc with Erica Bree Rosenblum at the University of Idaho and will work on local adaptations and selection in desert lizards in New Mexico. Moving from a tropical lowland forest to a temperate desert should prove an interesting and engaging contrast.

Jeanne’s completion of her Ph.D. was not witnessed by her mother, who passed away two years prior. It is for her mom and dad, the two people who foremost nurtured Jeanne’s growth and supported her interests, that this thesis is dedicated.
To my parents, Anne L. and Arthur Robertson
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It takes a village to do a Ph.D. I have many people to thank for their unique and considerable contributions to the inception, implementation and production of this thesis. First and foremost, Kelly R. Zamudio, deserves a special award for being my major professor. She is a generous mentor, friend, and collaborator. Her standards of excellence and scientific rigor inspired and challenged me to achieve beyond my own vision of what I believed was possible. She brings intellectual curiosity, spirit, and evolutionary theory to life, and remains one of the great role models in my life.

Harry W. Greene’s influence is indelible. Our exchange of thoughts and ideas took shape as I matured as a scientist, and each of those iterations led to important insights and forward movement. Casual conversation and ‘jam sessions’ about the natural history of frogs, lizards and snakes, led me to some of the broader questions of evolutionary biology that I attempted to tackle in my thesis and continue to ponder. I am grateful for his time, energy and investment.

The Zamudio Lab is a dynamic, molecular discoteca, full of intense, bright and enthusiastic graduate students, post-docs, lab managers, sabbatical visitors and undergraduate ‘herp nerds’. The Zamudio Lab graduate students form a charismatic group who foster a collaborative, inventive, creative approach to science and history. I thank each one for their thoughtful comments, spontaneous discussions, and editing of multiple drafts of manuscripts: Cici Coen, Lauren Chan, Kurt Galbreath, Anna Savage, Angie Stevenson, and Elizabeth Kuperberg. Three post-docs, Kyle Ashton, Jim Austin and Jon Richmond and sabbatical visitors Peter Rosenbaum and Andrew DeWoody, brought novel ideas, techniques, analyses and style to the lab. The face of the KZ Lab has changed over time, but each group provided a constant flow of support, good music and good times – for keeping everyone and everything under control, I thank Christine Voyer, Angie Stevenson, and Katie Duryea.
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Faculty members in the department of Ecology and Evolutionary Biology have made important contributions to my development as a scientist. André Dhondt was a member of my dissertation committee and brought a unique perspective that vastly improved the scope of my thesis. He inspired me to think broadly. Monica Geber was an unofficial committee member who has enriched my life with friendship and impromptu discussion about science and life—she is a model of academic excellence and integrity. Rick Harrison has been a special and comforting presence and I’ve appreciated his intensity and honesty. Peter Marks and Dick Root influenced the way I observe nature and translate those observations into research questions. Although not a faculty member, Steve Bogdanowicz made microsatellite library development and optimization a reality and was always ready to jump into the molecular trenches next to me.

For tremendous support, patient help with statistical analyses, and patient help with my writing, I thank Wai-Kwong Wong, Françoise Vermeylen and Giles Hooker, and Barbara LeGendre, respectively. The administrative and building staff are often the first to greet you in the morning. For keeping all things administrative smooth and running, and for the occasional hug, I thank: DeeDee Albertsman, Luanne Kenjerska, Linda Harrington, Rosie Brainerd, Janeen Orr, Patty Jordan, John Howell, Gary Oltz, Brian Mlodinski, and Tim Larkin. Special thanks to Al Hand, Bill Dyer, Sandy Hand and Terry Hamilton for being a part of my everyday in Corson Hall.

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CHAPTER ONE
SELECTION FOR COLOR PATTERN IN TWO LINEAGES OF CREPUSCULAR TREEFROGS: EVIDENCE FOR SPECIES RECOGNITION, APOSEMATIC COLORATION AND CONVERGENCE

ABSTRACT

Most species of frogs exhibit color pattern (CP) polymorphisms. Brightly colored, diurnal frogs have been subject of extensive study of color pattern, resulting in a solid evolutionary framework to test the adaptive significance of coloration in frogs. However, most frogs are nocturnal, many of which are also brightly colored, and the significance of color pattern, has been largely understudied and overlooked. I studied brightly colored, nocturnal treefrogs representing two lineages of treefrog, the phyllomedusines and dendropsophines. Three independent lines of evidence confirm a surprising role of color pattern as a social signal for phyllomedusines: tests of sympatry, visual system discrimination of color pattern, and the disassociation between color pattern and genetic distance. In contrast, color pattern polymorphisms of the Dendropsophinii frogs were consistent with the Convergent Niche Hypothesis, indicating a role of the environment in the maintenance of coloration. Amphibians are experiencing massive and devastating population declines and species extinctions, yet there is much to learn about basic social behavior of anurans. I suggest a general framework for testing hypotheses of the adaptive significance of color pattern that can be applied to most community of anurans.
INTRODUCTION

Frogs are the most successful lineage of extant amphibians. The number of frog species (more than 5,453) is 10 and 31 times larger than the number of salamander and caecilian species, respectively (Frost 2007). In addition to a nearly global distribution, frogs exhibit tremendous reproductive and life history diversity (Wells 1977), are important members of local ecological communities as both prey and predator, and serve as a model organism in developmental biology, evolution, immunology (Rollins-Smith and Conlon 2005; Woodhams et al. 2007) and chemical pharmacology (Macfoy et al. 2005; Saporito et al. 2007). Amphibian populations are experiencing massive and global declines: in the past two decades over 170 species have gone extinct and 32% are currently listed as threatened (AmphibiaWeb 2007). The loss of frog species will have a devastating impact on both ecosystem and human health, as well signifying the loss of cultural and aesthetic beauty, cherished by ancient and contemporary human civilizations.

Most species of frogs exhibit color pattern (CP) polymorphisms (Hoffman and Blouin 2000). Brightly colored, diurnal frogs (members of Dendrobatidae, Brachycephalidae, Atelopus, Hyloides) have been subject to extensive study of color pattern, resulting in a solid evolutionary framework to test the adaptive significance of coloration. However, most frogs are nocturnal, and many are also brightly colored: the significance of color pattern in these frogs have largely been understudied and overlooked. The precipitous decline of amphibian populations has many consequences, one of which is the curtailing of significant advances in behavioral and evolutionary biology. Mate choice and behavioral studies are required to fully understand adaptive coloration and selective forces underlying the evolution of populations and lineages. Yet these studies are time-intensive and would more effective and efficient after initial consideration of multiple hypotheses. I studied
populations of brightly colored, nocturnal frogs and found a surprising role of color pattern in social signaling (Table 1.1). In this paper, I suggest a general framework for testing hypotheses of the adaptive significance of color pattern that can be applied to any anuran community.

Animal coloration evolves through the interactions of natural selection favoring crypsis to avoid predator detection and sexual selection favoring conspicuousness for conspecific recognition. A color pattern signal is considered cryptic if the combined effect of shape, size and brightness represent a random sample of the environment (Cott 1940; Endler 1982). Selection for cryptic coloration often favors several key features including countershading where the dorsal coloration in darker relative to a lighter ventral region (Hailman and Jaeger 1974) as well as disruptive coloration, such as stripes and spots that have high contrast with other parts of the animals body (Schafer & Stobbe 2006; Stevens 2006). Natural selection on coloration for habitat background matching has been well documented in natural populations, including rodents (Hoekstra et al. 2004), lizards (Thorpe and Baez 1993; Thorpe 2002; Rosenblum 2006; Rosenblum et al. 2007; Stuart-Fox et al. 2007), frogs (Pyburn 1961; Nevo 1973; Stewart 1974; Hoffman and Blouin 2000), insects (Kettlewell and Conn 1977; Sandoval and Nosil 2005; Nosil et al. 2006) and snakes (King and Lawson 1995).

Conspicuous coloration, characterized by markings of maximal contrast in size, brightness and/or color, can be a visual signal to conspecifics (intrasexual competition or mate choice) or aposematic warning signal to predators indicating that a predation attempt would be unprofitable (e.g., toxicity, unpalatability; Endler 1980; Kuchta 2005; Darst and Cummings 2006; Darst et al. 2006). Attributes of conspicuous CP include brightly colored regions of the body, that may or may not be hidden at rest, or disruptive markings such as spots and stripes. The most well-studied anuran lineage
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<td><em>H. lemur</em> (hle)</td>
<td>no</td>
<td>yellow</td>
<td>hle</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>P. dacnicolor</em> (pda)</td>
<td>no</td>
<td>none</td>
<td>pda</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>C. calcalifer</em> (cca)</td>
<td>yes</td>
<td>yellow</td>
<td>cca</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

The color of the flank and presence of vertical stripes is indicated for each species. Because of the disjunct distribution of two basic color morphs for *A. callidryas*, the Caribbean (CAR) and Pacific (PAC) morph is analyzed separately. Tabulation of similar (+) or different (-) color pattern for each taxon pair (stripe, color) and whether the pair is sympatric (shaded box) reveals that sympatric taxa are always divergent in at least one color pattern character.
that exemplifies conspicuous color pattern is the Neotropical poison-dart frogs (Dendrobatidae), a family that contains many brightly colored, polymorphic species (Summers and Clough 2001; Summers et al. 2003; Roberts et al. 2007; Wollenberg et al. 2008). Conspicuous coloration effectively minimizes predation due to assumed aposematism (Summers and Clough 2001; Darst et al. 2006). However, behavioral studies showed that sexual selection also underlies color differences among poison-dart frog populations (Summers and Clough 2001; Siddiqi et al. 2004; Summers et al. 2004; Reynolds and Fitzpatrick 2007). Thus, for brightly colored and toxic animals, conspicuous signals can serve multiple functions, for both species recognition and predator deterrence (Summers et al. 1999; Reynolds and Fitzpatrick 2007).

Color pattern often has cryptic and conspicuous features, a duality that must be considered while inferring the adaptive significance of coloration (Endler 1982). For example, dwarf chameleons exhibit cryptic coloration for optimal background matching to avoid predator detection, but also possess the capacity for rapid color change to signal conspecifics (Stuart-Fox et al. 2007; Stuart-Fox and Moussalli 2008). Anoline lizards provide another example of dual color pattern. Body coloration is cryptic while at rest but conspicuous when used for conspecific communication: male lizards have extendable throat ‘fans’ (dewlaps) that are brightly colored, species-specific signals used for territorial displays and mate attraction. These examples illustrate how composite color pattern evolves in response to both natural and sexual selection pressures, a phenomenon widespread across animal taxa (Cott 1940; Endler 1980; West-Eberhard 1983; Gomez and Thery 2007; Gray and McKinnon 2007; Kingsolver and Pfennig 2007; Stuart-Fox and Moussalli 2008).

Color pattern polymorphisms are known from at least 225 species of anurans (Hoffman and Blouin 2000) and thus are ideal taxa for examining the microevolutionary forces that underlie the maintenance of CP within and/or among
populations. The two focal taxa in this thesis, *Agalychnis callidryas* (red-eyed treefrog) and *Dendropsophus ebraccatus* (hourglass treefrog) are wide-ranging Neotropical frogs contained within the large treefrog family (Hylidae), in the Phyllomedusinae subfamily, and Dendropsophinii tribe, respectively. These taxa share many characteristics, including, (1) a broad geographic distribution, ranging from Central Mexico to Colombia (2) a specialized reproductive mode (oviposit eggs primarily on leaves overhanging water) (3) prolonged reproductive season in which males aggregate in temporary pools of water, (4) color pattern polymorphisms and (5) lack of sexual dimorphism for CP. Two distinctions make these species ideal for a comparative study of color pattern: First, *A. callidryas* exhibits marked regional differentiation, while *D. ebraccatus* does not (Savage and Heyer 1967; Savage 2002); Second, *A. callidryas*, like many phylomedusine frogs, is known to contain toxic skin secretions effective for predator defense (Warburg 1965; Sazima 1974; Mignogna et al. 1997; Conlon et al. 2007). Based on studies of other chemically-defended and brightly colored frogs (e.g., diurnal frogs in the Dendrobatidae and Brachycephalidae families (Pombal et al. 1994) it is therefore possible that CP in *A. callidryas* also plays an aposematic role. However, the red-eyed treefrog (and all other phylomedusines) are nocturnal and thus are active at a time when most potential predators cannot distinguish color.

The adaptive significance for coloration in these two frogs are unknown, although two competing hypothesis may lend insight into the selective regimes that underlie CP polymorphisms. The Species Recognition Hypothesis predicts that sympatric species are divergent for traits used as social signals. This hypothesis, formalized by Rand and Williams (1970) was based on dewlap and body characteristics (size, color and shape) of island anole lizards and found that multiple, divergent signals are optimally effective in species discrimination. Several
community-level analyses found that co-distributed species exhibit the strongest divergence in social signal and that divergence increases among closely related taxa (Losos 1985; Harmon et al. 2005). Mate recognition must play a clear role if the hypothesis is correct that divergence of social signals among closely related taxa underlies incipient speciation processes (West-Eberhard 1983; Shaw 1996; Seehausen et al. 1999; Masta and Maddison 2002; Gray and McKinnon 2007; Ritchie et al. 2007).

Alternatively, the Convergent Niche Hypothesis (Grinnell 1924) predicts that multiple, syntopic species will exhibit similar color pattern characteristics if those characters maximize survival in a particular visual environment (Warburg 1965; Rand and Williams 1970; Stewart 1974; Endler 1982). In this case, color pattern is not used for species recognition and/or mate choice, but is an effective signal for predator deterrence, indicating a role of the environment in the evolution of CP (Endler 1982). Convincing support for this hypothesis is illustrated through careful study of Anolis lizards (Harmon et al. 2005). Phenotypic similarity among closely related species could indicate strong selection across the evolutionary history of that group.

The objective of this study was to determine whether the patterns of color polymorphism exhibited by phyllomedusine frogs (including A. callidryas) and Dendropsophinii frogs (including D. ebraccatus) support the Species Recognition Hypothesis or Convergent Niche Hypothesis. Towards this goal, I quantified the likelihood that sympatric taxa are more likely to diverge in social signals than taxa whose geographic range do not overlap. I used Matrix Correspondence Tests to determine if color pattern has a phylogenetic signal by testing the hypothesis that closely related taxa are more similar in coloration than distant congeners. Next, I determined whether CP in the red-eyed treefrog (phyllomedusine) was conspicuous, and could be a conspecific signal by measuring the contrast luminosity of color
patches based on the visual system of *A. callidryas*. For *A. callidryas*, I addressed an untested question of whether bright coloration in a nocturnal frog has evolved as an aposematic trait, species recognition trait, or both. I found corroborating evidence from all tests that color pattern polymorphisms for *A. callidryas* support the Species Recognition Hypotheses but that CP in *D. ebraccatus* is better explained by the Convergent Niche Hypotheses. Based on the functional significance and mode of selection acting on color pattern, I then discuss the possibilities of predicting the consequences of CP for population genetic structure.

**METHODS**

*Study Species*

My goal was to form testable hypotheses of the probable role of color pattern through examination of the ecological community and lineage phylogenetic history. Most phyllomedusine frogs have green dorsal coloration with brightly colored limbs and flanks (hues of yellow, orange, blue), and a few have vertical flank stripes. *Agalychnis callidryas* is one of nine Central American phyllomedusine frogs and the only taxon to exhibit regional differentiation in flank and limb coloration, and flank stripe pattern (Savage and Heyer 1967; Robertson and Robertson 2008). *Dendropsophus ebraccatus* is one of nine Central American taxa contained in the Dendropsophinii tribe (Faivovich et al. 2005). Color pattern for *D. ebraccatus* is highly variable and characterized by yellow, gold and brown blotches and spots with the dominant dorsal pattern in most populations resembling an hourglass shape (Duellman 2001).
Test of Sympatry

Each taxon was coded for color and pattern. For phyllomedusines, I coded basic flank color (blue, yellow or orange) and the presence of vertical flank stripes. Coloration among Dendropsophii included dorsal ground colors (grey, green, yellow or brown) and the shape of the markings (spots, blotches, stripes). Because of the high levels of polymorphisms among dendropsophines, I determined that two species exhibited a similar CP if any of the multiple forms were present for either species. For example, D. ebraccatus and Tlalocohyla loquax were coded as a similar phenotype because one of the four major polymorphic states in D. ebraccatus (plain yellow) is similar to the single yellow form of T. loquax.

Sympatry was determined by examining range distribution maps (Duellman 2001; Savage 2002). For both species, I tested whether two sympatric taxa were more likely to exhibit divergent signals than allopatric taxa. In the first test, I based signal divergence on color (hue) alone. In the next test, signal divergence was based on pattern. In the last test, I used information from both signals and determined that two species were divergent in CP if they differed for at least one of the two traits. Significance was determined using a Fisher’s exact test in R (Team 2005). A significant result provides support for the Species Recognition Hypotheses, whereas no association indicates CP is consistent with the Convergent Niche Hypothesis.

Visual discrimination of color pattern (Agalychnis callidryas)

Color perception depends on the properties of the signal, light conditions, and the sensory capabilities of the intended receiver (Lythgoe and Patridge 1991; Endler 1992). While human observers readily recognize regional color differences for A. callidryas as hues of red, violet, orange, green and blue, the signal must be evident to conspecifics for the color patch to be used as a social signal and evolve through sexual
selection (Endler et al. 2005). The red-eyed treefrog must possess the visual system to
detect and discriminate the signal. That is, the signal must be highly contrasted to
adjacent body regions and background habitat. To test this hypothesis I evaluated the
relative conspicuousness of the signal based on the spectral sensitivities of the visual
system of A. callidryas.

First, I characterized the visual pigments of A. callidryas using
microspectrophotometry. Subjects were dark-adapted overnight to maximize the visual
pigment sensitivity to light. The retinal cell tissue was dissected and viewed with
infrared illumination to quantify the absorbance spectra for photoreceptor cells. The
configuration of visual pigments were then used to construct a camera filter with
spectral sensitivities matching those of the frog, allowing for a direct measure of the
contrast luminosity of individual frogs against a natural background.

I took photographs of frogs from each of the five regions (representing five
distinct phenotypes) in the field. Photographs were imported into Photoshop and
transformed into greyscale images because color discrimination is based on the
contrast luminosity of a ‘target’ relative to the ‘background’, and not based on hue. I
used the color picker function in Photoshop to measure the brightness of several
‘target’ regions of the red-eyed treefrog body (flank, leg, ventral region, vertical stripe,
eye) as well as several points from the ‘background’, including the body and the leaf it
was sitting upon. For each frog, I measured the contrast between the frog body part
and the background and tested for significant differences in luminosity using the
following equation: \[ 1.96 \geq \frac{L_t - L_b}{\sqrt{\sum \sigma (L_{t\text{avg}})}} \], where \( L_t \) = luminosity of the target (color
patch), \( L_b \) = luminosity background (Lythgoe and Patridge; 1991). A contrast ratio
greater than 1.96 indicates sufficient discrimination based on the visual spectral
sensitivities of the species (Lythgoe and Patridge 1991).
I used an Ocean optic 2000 spectroradiometer (integration time = 50 msec) with a 400 micron bifurcated fiber laser and standardized against a white-grey card to determine the wavelength of color reflected by *A. callidryas*. I also determined if there were markings in the UV spectrum that would be visible to potential avian predators, indicating a role of natural selection in determining an color pattern that is not apparent to human observers (Thorpe and Richard 2001).

*Evolutionary patterns of color pattern*

I used matrix correspondence tests (MCT) to evaluate the association between genetic relatedness (mtDNA) and two components color pattern (hue and stripes). Matrix Correspondence Tests use repeated randomization and recomputation to test for the correlation between two distance matrices by comparing the individual pairwise distance for each parameter (Manly 1986). The randomized values provide a null distribution with which to test the hypothesis of no association. Significance values were determined by comparing the observed and expected z-statistic, generated by 10,000 permutations.

I constructed a pairwise genetic distance matrix based on the patristic distance, implemented in TreeEdit Version 1.0a10 (Rambaut and Charleston 2001). I examined two color pattern traits, hue and stripes, to account for the possibility that each trait varies under different selection regimes. I constructed pairwise distance matrices based on Euclidian distance of hue, measured in degrees. A distance matrix for stripes was based on a binary code, 1 (stripe) and 0 (no stripes). Because *A. callidryas* is the only phyllomedusine to exhibit within-taxon polymorphisms, I conducted two separate tests by coding *A. callidryas* flank coloration as blue and orange. A non-significant correlation indicates the genetic independence of color pattern among phyllomedusines.
RESULTS

Tests of Sympatry

For phyllomedusines, I found that sympatric taxa were highly divergent in CP when considering the combined signal (color + stripe; \( p = 0.003 \)), but were not divergent in flank coloration alone (\( p = 0.495 \)) or when considering the presence of vertical stripes alone (\( p = 0.085 \)). These findings are consistent with the prediction that multiple visual signals act in concert for species discrimination (Rand and Williams 1970). Thus, sympatric species are more likely to diverge in color and pattern than allopatric species, providing supporting evidence for the Species Recognition Hypothesis but not the Convergent Niche Hypothesis.

Many Dendropsophini taxa are yellow or brown and polymorphic for dorsal pattern (Table 1.2). Sympatric taxa were not divergent in any of the following CP combinations: color alone (\( p = 0.200 \)), dorsal pattern alone (\( p = 0.738 \)) or the combined signal of color and pattern (\( p = 0.741 \)). The sympatric distribution of a similar color pattern supports the Convergent Niche Hypothesis for Dendropsophini frogs and indicates that the evolution of this type of color pattern is more likely associated with environmental features (e.g., predator pressures, crypsis, or conspicuous predator avoidance) than species recognition or mate choice.

Visual discrimination of color pattern (Agalychnis callidryas)

*Agalychnis callidryas* possesses the visual system to discriminate both the hue and vertical stripes characteristic of the species. I identified spectral properties from three photopigment spectral classes, including two green rods having peak absorption at 515 nm and 525 nm and one blue cone having peak absorption at 437 nm. I confirmed that this species does not have infrared or UV vision.
TABLE 1.2. Color pattern for nine Dendropsophinii species in three genera (*Dendropsophus, Isthmyohyl, Tlalocohyla*) in Central America.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Color</th>
<th>Pattern</th>
<th>deb</th>
<th>dmi</th>
<th>dph</th>
<th>dsa</th>
<th>sel</th>
<th>sbo</th>
<th>sst</th>
<th>ips</th>
<th>tlo</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. ebraccatus</em> (deb)</td>
<td>yellow</td>
<td>brown spots, blotches, or plain</td>
<td>deb</td>
<td>+/+</td>
<td>+/+</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td><em>D. microcephalus</em> (dmi)</td>
<td>yellow</td>
<td>spots, blotches, stripes, plain</td>
<td>dmi</td>
<td>+/+</td>
<td>+/-</td>
<td>+/-</td>
<td>+/+</td>
<td>+/+</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td><em>D. phlebodes</em> (dph)</td>
<td>yellow/tan</td>
<td>brown markings</td>
<td>dph</td>
<td>-/+</td>
<td>+/+</td>
<td>+/-</td>
<td>+/+</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td><em>D. sartori</em> (dsa)</td>
<td>brown, grey</td>
<td>dark blotches</td>
<td>dsa</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td><em>S. elaeochroa</em> (sel)</td>
<td>yellow, tan, olive green</td>
<td>dark striped markings</td>
<td>sel</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td><em>S. boulengeri</em> (sbo)</td>
<td>green/tan</td>
<td>dark stripes</td>
<td>sbo</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td><em>S. staufferi</em> (sst)</td>
<td>grey</td>
<td>dark striped markings</td>
<td>sst</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
</tr>
<tr>
<td><em>I. pseudopuma</em> (ips)</td>
<td>yellow, brown, green</td>
<td>blotches, stripes, plain</td>
<td>ips</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
</tr>
<tr>
<td><em>T. loguax</em> (tlo)</td>
<td>yellow</td>
<td>plain</td>
<td>tlo</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
</tr>
</tbody>
</table>

The dorsal color and pattern is described for each species. Tabulation of similar (+) or different (-) color pattern for each taxon pair (color, pattern) and whether the pair is sympatric (shaded box) reveals no association between divergence in color pattern and sympathy.
The vertical stripes and ventral aspect of the frog showed the greatest contrast luminosity relative to both the background and the rest of the frog body, indicating that these regions are likely important visual signals for conspecific recognition (Table 1.3). The luminosity of ‘orange’ and ‘blue’ were also sufficiently contrasting and could be discriminated by *A. callidryas* (Table 1.3).

I found no evidence of markings that reflected light in the UV spectrum. The ‘orange’ coloration reflected a true orange. However, the ‘blue’ reflected light in the green wavelength. Thus, for *A. callidryas* the ‘blue’ observed for some populations is actually the same pigment as the green covering the frog back and posterior surface of limbs, while the ‘orange’ coloration requires the presence of a new pigment.

*Evolutionary patterns of color and toxicity*

Matrix Correspondence Tests revealed that flank color and the presence of flank stripes cannot be predicted based on genetic distance among phyllomedusine frogs tested in this study. Specifically, I found no association between genetic distance and orange flanks (r = 0.479, p = 0.061), blue flanks (r = 0.366, p = 0.073), or flank stripe (r = 0.359, p = 0.108). Significance was adjusted following Bonferroni correction, with the probability of a type 1 error set to alpha = 0.012.

**DISCUSSION**

Our data confirm that pattern polymorphisms in phyllomedusine frogs are signals used for species recognition and thus could evolve through sexual selection. This conclusion is based on three independent lines of evidence: tests of sympatry, visual discrimination of color pattern, and the disassociation between color pattern and genetic distance. For sexual selection to drive signal divergence among populations,
<table>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>chg</td>
<td>Southeast CR</td>
<td>orange</td>
<td>blue</td>
<td>2.50</td>
<td>3.42</td>
<td>8.74</td>
<td>25.74</td>
<td>-16.79</td>
<td>-9.19</td>
<td>na</td>
</tr>
<tr>
<td>val</td>
<td>Panama</td>
<td>orange</td>
<td>blue</td>
<td>0.47</td>
<td>-4.05</td>
<td>-1.03</td>
<td>2.59</td>
<td>-8.48</td>
<td>-7.21</td>
<td>7.07</td>
</tr>
<tr>
<td>gam</td>
<td>Panama</td>
<td>orange</td>
<td>blue</td>
<td>-0.29</td>
<td>3.39</td>
<td>1.74</td>
<td>13.70</td>
<td>-10.06</td>
<td>-6.08</td>
<td>4.00</td>
</tr>
<tr>
<td>siq</td>
<td>Northeast CR</td>
<td>blue</td>
<td>blue</td>
<td>6.54</td>
<td>7.9</td>
<td>30.26</td>
<td>16.29</td>
<td>5.04</td>
<td>8.16</td>
<td>na</td>
</tr>
<tr>
<td>siq</td>
<td>Northeast CR</td>
<td>blue</td>
<td>blue</td>
<td>-1.81</td>
<td>1.75</td>
<td>18.52</td>
<td>5.21</td>
<td>-2.99</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>lse</td>
<td>Northeast CR</td>
<td>blue</td>
<td>blue</td>
<td>0.70</td>
<td>-4.96</td>
<td>na</td>
<td>15.03</td>
<td>-23.97</td>
<td>-9.16</td>
<td>na</td>
</tr>
<tr>
<td>car</td>
<td>Northwest CR</td>
<td>orange</td>
<td>brown</td>
<td>2.04</td>
<td>3.81</td>
<td>33.07</td>
<td>15.85</td>
<td>-12.13</td>
<td>-8.91</td>
<td>na</td>
</tr>
<tr>
<td>car</td>
<td>Northwest CR</td>
<td>orange</td>
<td>brown</td>
<td>4.24</td>
<td>0.20</td>
<td>38.64</td>
<td>22.97</td>
<td>-17.23</td>
<td>-15.40</td>
<td>na</td>
</tr>
</tbody>
</table>

The test value for significant difference in brightness between ‘target’ and ‘background’ areas is a value > 1.96 (bolded). na = not available. For individuals with different leg and flank coloration, the contrast between those two regions of the frog are flank:leg.

TABLE 1.3. Brightness contrast measures for nine *Agalychnis callidryas* sampled from five regions in Costa Rica and Panama.
and eventually lead to speciation, several criteria must be met: 1) signals are perceived by conspecifics 2) signals must differ among populations and result from selection and not drift, 3) females must prefer native signals over non-native signals (Boul et al. 2007). I have determined that CP is highly regionalized and that the visual system of this species adequately discriminates CP. In this thesis, I use molecular markers (mtDNA and ncDNA) to confirm that population differentiation cannot be explained by non-adaptive processes alone, invoking the role of selection. Subsequent behavioral studies are required to confirm that female choice is based on CP.

Many chemically defended amphibians exhibit conspicuous, aposematic coloration that effectively deters predators (Summers and Clough 2001; Pires et al. 2002; Darst et al. 2006; Conlon et al. 2007). However, because signal divergence among sympatric taxa was evident, aposematic coloration cannot be the only explanation for bright coloration. It is likely that the conspicuous coloration could serve dual purposes (mate choice and predator deterrence) for the red-eyed treefrog, as shown for other aposematic frogs (Siddiqi et al. 2004; Summers et al. 2004; Rudh et al. 2007).

For the Dendropsophinii frogs, color pattern polymorphisms were consistent with the Convergent Niche Hypothesis. Blotches and spots (widespread across members of the clade, including Dendropsophus, Isthmyohyl, and Tlalocohyla species) are a form of disruptive coloration utilized by insects and vertebrates for crypsis against a heterogeneous background. The maintenance of a relatively conserved color pattern within a tribe indicates that this phenotype has been under selection and that this group has not undergone adaptive and divergent radiation for this particular phenotype. Our analyses indicate that CP in D. ebraccatus serves as a signal to escape predation, and thus could evolve through natural selection regimes. Studies of the visual system of predators against the natural background are required to determine
whether the CP in the hourglass treefrog is conspicuous or cryptic. I have no evidence that D. ebraccatus is chemically defended, but if this were true, it would support the notion that coloration in this species is conspicuous.

**Color pattern and Genetic Structure**

Is it possible to predict the genetic population structure of a taxon based on the adaptive function of its coloration and mode of selection? The spatial distribution of phenotypic traits reflects a balance between divergent localized selection, drift and the homogenizing effects of gene flow (Endler 1973, 1980; Gray and McKinnon 2007). Predictions of genetic structure are initially based on the biogeographic history of populations, a species dispersal capacity, and the connectivity of populations. In the absence of localized, divergent selection, genetic and phenotypic distance measures should be congruent such that populations experiencing gene flow will be similar in coloration (Wright 1937; Ritchie et al. 2007; Roberts et al. 2007). Departures from this null model indicate a role of localized selection (Endler 1982; Lenormand 2002; Saint-Laurent et al. 2003; Price 2006; Harper and Pfennig 2008). Whether the causative agent of phenotypic diversification among populations is a predator or conspecific, the combined effects of geographic history, genetic drift, selection and the natural history traits of the species results in a mosaic of differentiated populations.

*Thesis overview: color pattern, genetic structure, selection, and historical biogeography*

The adaptive significance of coloration was not the objective of this thesis. However, quantification of the nature and geographic distribution of CP within and among populations is the requisite first step for subsequent hypothesis testing on the mode of selection on maintaining CP (Endler 1982; Gray and McKinnon 2007). The
objectives of this thesis were to quantify the geographic patterns of phenotypic and genetic diversity in two focal taxa, across 21 populations in Costa Rica and Panama to determine whether: 1) spatial patterns of phenotypic and genetic diversity were congruent 2) landscape features limited gene flow 3) the complex biogeographic history of Central American Isthmus populations for two frogs have resulted in a similar evolutionary history of vicariance and dispersal and 4) gene flow patterns explained the geographic distribution of phenotypic diversity. I examined the geographic distribution of genetic and phenotypic diversity through a historical and spatial approach to understand the evolutionary processes driving the maintenance of polymorphisms across a complex landscape. I focused on evaluating the role of gene flow in determining the population structure of both taxa and determined cases where gene flow processes alone cannot explain the distribution of phenotypic diversity. It is in these instances that I could invoke different modes of selection to sustain polymorphism for both taxa. This approach provides a comprehensive understanding of the historical and microevolutionary processes that underlie biological diversity.
LITERATURE CITED

AmphibiaWeb: Information on amphibian biology and conservation. [web


activity from skin secretions of the lemur leaf frog *Hylomantis lemur* (hylidae:


Darst, C. R., and M. E. Cummings. 2006. Predator learning favours mimicry of a less-

Darst, C. R., M. E. Cummings, and D. C. Cannatella. 2006. A mechanism for diversity
in warning signals: Conspicuousness versus toxicity in poison frogs. Proc.
Natl. Acad. Sci. USA 103:5852-5857.

Duellman, W. E. 2001. The hylid frogs of Middle America. Society for the Study of
Amphibians and Reptiles, Ithaca, New York.


---. 1980. Natural selection on color patterns in *Poecilia reticulata*. Evolution 34:76-
91.

---. 1982. Convergent and divergent effects of natural selection on color patterns in 2
fish faunas. Evolution 36:178-188.

139:S125-S153.

systems and the evolution of color patterns: Sensory processing illuminates

Wheeler. 2005. Systematic review of the frog family Hylidae, with special


CHAPTER TWO
SPATIAL AND TEMPORAL PATTERNS OF PHENOTYPIC VARIATION IN A NEOTROPICAL FROG

ABSTRACT

Studies of the spatial and temporal patterns of phenotypic diversity help to elucidate the fine-scale evolutionary and ecological mechanisms underlying geographic differentiation. The red-eyed treefrog, *Agalychnis callidryas*, is a widespread Neotropical frog that exhibits a broad range of coloration and flank-stripe pattern polymorphism. The goal of this study was two-fold: first, to investigate the stability of polymorphisms over a 38-yr period; and second, to evaluate biogeographic hypotheses of diversification among lower Central American populations through quantification of phenotypic diversity on a fine geographic scale. We quantified color, categorized flank-stripe pattern from digital photos taken during field sampling, and measured body size for each individual. We compared the regional frequency of each flank-stripe pattern in 2005 to the frequency distribution from a previous study of the same sites in 1967 using logistic regression analyses. We determined the geographic signal of leg coloration by employing linear discriminant function analyses to generate a classification matrix based on co-variance similarities, and by comparison of the average hue values within and among regions. We found a temporal shift in the frequency of flank-stripe patterns in three of four regions over 38 years. Based on measures of leg coloration, the frequency distribution of flank-stripe patterns, and body size, we conclude that *A. callidryas* populations are easily distinguishable at a regional scale. *Agalychnis callidryas* exhibits regional differentiation in all phenotypic traits measured in this study, supporting the role of three major biogeographic barriers
to gene exchange. We found evidence of a putative contact zone between polytypic regions in Costa Rica. In addition, we report temporal instability of the relative frequency of stripe patterns located on the flanks. The ecological and evolutionary mechanisms that may underlie this variation include sexual selection and predator avoidance.

**INTRODUCTION**

Spatial and temporal patterns of phenotypic diversity and the underlying ecological and evolutionary processes that produce them provide important insights into the biogeographic history of a species (Grinnell 1924; Endler 1973; Velez and Feder 2006). All taxa exhibit some level of individual or population variation, but some species are highly polytypic across their range, or exhibit geographic clines in body size, behavioral, coloration, and ornamentation (Nevo 1973; Gray 1983; Brooks and Endler 2001; Storz et al. 2001). Phenotypic diversity may vary among regions due to demographic factors (e.g., effective population size), diversifying selection, historical biogeography, or the isolating effects of restricted gene flow (Grinnell, 1924; Pyburn, 1961a; Endler, 1973; Hairston, 1979). Tracking this diversity over time allows us to observe the relative stability of these demographic, selective, or migration forces acting upon populations (Grinnell 1924; Pyburn 1961a; Holt et al. 2004; Blanco et al. 2005; Prieto et al. 2005; Grant and Grant 2006). Studies of spatial and temporal variation provide insights into taxon diversification, and ultimately, processes leading to speciation (Irwin et al. 2001).

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

Here, we investigate patterns of phenotypic diversity in the red-eyed treefrog, *Agalychnis callidryas* Cope 1862 (Anura: Phyllomedusa: Hylidae), a species distributed from Central Mexico to Panama that exhibits striking regional differentiation in flank and leg coloration (Savage and Heyer 1967; Duellman 2001). *Agalychnis callidryas* is one of few Neotropical frogs to exhibit low within-population variation but high among-population variation (Summers et al. 2003; Richards and Knowles 2007). These color differences are sufficiently dramatic that Northeastern and Western Costa Rican populations were once considered different species (Funkhouser 1957; Savage and Heyer 1967). The red-eyed treefrog exhibits sexual dimorphism in size (females are larger and heavier), but no sexual dimorphism in coloration or pattern. Phenotypic diversity of this species was studied almost 38 years ago (Savage and Heyer 1967), permitting a study of the geographic distribution of phenotypic diversity and its stability over time.
In this study, we quantify the current geographic distribution of three phenotypic traits in populations of *A. callidryas* in Costa Rica and Panama and examine temporal variation in one of the traits, flank-stripe pattern, after 38 years. These study populations represent 25% of the geographic range of the species, yet contain all of the known color variation, thus providing an excellent opportunity to examine spatial and temporal processes acting at a fine geographic scale. We examine flank-stripe pattern, color differentiation, and body size among focal populations.

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

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

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

**MATERIAL AND METHODS**

*Study Sites*

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

Sampling sites were selected to test four biogeographic hypotheses of regional differentiation in the red-eyed treefrog. We compare diversity patterns between the Pacific and Caribbean regions to test the isolating role of the Cordillera de Talamanca (Figure 2.1). For the second biogeographic barrier (Gulfo Dulce), we sampled populations from both the Osa and Burrica Peninsula (Figure 2.1). Finally, we compared Northeast and Southeast CR/PA populations to test a putative break at Limón, CR. To investigate a possible contact zone between Northeast and Northwest CR populations, we sampled two mid-elevation sites situated along this potential corridor (Til and Sra).

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

**Flank-Stripe Pattern**

*Agalychnis callidryas* shows bright, contrasting flank-stripes, usually white to pale yellow, overlaying the background color. We implemented the scoring system designed by Savage and Heyer (1967) to categorize individuals as possessing one of five flank-stripe pattern types: A, AB, B, BC, and C (Figure 2.2). In 2005, we discovered frogs with two novel combinations of the three basic types; for these individuals, we modified the Savage and Heyer protocol and categorized them as AC or ABC (Figure 2.2). Individuals sampled in both time periods often exhibited different patterns on the two sides of their body (Savage & Heyer 1967). Due to high rates of asymmetry, we analyzed only the left side of the body.

**Measuring Coloration**

In life, red-eyed tree frogs are bright green dorsally, have large red eyes and orange-red feet and hands. Coloration of flanks and limbs are very similar for an individual in most regions, thus we only measured and report leg color in this study.

Many studies of coloration use spectral reflectance (Summers et al. 2003; Hofmann et al. 2006; Vercken et al. 2006), which yields precise measures of hue, saturation, and brightness at focal points of interest. This technique yields highly accurate results for quantifying color, especially when hue does not vary within an
Grey shaded region shows topographic relief. Sites sampled in 2005 are close to localities sampled in a previous study of polymorphism (Savage & Heyer, 1967). Inset shows detailed view of Southwestern CR. See Appendix 1 for sample locality abbreviations.

**FIGURE 2.1.** *Agalychnis callidryas* Cope 1862 sampling localities from four regions in Costa Rica (CR) and Panama (PA): Northeastern CR (NE CR), Southeastern CR/PA (SE CR/PA), Western CR, and Central PA.
Pattern A individuals have a horizontal line connecting all vertical stripes, in pattern B, the vertical stripes are disconnected, and each is ‘T’ shaped, and in pattern C the disconnected vertical stripes have no ‘T’ shape. Individuals with a combination of these three basic pattern types are characterized as AB (both A and B stripes) or BC. This study identifies two novel pattern types, ABC and AC, not observed by Savage and Heyer (1967).

FIGURE 2.2. Flank pattern variation in *Agalychnis callidryas.*
individual. The greatest advantage of our method of quantifying color is that we are able to accurately measure multiple hues occurring across the entire surface area of an organism, as opposed to sub-sampling color patches. The use of the grey-card color standard provides necessary and sufficient standardization to accurately differentiate hue. In addition, the use of digital photography is increasingly utilized to document animal coloration (Stevens et al., 2007; Richards and Knowles 2007; Touchon & Warkentin, personal communication) digital cameras are a cost-effective alternative for quantifying color variation and are easily used in field conditions.

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

The number of dominant leg colors of *A. callidryas* varies regionally; individuals from some populations are virtually monochromatic (e.g., blue), others contain two dominant colors (e.g., blue and orange), while others contain a continuum of multiple hues (e.g., reddish blue through greenish blue). To avoid a sampling bias, we therefore selected the entire posterior surface of the leg in ImageJ (as opposed to focal subsampling) to acquire a frequency histogram of the number of pixels for each hue (0 – 255), corresponding to 8-bit hue values of 360. We were careful to exclude sampling the green portion of the leg common to all individuals (see Figure 2.3 for example). We transformed the ImageJ hue data (which ranges from 0-255) to the more conventional standard measure of hue with a range of 0-360. Because of the broad range of leg coloration in the red-eyed treefrog, we divided the 360-degree color
spectrum into 8 equal color bins, each spanning 45 degrees (Figure 2.3). Each color bin has a central hue, surrounded by a gradient of neighboring hues. The eight color bins in this study are named according to the central hue for that bin (measured in degrees): red (-337.5 – 22.5), orange (22.6 – 67.5), yellow (67.6 – 112.5), green (112.6 – 157.5), light blue (157.6 – 202.5), dark blue (202.6 – 247.5), purple (247.6 – 292.5) and violet (292.5 – 337.5). The standard hue definition of pure red is zero, therefore the red bin spans 22.5 degrees on each side of zero degrees. Our transformations from the ImageJ data also eliminated any bright white overexposed (blown-out) regions of the photograph that come from light reflection of the wet body of the frog.

STATISTICAL ANALYSES

Flank-Stripe

We measured both temporal and spatial variation in flank-stripe pattern diversity. For the temporal analyses, we tested whether the relative frequency of each pattern differed between 1967 and 2005 within each region using a chi-square contingency test in JMP (Vers. 5.1.2). Due to low sample sizes in some categories, we performed an exact test for a measure of significance of the chi-square test (StatXact Ver. 4, 1998, Cytel Software Corporation, Cambridge, MA 02139). Because the 1967 study did not contain AC or ABC phenotypes, we analyzed temporal patterns in two ways: first, we excluded AC and ABC individuals from 2005 for a direct comparison with the 1967 dataset; second, we consider the possibility that ABC and AC patterns were actually present in 1967, but scored as AB or A, respectively. We repeated the analyses with those individuals in the study but rescored accordingly.
Western CR exhibits a clinal change in coloration, therefore we provide photo images of the northernmost population (Playa Bandera) and southernmost population (Pavones). For each photograph, the corresponding LEG-AREA histogram obtained from hue analyses in ImageJ is transformed to traditional 360 color range. A thin black line outlining the thigh shows color selection analysed in ImageJ. Histogram bars are coloured based on corresponding 360 color range. Color bins are: red (RE), orange (OR), yellow (YE), green (GR), light blue (LB), dark blue (DB), purple (PU), violet (VI). The exact numerical range for each color bins are in the text.

FIGURE 2.3. Color polymorphism in the posterior surface of the thigh of *Agalychnis callidryas* from four regions throughout Costa Rica and Panama.
For the spatial analyses, we tested whether the distribution of flank-stripe patterns differed both within and among regions sampled in 2005 (using chi-square contingency tests and exact test for correction of low sample sizes). In addition, we applied these analyses to the 1967 dataset to test for patterns of differentiation among regions sampled in the Savage and Heyer study (1967).

**Coloration**

We tested for differences in leg color within and among regions sampled in 2005. We analyzed leg coloration in two ways. First, we measured the percent of leg area in each color bin, hereafter referred to as LEG-AREA; second, we compared the mean population hue for each of 8 color bins. To test for population and regional differences in LEG-AREA, we used linear discriminant analysis, which compares each individual to the group multivariate mean (JMP Vers. 5.1.2). A classification matrix shows the number of individuals correctly assigned to source populations and the number of individuals misclassified to an alternate population, based on LEG-AREA alone. We used chi-square tests to determine whether individual assignment was random with respect to source populations for each population and each region. Accurate assignment indicates that LEG-AREA has diagnostic value for regional identification and differentiation. In some cases, LEG-AREA was insufficient to unambiguously assign individuals to the correct population and/or region. For these cases, we compared the average hue among regions using Kruskal-Wallis non-parametric comparison of means implemented in JMP Vers. 5.1.2. We used discriminant analysis to test the independence of flank pattern and leg coloration. We tested the correlation between flank pattern and leg coloration (using chi-square contingency tests and exact test for correction of low sample sizes) by categorizing leg color into four bins: blue, blue and orange, orange, purple. We also used discriminant
function analysis to test whether flank pattern is predicted for each individual (regardless of population of origin) based on LEG-AREA of blue, orange, and purple.

**Body Size**

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

**RESULTS**

*Flank-Stripe*

*Temporal Pattern*

The regional distribution of flank-stripe patterns has changed significantly over the last 38 years in all regions except Western CR (significance of exact test of chi-square: $p = 0.0902$; Northeastern CR, $p < 0.001$; Southeastern CR/PA, $p < 0.001$; Central Panama, $p < 0.001$; Figure 2.4). Repeating those analyses with ABC/AC individuals rescored as AB and A, respectively, did not change those results. Changes in this phenotype over time include a shift in the frequency of the dominant flank pattern: A to AB in Northeastern CR; AB to BC in Southeastern CR/PA; AB to C in Panama. In addition, we observed the loss and gain of patterns, including, a gain of AB in Western CR, a gain of BC in Northeastern CR, a gain of C and loss of A in the Southeastern CR/PA, and loss of A and B in Panama. Therefore, although populations continue to be significantly distinct based on flank-stripe patterns, these patterns are not static even over relatively short time frames.
Sampled in 1967 (grey bars) and 2005 (black bars). All regions except Western CR show temporal change in flank-stripe frequencies. Sample sizes provided in supplemental material SI Table 1.

**FIGURE 2.4. Temporal and spatial variation in the flank-stripe pattern in *Agalychnis callidryas* across four regions.**
Spatial Pattern

In 2005, all regions are distinguished from each other by different dominant pattern(s) ($X^2_{df=18} = 384.08, p < 0.001$): pattern C in Western CR; pattern AB in the Northeastern CR; BC in the Southeastern CR/PA, and a high proportion of both BC and C in Central Panama (Figure 2.4). Western CR is readily distinguishable from the other three regions based on the near fixation of pattern C and the absence of novel types ABC and AC, which are present in all other regions. These results are similar to our analyses of among-region variation in 1967 dataset ($p < 0.001$).

We detected flank-pattern differences between the two Central Panama populations ($X^2_{df=2} = 23.458, p < 0.001$), but no differences among populations within the other three regions: Western CR ($X^2_{df=3} = 6.36, p = 0.095$); Northeastern CR ($X^2_{df=6} = 6.324, p = 0.38$), Southeastern CR/PA ($X^2_{df=6} = 11.409, p = 0.076$).

COLORATION

Among-Region Variation

Our results show regional differentiation in leg coloration. The only individuals in the study with completely blue legs were found in Northeastern CR (Figure 2.5). Individuals with bi-colored legs (red/orange and blue; Figure 2.5) occurred in two regions, Southeastern CR/PA and Central PA. Western CR contains the only populations with solely red/orange legged individuals.
For each population, individuals are aligned along the horizontal axis. LEG-AREA for each individual is represented in a stacked, vertical histogram. Populations are arranged in a West to East direction for the three Caribbean regions and arranged in a North to South direction for the Pacific region. Sample sizes (x) are provided for each population.

FIGURE 2.5. The proportion of the leg (measured as % pixels) assigned to each of eight color bins (LEG-AREA) for 12 populations of *Agalychnis callidryas*. 
We observe a west–east transitional change in coloration among populations along the Caribbean coast; the western-most site contains individuals with completely blue legs while individuals from the eastern-most populations have primarily red/orange legs with some light blue coloration (Figure 2.5). Between these two regions, individuals exhibit a near equal mix of red/orange and dark blue coloration on their legs.

Discriminant function analyses on LEG-AREA resulted in classification of most individuals to the source region ($X^2_{df=16} = 530.82, p < 0.001$; Table 2.1). Discriminant function analyses classified Northeastern CR individuals correctly in all cases (Table 2.1). Some individuals from Western CR were misclassified as Central PA, and vice-versa. Individuals from Southeastern CR/PA were assigned incorrectly to Central PA, but with no reciprocity in misclassification (Table 2.1). To distinguish among these regions (with misclassified individuals), we compared the average regional hue in each of 8 color bins. First, we compared Pavones (Western CR population with a high misclassification rate) to three regions: Southeastern CR/PA, Central Panama, and the other populations in Western CR. We found Pavones was distinguishable from the others based on three color bins: red ($X^2_{df=3} = 101.11p < 0.001$), orange ($X^2_{df=2} = 49.23, p < 0.001$), and violet ($X^2_{df=3} = 27.38, p < 0.001$; Table 2.2). Next we compared Western CR (excluding Pavones) and Central Panama and found average hue differences in 2 color bins: red ($X^2_{df=1} = 16.27p < 0.001$) and yellow ($X^2_{df=1} = 7.96p =0.004$; Table 2.2). Finally, a comparison between Southeastern CR/PA and Central PA revealed differences in average red ($X^2_{df=1} = 30.07, p < 0.001$), light blue ($X^2_{df=1} = 19.33, p < 0.001$), and dark blue ($X^2_{df=1} = 11.12, p = < 0.001$) bins (Table 2.2).

The association between flank pattern and leg coloration varies regionally (Figure 2.6): flank pattern C (occurs primarily in Western CR) contains mostly orange
legged-individuals, whereas flank pattern A (Northeastern CR) is observed with only blue-legged individuals. However, the other flank patterns co-occur with 3 of 4 leg color types. There is a correlation between leg color and flank pattern when considering all individuals in the study (Rsquare = 0.479, $X^2_{df=18} = 368.52$, p < 0.001). This correlation is largely due to the near fixation of flank pattern C in Western CR (dominated by orange legs; Figure 2.6). We repeated this analysis after removing individuals with flank pattern C and found a weaker correlation (Rsquare = 0.271, $X^2_{df=8} = 91.409$, p < 0.001). Overall, these findings are consistent with results from the discriminant function analyses which correctly assigned individuals to flank pattern based on LEG-AREA ($X^2_{df=16} = 361.59$, p < 0.001).

**Within-region variation**

We detected among-population differentiation in coloration for all regions. This is most evident in Western CR, where populations exhibit a north-south cline in coloration (LEG-AREA: $X^2_{df=9} = 77.54$, p = 0.001; Figure 2.5): individuals have red/orange legs in Playa Bandera; Sierpe contains red/orange/green legged individuals; Campo contains red/orange/green/light blue legged individuals; and Pavones (the most southern Western CR site) contains individuals with coloration in almost all color bins (Figure 2.5).
### TABLE 2.1. Classification matrix based on discriminant function analyses of LEG-AREA.

<table>
<thead>
<tr>
<th>Population $i$</th>
<th>SE CR/PA</th>
<th>Central PA</th>
<th>Western CR</th>
<th>NE CR</th>
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<tr>
<td></td>
<td>Alm</td>
<td>ChG</td>
<td>Man</td>
<td>ElV</td>
<td>Gam</td>
</tr>
<tr>
<td>SE CR/PA</td>
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<td></td>
<td></td>
<td></td>
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<td>[2]</td>
<td>[1]</td>
<td>[1]</td>
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<tr>
<td>ChG</td>
<td>[6]</td>
<td>[6]</td>
<td>[5]</td>
<td>[1]</td>
<td>[2]</td>
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<tr>
<td>Man</td>
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<td>Central PA</td>
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<tr>
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<td>[0]</td>
<td>[2]</td>
<td>[11]</td>
<td>[5]</td>
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<tr>
<td>Gam</td>
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<td>[0]</td>
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<td>[10]</td>
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<td>[3]</td>
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<td>NE CR</td>
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</table>

The number of individuals sampled from population ($i$) on vertical axis, into population ($j$) on horizontal axis. The dark-grey shaded boxes show the number of individuals correctly assigned to the source population; the light-grey bars show correct assignment to source region. The unshaded boxes show the number of incorrectly assigned individuals. Total sample size per population, N. See Appendix S1 for population abbreviations. SE = Southeastern, NE = Northeastern. N = sample size.
### TABLE 2.2 The mean region and population hue for 8 color filters.

<table>
<thead>
<tr>
<th>Region</th>
<th>RE</th>
<th>OR</th>
<th>YE</th>
<th>GR</th>
<th>LB</th>
<th>DB</th>
<th>PU</th>
<th>VI</th>
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<tr>
<td>Western CR</td>
<td>10.89</td>
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<td>PlB</td>
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<td>183.71</td>
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</table>

Each color filter spans 45 degrees, see text). See Appendix S1 for population abbreviations and Figure 3 for sample sizes. Color bin abbreviations: red (RE), orange (OR), yellow (YE), green (GR), light blue (LB), dark blue (DB), purple (PU), violet (VI).
All Northeastern CR populations contain individuals with blue leg coloration; however, La Selva is distinct from San Ramon and Tilarán in both analyses of LEG-AREA (p < 0.001) and average hue in two color bins, dark blue ($X^2_{df=2} = 14.28$, p = 0.0008) and purple ($X^2_{df=2} = 12.06$, p = 0.0024; Table 2.2). Southeastern CR/PA populations contain individuals with bi-colored legs (red/orange and blue); based on LEG-AREA, most individuals were correctly assigned to their source population ($X^2_{df=4} = 23.77$, p < 0.001; Table 2.1). Further, we detected fine-scale hue differences among Southeastern CR/PA populations in 5 color bins: red ($X^2_{df=2} = 11.84$, p = 0.002), orange ($X^2_{df=2} = 10.84$, p = 0.004), yellow ($X^2_{df=2} = 7.69$, p = 0.02), light blue ($X^2_{df=2} = 9.38$, p = 0.009), and violet ($X^2_{df=2} = 7.6$, p = 0.02; Table 2.2). Central PA populations are distinct from each other based on both LEG-AREA ($X^2_{df=1} = 9.14$, p = 0.01; Table 2.1) and mean hue value in the dark blue color bin ($X^2_{df=2} = 6.12$, p = 0.013; Table 2.2).

**BODY SIZE**

Male body size varies regionally ($X^2_{df=3} = 158.06$, p < 0.0001); individuals are biggest in Northeastern CR, smallest in Panama and of similar intermediate size in Southeastern CR/PA and Western CR (Appendix S1). Unfortunately, we could not obtain the raw body size data from the 1967 study to compare SVL over the course of 38 years. Savage and Heyer (1967) report that male body size is (similarly) largest in Northeastern CR but of smaller, equal size in the other three regions. However, without raw data we cannot conclusively comment on any directional change in body size in these regions. Female body size in 2005 also varies regionally ($X^2_{df=2} = 28.51$, p < 0.0001), but exhibits a slightly different pattern than the males. Although females are also largest in Northeastern CR, they are of intermediate body size in Central
Panama and smallest in Western CR (Appendix S1). We lack body size data for females from Central Panama to include in the analysis.

**DISCUSSION**

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

Most anuran color patterns are genetically inherited (Pybrun 1961b; Hoffman & Blouin, 2000). Amphibian skin coloration is controlled primarily by two groups of pigment cells, melanophores and chromatophores (Hoffman and Blouin 2000). Color change over a short period of time (seconds to minutes) is associated with physiological changes due to temperature, humidity, and ambient light, whereas changes over generations (e.g., due to natural selection) is typically associated with changes between greys to browns (Hoffman and Blouin 2000) For *A. callidryas*, the shade of green on the dorsum can change rapidly with light exposure due to intracellular transport of pigment cells (Schliwa and Euteneuer 1983). However, the coloration along the flanks, thighs and upper arms does not change with environmental cues (personal observation). Therefore, we consider color and pattern as variable, genetically inherited traits because it is unlikely that the full-color spectrum differentiation among *A. callidryas* populations is due to local environmental factors.
Overall, Northeastern CR populations are distinguished by their large body size, blue legs and dominant flank-stripe pattern A. Southeastern CR/PA populations are intermediate in size, have bi-colored legs (orange and dark blue), and a dominant B stripe. Central Panamanian frogs are small, have predominantly orange legs with a small percent of light blue, and contain flank-stripe patterns B and C. Western CR populations are of intermediate body size, exhibit a north-south clinal change in coloration, and are unique in being nearly monomorphic for C flank stripe-pattern.

Two of the phenotypic traits measured in this study are correlated (leg coloration and flank-pattern). The strength of the correlation varies among flank-pattern. For example, flank-patterns A and C are tightly correlated with coloration, but the same is not true for other patterns (Figure 2.6). We suggest that these two traits loosely co-evolve over spatial and temporal scales. In North Atlantic CR, all individuals are fixed for blue legs. While flank-pattern A is found only in blue-legged individuals, a large percent of these individuals with blue legs have alternate flank-patterns. Similarly, individuals sampled from Western CR exhibit near-fixation for flank-pattern C but with variation in leg coloration (Figure 2.5). Thus, one region (North Atlantic) shows a near-fixation for blue leg coloration (with variable flank-pattern) while Western CR shows near-fixation for flank-pattern (with variable leg-coloration).
Leg color bins are as follows: blue (black bars), blue and orange (white bars), orange (dark grey bars), violet (light grey bars). Leg color is positively correlated for flank pattern (p < .001).

FIGURE 2.6. The proportion of each of four leg color bins for seven flank pattern types in *Agalychnis callidryas* individuals.

SPATIAL VARIATION

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

**Biogeography**

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

We found evidence to support the isolating effect of all three biogeographic barriers (Cordillera de Talamanca, Limón, Osa Peninsula) and one putative contact zone (Northeast-Northwest) in influencing the distribution of phenotypic diversity in *A. callidryas*. The Cordillera de Talamanca asserts a strong barrier to gene exchange between Caribbean and Pacific populations for other terrestrial amphibians and reptiles (Zamudio & Greene, 1997; Crawford, 2003) and likely explains the divergence in coloration and flank-stripe in *A. callidryas* (Figures 2.4-2.5).

Both leg coloration and flank-pattern differentiates the two regions separated by Limón (Figures 2.4-2.5) supporting the hypothesis that Limón is a biogeographic break for some terrestrial organisms. The addition of other characters (molecular,
behavioral al) to this dataset will greatly contribute to understanding the nature of this biogeographic break.

Populations in Western CR showed the most intra-region variability among all the sampled regions, exhibiting a north-south clinal change in coloration (Figure 2.5). The southernmost population, Pavones, containing the most color polymorphism, is isolated from the other three Western CR populations by the Golfo Dulce. Thus, it is possible that Pavones and the other Western CR populations evolved in allopatry.

We found evidence to support the hypothesis of a putative contact zone (Northeast-Northwest) at the junction of three non-contiguous mountains east of the Talamanca Mountains. Savage and Heyer (1967) suggested low levels of historical gene flow connected these two regions. Our more extensive sampling of flank pattern corroborates this pattern that low-elevation mountain-passes provide habitat corridors within the physiological tolerance of *A. callidryas*, and facilitate passage for dispersal between these two regions (Figure 2.4). For example, the dominant flank pattern observed in Tilarán was AC, a combination of A and C flank-stripes. This mid-elevation site may be a site of historical gene flow between Northeastern CR ‘A’ and Western CR ‘C’ forms, or alternatively, may reflect ancestral polymorphisms. However, breeding studies are required to confirm that AC is a hybrid form. We maintain caution in using flank-pattern analyses alone to make predictions about gene flow patterns because leg coloration clearly distinguishes these two regions.

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

**Signaling**

Phenotypic signals used for communication (visual and acoustic) co-evolve with the sensory systems of conspecifics and predators, and therefore are a balance between sexual selection (Endler 1980; Tuttle and Ryan 1981; Endler 1992) and natural selection (Hoekstra et al. 2004). Therefore, it is useful to consider the type of color pattern (cryptic and aposematic) and its employment (predator avoidance and/or visual signaling for mate-choice) as co-evolutionary forces that shape the directional selection of phenotypic diversity (Endler 1992; Maan et al., 2004; Endler & Mielke, 2005).

Organisms which possess no inherent chemical defence typically display cryptic coloration and/or behavioral al crypsis such that their color pattern matches a random sample of their environment. Many anurans exhibit cryptic color polymorphism polymorphism (Savage and Emerson 1970; Nevo 1973; Sazima 1978; Morey 1990). We know that this is true for *A. callidryas*: the green dorsal coloration reflects in the infrared range (700 – 900 nm), perfectly matching leaf reflectance (Schwalm et al. 1977). *Agalychnis callidryas* takes retreat under leaves during the day, thus effectively hiding from diurnal predators. However, the variation we quantified in this study (reds, oranges, and blues) is not typically associated with crypsis, and we suggest that the differences in bright coloration or contrasting color pattern may have evolved as aposematic coloration. Aposematism occurs in organisms that advertise chemical defenses (such as distasteful/poisonous toxins) to
potential predators through flashy and/or bright colors (Siddiqi et al. 2004). A classic example among anurans are a number of species in the poison arrow family, Dendrobatidae (Summers et al. 2003). One species, Dendrobates pumilio, is highly color polymorphic and utilizes aposematism to avoid diurnal predation (Siddiqi et al. 2004). Similar to poison arrow frogs, phyllomedusine frogs contain noxious skin peptides (Cei and Erspamer 1966) which stimulate regurgitation by snake predators (Sazima 1974). Aposmetism in crepuscular amphibians is poorly understood. However, a behavioral al study showed reduced predation rates on a brightly colored crepuscular salamander (Ensantina e. xanthoptica; (Kuchta 2005), suggesting that behavioral and vision studies are critical to determine whether crepuscular/nocturnal predators (birds, snakes, spiders) possess the visual system required for identifying and discriminating A. callidryas from non-toxic prey items. It is possible that A. callidryas utilizes a combination of crypsis and aposematism as a defense mechanisms.

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

**TEMPORAL VARIATION**

We observed a significant shift in the dominant flank-stripe pattern since data collected 38 years ago in 3 of the 4 regions (Figure 2.4). The only region that remained static was Western CR, a region that was and remains monomorphic for flank-stripe pattern C. Temporal studies of changes in genetic and phenotypic variation are uncommon (Barcia et al. 2005; Prieto et al. 2005; Ray and King 2006). These studies have differed in results, with some reporting large shifts (Barcia et al. 2005; Prieto et al. 2005) while others observed no change over time (Blanco et al. 2005; Rendell and Whitehead 2005). In the cases of limited change, the authors have argued that large effective population size limits genetic drift and stabilizes polymorphisms over time (Blanco et al. 2005).

In our study, temporal variation in flank-stripe pattern may reflect directional selection due to changes in mate choice criteria or predator behavioral (Pyburn 1961b). Alternatively, landscape changes, either natural or anthropogenic, might alter gene flow patterns and effectively swamp localized selection (Lenormand 2002). The stability of flank-stripe pattern in Western CR could be due to large effective population sizes (Ne), or to historical fixation of a single pattern in a region that receives no migrants from other more variable regions. We have no reason to believe Ne is higher in Western CR relative to other regions. The combination of these two temporal patterns (static in Western CR and dynamic in 3 regions) suggests that *A. callidryas* is not subjected to the same ecological and evolutionary history across it’s range. We are currently using mitochondrial and nuclear genetic markers to examine
historical and contemporary patterns of gene flow among these populations/regions for comparison with phenotypic variation in this species.

CONCLUSIONS

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

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

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

We thank the staff of the Smithsonian Tropical Research Institute (STRI), especially M. Leone and O. Arosemena and the Organization for Tropical Studies (O.T.S.) for support with this project. We thank the Ministry of the Environment (ANAM) in Panama and the Ministry of the Environment and Energy (MINAE) in Costa Rica for granting research, collecting, and export permits. Our gratitude extends to C. Dardia of the Cornell University Museum of Vertebrates and G. Chaves of the Museum of Vertebrates, University of Costa Rica, San Jose. We are especially grateful for the field assistance of A. Vega, L. Davis, B. Hilje, C. Vriesendorp, M. Williams, A. L. Robertson. For statistical consulting, we acknowledge F. Vermeylen and G. Hooker. L. Chan, K. Galbreath, H. Greene, B. LeGendre, A. Stevenson, J. Degenhardt, C. Prado, J. Richmond, J. Sigala-Rodríguez and K. Zamudio, commented on previous versions of this manuscript and A. Vega, B. Kohlmann, and F. Bolaños contributed through valuable discussion. We thank J. Somera for his scientific illustration of *A. callidryas*. Funding for this study was provided by the NSF Doctoral Dissertation Enhancement Program, Tinker Foundation and the Einaudi Center, Latin American Studies Department, Cornell University, Department of Ecology and Evolutionary Biology, Cornell University, O.T.S and STRI.
APPENDIX 2.1. Sampling populations of *Agalychnis callidryas* from four regions in Costa Rica and Panama.

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<th>Population</th>
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<th>1967 N</th>
<th>Male SVL (mm)</th>
<th>Female SVL (mm)</th>
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</thead>
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<td>77</td>
<td>43.58, 2.60, 67</td>
<td>na</td>
</tr>
</tbody>
</table>

Sampling size for flank-stripe pattern analyses for each population, and the total number of individuals sampled per region for 1967 study. Body size varies among regions in 2005: measures of snout-vent-length (SVL; mean, std, x) provided for males and females per population and averaged for each region. na= not available. Geographic coordinates (GIS) are Latitude (Lat), Longitude (Long), elevation (El, m).
LITERATURE CITED


CHAPTER THREE
VICARIANCE AND LOCALIZED SELECTION CONTRIBUTE TO
PHENOTYPIC DIVERSIFICATION IN A POLYTYPIC FROG

ABSTRACT
Spatial patterns of phenotypic diversity reflect the relative roles of gene flow and selection in determining geographic variation within a species. In this study we quantified color differentiation and genetic divergence among 20 populations of the red-eyed treefrog (*Agalychnis callidryas*) in Central America. Phylogenetic analyses revealed high population structure with five well-supported mitochondrial DNA clades. We infer from our phylogeny that geographic barriers have played a large role in isolating *A. callidryas* populations. The two phenotypic characters measured in this study did not co-vary across isolated population groups: flank coloration easily distinguished Caribbean from Pacific individuals, while leg coloration exhibited a more complex pattern. The incongruence between geographic, genetic and phenotypic diversity at broad spatial scales indicates that the relative strength of gene flow and selection is not equal across all geographic barriers, resulting in three general patterns: 1) phenotypic differentiation in the presence of historical gene flow, 2) phenotypic uniformity across genetically differentiated regions 3) and co-variation of genetic and phenotypic characters. These patterns indicate that spatially-varying localized adaptations also contribute to color differences. Our study underscores the fact that selection gradients vary across relatively small spatial scales, even in species that occupy relatively homogenous environments.
INTRODUCTION

Studies of geographic variation among populations of widespread species inform evolutionary biologists of the historical and current processes that underlie population differentiation (Ford 1971; Brown et al. 1996). The spatial distribution of divergent phenotypes provides insight into the relative roles of natural and sexual selection, gene flow, and vicariance in the diversification of a species (Grinnell 1924; Slatkin 1985b). Many taxa exhibit some individual or population variation, although the spatial distribution of genetic and phenotypic diversity varies substantially among species (Hoffman and Blouin 2000; Gray and McKinnon 2007). For some species geographic clines in body size (Brown and Thorpe 1991; Storz 2002), behavior (Thompson 1990; Prohl et al. 2006), color pattern (McDiarmid 1968; Stewart 1974; Hoffman and Blouin 2000; Woolbright and Stewart 2008), life history traits (Dhondt et al. 1990) or ornamentation (Nevo 1973; Gray 1983; Brooks and Endler 2001a; Storz et al. 2001) result from selection acting on continuous traits across selective gradients (Endler 1973; Storz et al. 2001; Storz 2002), genetic drift, or both (Hoffman et al. 2006). In contrast, far fewer species are highly polytypic across their range. In those cases, divergence usually results from isolation due to dispersal barriers that is reinforced by local adaptation and genetic drift (Summers et al. 2003; Fuller et al. 2004; Maan et al. 2006b; Boul et al. 2007). These two patterns of spatial variation are not exclusive; some species exhibit phenotypic divergence among regions (due to dispersal barriers) as well as clinal variation within regions (Endler 1973).

The interaction between barriers to gene flow and local natural selection determines the extent of phenotypic differentiation among localities and the rate and direction of phenotypic change over time (Grinnell 1924; Slatkin 1985a; Lenormand 2002). Thus, geographic patterns of genetic and phenotypic diversity can be used to infer the evolutionary processes acting upon populations. For example, high levels of
gene flow, either due to high dispersal capacity or absence of isolating barriers, combined with limited localized selection results in widespread genetic and phenotypic homogeneity. In contrast, strong localized divergent selection and regional barriers to gene flow can result in highly structured populations (King and Lawson 1995; Boul et al. 2007), especially when populations are small (Hofmann et al. 2006). Congruence in spatial patterns of genetic and phenotypic diversity provides evidence that similar microevolutionary processes have shaped both characters during the history of that species. In contrast, incongruence between genetic and phenotypic characters can result from restricted gene flow in the absence of divergent selection or from strong localized ecological selection in the presence of gene flow (Endler 1973; Gray 1983; Dallimer et al. 2003; Hoekstra et al. 2005; Jordan et al. 2005; Prohl et al. 2006; Rosenblum 2006).

The red-eyed treefrog, *Agalychnis callidryas*, is a common Neotropical treefrog broadly distributed from Central Mexico to Colombia (Duellman 2001; Savage 2002); this species exhibits regional phenotypic differentiation (Savage and Heyer 1967; Duellman 2001; Robertson and Robertson 2008), making it an ideal species for studying evolutionary mechanisms that contribute to geographic variation. Combined, color pattern differences within this species are sufficient to distinguish frogs from five biogeographic regions in Costa Rica and Panama with high accuracy (Robertson and Robertson 2008). Despite striking color divergence, different regions do not vary noticeably in habitat, elevation, or visual environment. It is therefore unlikely that regional color variation results from differences in localized ecological selection, as observed for many polytypic species that vary along steep environmental gradients (Kettlewell and Conn 1977a; Thorpe and Brown 1989; Hoekstra et al. 2005; Jordan et al. 2005; Rosenblum 2006). However, subtle regional differences in sexual selection or predator pressures could potentially drive the observed patterns of
phenotypic divergence (Brooks and Endler 2001b; Price 2006; Gosden and Svensson 2008).

In this study we compare patterns of genetic and phenotypic character distribution to test hypotheses about the role of selection and barriers to gene flow in the origin and maintenance of the highly regionalized diversity among red-eyed treefrog populations. We quantified variation in flank and leg color and compared it to the distribution of genetic lineages reconstructed from mitochondrial DNA sequences for populations throughout Costa Rica and Panama. Our sampling represents approximately 25% of the total geographic range of the species and encompasses all of the known color variants (Duellman 2001). Our specific objectives were to: 1) test the role of geographic factors, specifically isolation due to geographic distance and/or geographic barriers in structuring genetic diversity; 2) test whether regional color variation could also be explained by the same geographic factors; 3) test the null hypothesis that spatial patterns of phenotypic and genetic diversity were congruent. Incongruence, for example, high phenotypic discontinuity in the absence of genetic breaks, indicates that divergent polymorphisms are not solely the result of restricted gene flow among populations, and that selection or drift have contributed to divergence.

MATERIAL AND METHODS

Field Sampling

We quantified patterns of genetic and phenotypic variation among 20 red-eyed treefrog populations throughout Costa Rica (CR) and Panama (PA) (Figure 3.1). Previous phenotypic analyses identified five distinct regions diagnosable by leg color and pattern (Robertson and Robertson 2008). Three of these regions are located on the Caribbean versant of Central America, east of the central Cordillera de Talamanca.
(Northeastern CR, Southeastern CR/PA, and Central PA). The other two regions are located on the Pacific versant, west of the Talamancas (Northwestern CR and Southwestern CR; Figure 3.1, Table 3.2). We expanded upon the earlier study (Robertson and Robertson 2008) to include more populations, an additional measure of phenotypic diversity (flank coloration), and an analysis of genetic differentiation among the same populations to serve as a comparative framework for the phenotypic data. We conducted field surveys during the breeding seasons (May – August) of 2003, 2004, and 2005. At each site, we captured 10 – 26 individuals and collected data on body size (snout to vent length and mass) and coloration. We photographed every individual using a Nikon Coolpix 5700 against a black-white-grey card for color standardization. For each individual, we photographed three areas of the body: posterior surface of the thighs, left flank, and right flank of the body.

Up to three individuals per populations were preserved as vouchers and deposited at the Cornell University Museum of Vertebrates (CUMV: 14093,14206-11,14228,14231-33) and the University of Costa Rica, San José (UCR accession numbers: 19100-101, 19213). All photographs have been archived at the CUMV. Non-voucheried individuals were photographed, toe-clipped for genetic material and released at site of capture.
The shading of topological relief corresponds to elevation: dark grey (>1300 m), light grey (300 – 1299 m), white (0 – 300 m). Elevation higher than 1300 m represents unsuitable habitat that exceeds the physiological tolerance of *A. callidryas*. Dry forest habitat between Southwestern CR and Central Panama prevents the occurrence of red-eyed treefrogs along Pacific coast of Panama. Biogeographic barriers tested in this study are shown: Cordillera de Talamanca, Caribbean Valley Complex, Bocas del Toro, Rio Parrita, and Osa Peninsula.

**FIGURE 3.1** Sampling localities for 20 populations of *Agalychnis callidryas* in five biogeographic regions. The Cordillera de Talamanca isolates the Pacific and Caribbean versants of Costa Rica and Panama.
### TABLE 3.1 Populations of *Agalychnis callidryas* from five regions in Costa Rica and Panama.

<table>
<thead>
<tr>
<th>Region</th>
<th>Province</th>
<th>Population</th>
<th>GIS (Lat, Long, El)</th>
<th>DFA N</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Southwestern CR</strong></td>
<td>Puntarenas, CR</td>
<td>Pavones (pav)</td>
<td>8.4204, -83.1069, 37</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Puntarenas, CR</td>
<td>Campo (cam)</td>
<td>8.6909, -83.5013, 35</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Puntarenas, CR</td>
<td>Sierpe (sie)</td>
<td>8.8892, -83.477, 17</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>Puntarenas, CR</td>
<td>Uvita (uvi)</td>
<td>9.1235, -83.7011, 26</td>
<td>24</td>
</tr>
<tr>
<td><strong>Northwestern CR</strong></td>
<td>Puntarenas, CR</td>
<td>Playa Bandera (plb)</td>
<td>9.5188, -84.3774, 23</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>Puntarenas, CR</td>
<td>Carara (car)</td>
<td>9.7256, -84.5313, 385</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Guanacaste, CR</td>
<td>Cabo Blanco (cab)</td>
<td>9.5805, -85.1246, 166</td>
<td>18</td>
</tr>
<tr>
<td><strong>Northeastern CR</strong></td>
<td>Guanacaste, CR</td>
<td>Tilarán (til)</td>
<td>10.5162, -84.9601, 637</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Alajuela, CR</td>
<td>San Ramon (sar)</td>
<td>10.2335, -84.5287, 638</td>
<td>14</td>
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<tr>
<td></td>
<td>Heredia, CR</td>
<td>La Selva (las)</td>
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<td>20</td>
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<td>Universidad de EARTH</td>
<td>10.2368, -83.567, 44</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Heredia, CR</td>
<td>Siquires (siq)</td>
<td>10.0546, -83.551, 574</td>
<td>0</td>
</tr>
<tr>
<td><strong>Southeastern CR/PA</strong></td>
<td>Limón, CR</td>
<td>Cahuita (cah)</td>
<td>9.7189, -82.8143, 16</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Limón, CR</td>
<td>Manzanillo (man)</td>
<td>9.6332, -82.6556, 2</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>Bocas del Toro, PA</td>
<td>Chiriquí Grande (chg)</td>
<td>8.9460, -82.1571, 21</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Bocas del Toro, PA</td>
<td>Almirante (alm)</td>
<td>9.1980, -82.3445, 13</td>
<td>10</td>
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<tr>
<td><strong>Central Panama</strong></td>
<td>Veraguas, PA</td>
<td>Santa Fé (sfe)</td>
<td>8.5070, -81.1141, 714</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>Coclé, PA</td>
<td>El Cope (cop)</td>
<td>8.6299, -80.592, 792</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Coclé, PA</td>
<td>El Valle (val)</td>
<td>8.6299, -80.1159, 866</td>
<td>21</td>
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<tr>
<td></td>
<td>Panamá, PA</td>
<td>Gamboa (gam)</td>
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<td>22</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td></td>
<td></td>
<td></td>
<td>336</td>
</tr>
</tbody>
</table>

Sample sizes for coloration used in discriminant function analyses (DFA) are listed per population, with exact locality and geographic coordinates (GIS: latitude (Lat), longitude (Long), elevation (El)). Population abbreviations correspond to locality on Figure 3.1.
POPULATION GENETIC VARIATION

We extracted whole genomic DNA from 125 individuals sampled throughout Costa Rica and Panama (Figure 3.1). Toe clips or liver were digested in standard lysis buffer with Proteinase K followed by purification using the Qiagen DNeasy Tissue Kit (QIAGEN, Valencia, California) following manufacturer’s protocols. We amplified a fragment of the mtDNA including the partial 16S rRNA, the complete NADH dehydrogenase subunit 1, and the adjacent flanking tRNA\textsuperscript{Met} (hereafter, referred to as ND1) using primers tmet-frog (5’TTGGGGTATGGGCCCAAAAGCT3’; Wiens et al. 2005) and a primer designed for Agalychnis callidryas (ACA-Int: 5’ACGTGATCTGAGTTCAGACCG 3’). PCR reactions were performed in a total volume of 25 µl, each containing 100 ng template DNA, 1X PCR Buffer, 0.75 mM dNTPs, 1.5 mM MgCl\textsubscript{2}, 1 uM primer, and 0.625 units of Taq polymerase. PCR conditions consisted of an initial 95 °C denaturation for 5 minutes, followed by 35 amplification 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 minute extension at 72 °C. Exonuclease (10 units) and SAP (1 unit) were used to remove unincorporated oligonucleotides and dNTPs with an incubation at 37 °C for 45 minute and denaturation at 90 °C for 10 minutes. We performed cycle sequencing reactions with Big Dye terminator sequencing components according to manufacturer’s protocol (Applied Biosystems, Perkin Elmer, Foster City, CA) using the same primers used for fragment amplification. Cycle sequencing reaction conditions were 25 cycles of 96 °C (30 sec), 50 °C (15 sec), and 60 °C (4 min). We sequenced gene fragments in both directions to resolve any 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 (Applied Biosystems, Foster City, California).
Electropherograms were checked by eye and fragments assembled into contiguous sequences using Sequencher 4.1 (GeneCodes, Michigan).

We aligned ND1 sequences using ClustalW (Thompson et al. 1994) in the MegAlign 6.1.2 program of the Lasergene sequence analysis software (DNASTAR, Inc., Madison, Wisconsin). We conducted multiple alignments using the 'slow/accurate' option. 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) to identify regions of ambiguous homology (Gatesy et al. 1993); positions that varied in alignment across this range were excluded as characters in phylogenetic analyses.

We estimated haplotype diversity ($h$) and nucleotide diversity ($\pi$) (Nei 1987) and the number of unique haplotypes using Arlequin 3.01 (Schneider et al. 2000). Overall genetic differentiation among regions was estimated using pairwise $F$-statistics ($\phi_{ST}$) and we compared estimates to a null distribution of no difference between regions to test for significance ($\alpha = 0.05$) using 10,000 permutations in Arlequin. Transition-transversion ratios, as well as the overall nucleotide frequencies were computed from the data.

We inferred a Bayesian phylogenetic topology using MrBayes (Huelsenbeck and Ronquist 2001). The best-fitting model of nucleotide substitution for our data was selected based on the Akaike Information Criterion as implemented in MrModelTest (Nylander 2004). Our Bayesian analyses consisted of two independent runs of four Markov chains, run for 10,000,000 iterations and with sampling every 1000th iteration. We applied default prior distributions in MrBayes with the exception of the $\alpha$ shape (exponential, mean = 1.0) and branch length parameters (exponential, mean = 0.1). We determined the appropriate number of burn-in samples and tested for
stationarity of parameter values using trace plots in the software package **TRACER** (Rambaut and Drummond 2005). Removal of 10% of the initial samples provided ample burn-in in both analyses.

**PHENOTYPIC VARIATION**

In life, red-eyed tree frogs are green dorsally, have large red eyes and orange or violet front and hind feet. This species is one of few anuran taxa that exhibits relatively low levels of phenotypic variation within populations, but high variation among populations (Hoffman and Blouin 2000; Savage 2002). To the human eye, variation in leg and flank coloration among populations is obvious, and includes hues of red-orange, yellow, blue and violet (Robertson 2008). Unlike the green dorsum, leg and flank colors do not change ontogenetically or with environmental cues (Schliwa and Euteneuer 1983); personal observation). Thus, color in those two body regions can be reliably measured for studies of character differentiation.

We imported photographs of each individual into Adobe Photoshop CS2 to correct for ambient light intensity and color by reference to the black-white-grey standard (QPcard 101) in the background of every photograph (Stevens et al. 2007). We quantified color as ‘hue’ in the HSB (hue, saturation, and brightness) realm because previous analyses of leg coloration confirmed that hue accurately represents variation when saturation values are high (McKenna et al. 1999; Robertson and Robertson 2008). Saturation is an index of the purity of a color; low saturation values correspond to ‘muddy’ colors because they contain a mixture of all three primary colors whereas highly saturated colors contain only one or two of three complementary colors. Thus, the error in hue measurements increases with decreasing saturation levels (McKenna et al. 1999). We used the Color Picker function in Adobe Photoshop CS2 to measure hue and saturation and conducted an homogeneity of
variance test within populations to compare the variance in hue for individuals with high (> 30 %) and low (≤ 30 %) saturation (implemented in JMP 7; SAS Institute Incorporated, 2006). If the variance between these groups differed significantly, this would validate previous findings that low saturation hues are unreliable, and thus must be excluded from further analyses (McKenna et al. 1999).

We measured leg and flank coloration of 14 – 26 frogs per population (Table 3.2). Dominant leg colors of *A. callidryas* vary regionally; individuals from some populations are monochromatic (blue), others contain two dominant colors (blue and orange), while others contain a continuum of hues (e.g., reddish blue through greenish blue). To quantify color, we followed the protocol of Robertson and Robertson (2008) and imported the color-corrected photographs into ImageJ 10.2 for downstream analyses. We selected the entire posterior surface of the leg in ImageJ to create a frequency histogram of the number of pixels of each hue. We then transformed the ImageJ hue data to the standard measure of hue with a range of 0-360 degrees and divided the color spectrum into eight equal bins (each spanning 45 degrees) named according to the central hue for each bin. For example, the standard hue definition of pure red is zero, therefore the red bin spans 22.5 degrees on each side of zero degrees. Final hue ranges for the eight color bins were: red (337.6 - 22.5), orange (22.6 – 67.5), yellow (67.6 – 112.5), green (112.6 – 157.5), light blue (157.6 – 202.5), dark blue (202.6 – 247.5), purple (247.6 – 292.5) and violet (292.5 – 337.5).

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

To test for population and regional differences in coloration, we used linear discriminant analysis implemented in JMP 7.0 to compare each individual response (across all variables) to the group multivariate mean. The following color parameters were included in the model: the average hue (leg and flank) for each of eight bins, the percentage of hue contained in each bin, and overall saturation level (31 – 100 %). We excluded from these analyses any individual whose leg and/or flank saturation was ≤ 30 % because the standard deviation of measured hue increases as saturation decreases, confirming the analytical prediction that hue is not a reliable measure when saturation is low (McKenna et al. 1999). To quantify the number of individuals correctly assigned and those misclassified to source populations we generated a classification matrix, and tested the significance of individual assignments using a chi-square test. This method predicts assignment based on multivariate analysis of variance. Significantly accurate assignment indicates that leg and flank coloration are highly diagnostic for the five regions examined. In addition, we used the quantification of hue and saturation (for both flank and leg) to construct pairwise Euclidian distance matrices for use in Matrix Correspondence Tests (MCTs).

MATRIX CORRESPONDENCE TESTS

We used Matrix Correspondence Tests (MCT) and partial MCT (pMCT) to examine the determinants of the geographic distribution of genetic and phenotypic diversity in red-eyed treefrogs. Matrix Correspondence Tests use repeated randomization and recomputation to test for the correlation between two distance matrices by comparing the individual pairwise distance for each parameter (Manly 1986). The randomized values provide a null distribution with which to test the
hypothesis of no association. Significance values were determined by comparing the observed and expected Z-statistic, generated by 10,000 permutations. We conducted pMCT when more than one independent variable was significant ($P < 0.05$) in the individual MCT. Partial MCT tests 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 and Rousset 2001; Castellano and Balletto 2002; Rousset 2002). We therefore compared the pairwise regression and partial regression analyses and interpreted our results in light of this possible limitation. We carried out MCT in two parallel analyses using phylogeny and coloration as dependent variables. We tested the association between genetic distance and geographic factors (proximity and barriers) as well as two measures of coloration (leg and flank) and the same geographic factors. Then, we tested for co-variation between genetic and phenotypic diversity. We conducted MCT analyses at three spatial scales: including all populations (Caribbean and Pacific), Caribbean populations only, and Pacific populations only.

To construct the genetic distance matrix, we calculated pairwise patristic distances from the Bayesian consensus topology using the program TreeEdit 1.0a10 (Rambaut and Charleston 2001). We constructed color matrices by calculating individual pairwise Euclidian distances based on the combined measures of coloration, including: saturation, average hue and the percentage of hue for each of eight bins. This methodology is similar to reducing multiple, uncorrelated variables into a single eigenvector value in a principal component analysis.

We used two measures of geographic distance: the first matrix contained the straight-line distance between all pairs of sites (proximitySTRAIGHT), the second matrix reflected distances of likely dispersal paths based on our knowledge of the physiology and habitat requirements of the species (proximityAROUND). The second
matrix accounted for the inhospitable habitats in the Cordillera de Talamanca and the dry Pacific landscape located between Southwestern CR and Central Panama (Figure 3.1), both areas that are not occupied by red-eyed treefrogs.

We used MCTs to test for isolation due to five known topographic or biogeographic barriers in Costa Rica and Panama: Cordillera de Talamanca, two barriers among Pacific populations (Osa Peninsula and Río Parritta), and two barriers among Caribbean populations (Caribbean Valley Complex and Bocas del Toro; Figure 3.1). To test for associations between genetic and phenotypic discontinuities and these five landscape features we created a “barrier matrix” using binary indicator variables (0,1) to designate whether populations occurred in the same (1) or different (0) regions with respect to each putative barrier. Three populations included in the mtDNA phylogeny (ear, siq, cah) were excluded from MCT analyses because of insufficient data for coloration (Table 3.2). We generated pairwise matrices and conducted analyses in the program R 2.4.1 (Team 2005).

RESULTS

Population Genetic Variation

The mitochondrial fragment used for analyses was 1149 bp in length, including 118 bp of the 16S gene and 1031 bp of NADH1. We sequenced this fragment in 125 A. callidryas and 1 outgroup taxon, A. saltator, and identified 75 unique haplotypes with 178 variable sites, of which 142 were parsimony informative; no insertions/deletions were detected. Haplotype (h) and nucleotide (π) diversity varied
### TABLE 3.2 Summary of within-population diversity of NADH1 sequences.

<table>
<thead>
<tr>
<th>Region</th>
<th>Population</th>
<th>N</th>
<th>No. Unique Haplotypes</th>
<th>$h$</th>
<th>Po</th>
<th>$\pi$</th>
<th>Pi</th>
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</thead>
<tbody>
<tr>
<td>Northeast CR</td>
<td>lse</td>
<td>10</td>
<td>6</td>
<td>0.95</td>
<td>15</td>
<td>0.0034</td>
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<tr>
<td></td>
<td>sra</td>
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<td>2</td>
<td>0.50</td>
<td>6</td>
<td>0.0026</td>
<td>3.00</td>
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<tr>
<td></td>
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<td>0.73</td>
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<tr>
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<tr>
<td>Southeast CR/PA</td>
<td>man</td>
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<td>5</td>
<td>4</td>
<td>0.90</td>
<td>5</td>
<td>0.0022</td>
<td>2.60</td>
</tr>
<tr>
<td></td>
<td>uvi</td>
<td>11</td>
<td>8</td>
<td>0.94</td>
<td>82</td>
<td>0.0345</td>
<td>38.54</td>
</tr>
<tr>
<td></td>
<td>pav</td>
<td>7</td>
<td>1</td>
<td>0.71</td>
<td>4</td>
<td>0.0009</td>
<td>1.14</td>
</tr>
<tr>
<td>Northwest CR</td>
<td>cab</td>
<td>7</td>
<td>3</td>
<td>0.75</td>
<td>43</td>
<td>0.0097</td>
<td>11.21</td>
</tr>
<tr>
<td></td>
<td>car</td>
<td>5</td>
<td>0</td>
<td>0.93</td>
<td>10</td>
<td>0.0026</td>
<td>3.00</td>
</tr>
<tr>
<td></td>
<td>ban</td>
<td>11</td>
<td>5</td>
<td>0.92</td>
<td>14</td>
<td>0.0038</td>
<td>4.36</td>
</tr>
</tbody>
</table>

Population sample sizes (N), Heterozygosity ($h$, with standard error), Number of polymorphic sites (Po), Nucleotide diversity ($\pi$, with standard error), Mean number of pairwise differences (Pi). Estimates from the population siq were not available due to small sample size.
TABLE 3.3. Pairwise $\phi_{ST}$ (below diagonal) and corrected average pairwise difference of mtDNA haplotypes (above diagonal) for 5 regions, representing 20 populations of *Agalychnis callidryas* in Costa Rica and Panama.

<table>
<thead>
<tr>
<th></th>
<th>Northeast CR</th>
<th>Southeast CR</th>
<th>Central PA</th>
<th>Northwest CR</th>
<th>Southwest CR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Northeast CR</td>
<td>4.11</td>
<td>3.709</td>
<td>19.465</td>
<td>34.043</td>
<td>54.089</td>
</tr>
<tr>
<td>Southeast CR</td>
<td>0.327*</td>
<td><strong>13.005</strong></td>
<td>14.879</td>
<td>29.480</td>
<td>49.272</td>
</tr>
<tr>
<td>Central PA</td>
<td>0.704*</td>
<td>0.533*</td>
<td><strong>13.019</strong></td>
<td>30.318</td>
<td>47.851</td>
</tr>
<tr>
<td>Northwest CR</td>
<td>0.863*</td>
<td>0.755*</td>
<td>0.754*</td>
<td><strong>6.804</strong></td>
<td>52.361</td>
</tr>
<tr>
<td>Southwest CR</td>
<td>0.806*</td>
<td>0.737*</td>
<td>0.735*</td>
<td>0.781*</td>
<td><strong>20.007</strong></td>
</tr>
</tbody>
</table>

The diagonal element (bolded) are the average pairwise nucleotide differences between haplotypes within each region. * = significance $P < 0.0001$.

among populations with high $h$ (mean ± SD = 0.846 ± 0.145; range = 50 – 100 %) and relatively low $\pi$ for most populations (mean ± SD = 0.006 ± 0.007; range = 0.0008 – 0.00345; Table 3.3). The average number of polymorphic sites between populations within regions varied from 4.11 (Northeast CR) to 20.00 (Southwest CR). The large genetic differences among populations in the Southwest CR region was driven by individuals from the population Uvita (uvi; Table 3.3); excluding that population, the degree of polymorphism in this region is 5.6, well within the range of the other
Values above branches are posterior probabilities. Phylogram is rooted with the outgroup taxon, *Agalychnis saltator*. Bars are coded by geographic regions in Figure 2.1. Population and regions correspond to those listed in Table 3.2.

**FIGURE 3.2** Bayesian consensus phylogram based on 1149 basepairs of the NADH1 mitochondrial DNA gene fragment.
regions. The average number of pairwise differences among regions was greatest when comparing Southwest CR to all other populations (Table 3.2). Pairwise $\Phi_{ST}$ values among regions were high and significant for all comparisons, ranging from 0.327 (Northeastern - Southeastern CR) to 0.863 (Northwestern-Northeastern CR; Table 3.3).

ModelTest 3.7 showed the model GTR + I + $\Gamma$ with unequal base frequencies (A=0.3076, C=0.22190, G=0.11182, T=0.35230; pinvar = 0.6311 and $\alpha = 2.1712$). The Bayesian topology including all haplotypes showed an overall pattern of regional differentiation and well supported regional clades (bootstrap values ranged from 89 – 99%); however, haplotypes from none of the regions formed a monophyletic group (Figure 3.2). The consensus topology showed an early divergence of the Pacific clade A (including only Southwestern CR populations) relative to the other four regions (Figure 3.2). Within Pacific clade A, samples from the Osa Peninsula (cam) were genetically distinct from other Southwestern CR populations. Haplotypes from the remaining regions fell within three clades (Pacific clade B, Caribbean clades A and B, and the Central Panama clade) united at their base by a polytomy: (Figure 3.2). Pacific clade B included individuals from Northwestern CR, but also four individuals from Uvita, the admixed Southwestern CR population. The other seven Uvita individuals were members of Pacific clade A (Figure 3.3). The Caribbean clade A contained individuals from three regions and was the only clade to contain individuals from both sides of the Cordillera de Talamanca. Caribbean clade B (sister to Caribbean clade A) contained individuals from Southeastern CR and Central Panama. The third major clade contained individuals exclusively from Central Panama (Figure 3.2).
PHENOTYPIC VARIATION

Prior to color analyses, we conducted an homogeneity of variance test within populations to compare the variance in hue for individuals with high (> 30%) and low (≤ 30%) saturation. We performed this test only on the three populations that contained both high and low saturation measurements (Figure 3.3; alm: $F = 13.98$, $P = 0.011$; pav: $F = 33.51$, $P < 0.0001$; cop: $F = 12.24$, $P = 0.005$). We detected unequal variance in the two groups; hue values in the high saturation group clustered tightly, whereas hue values for individuals with low saturation were scattered across the color spectrum. This validated the concern that measures of hue are unreliable when saturation levels are low (McKenna et al. 1999). Thus, we subsequently measured and analyzed hue only for individuals with saturation levels > 30%. This also permitted multivariate comparisons including both leg and flank color data. The total number of excluded individuals was small (8.2% of all individuals; average number of individuals per region = 3.8).

Divergence in flank coloration was most evident between Pacific (orange) and Caribbean (blue) populations (Figures 3.4-3.5). Pacific populations had mostly orange legs, but a few individuals from the Southwestern CR region exhibited some blue and green (Figure 3.4). In contrast, leg coloration among Caribbean regions varied from blue-violet (Northeastern CR) to populations with unequal proportions of blue and orange (Figure 3.4). The average leg coloration among Southeastern CR/PA individuals was approximately 65% orange and 35% blue; in contrast Central PA
Saturation ranges from 0 – 100 % among sampled individuals; the dotted line at 30 % represents the threshold saturation level for excluding individuals in the study of flank coloration.

**FIGURE 3.3.** Flank hue and saturation measures for individuals sampled at 17 sites in Costa Rica and Panama.
FIGURE 3.4. The proportion of the leg and flank (area measured as percent pixels) that falls within eight color bins (red, orange, yellow, green, light blue, dark blue, purple, violet) for 125 red-eyed treefrogs in the mtDNA analyses.

Individuals are represented on the horizontal axes and the proportional leg and flank color for each individual are represented as a vertical histogram. The color frequency graphs underscore the low within-population variation and high regional variation characteristic of this species. The black, bold lines show the three biogeographic barriers associated with flank coloration (Bocas del Toro), leg coloration (Caribbean Valley Complex) and both flank and leg coloration (Cordillera de Talamanca). The phylogenetic clades (light grey bars) show genetic admixture between neighboring regions and the incongruence between genetic structure and phenotypic divergence. Caribbean clade A includes one individual sampled from cab (dotted line).
FIGURE 3.5. Variation in the average (and standard error) percentage of leg (top) and flank (bottom) coloration measured as ‘orange’ (combined orange and red color bins) for 17 populations of *Agalychnis callidryas*. The greatest divergence in coloration occurs across the Cordillera de Talamanca.
individuals had a higher proportion of orange in the legs (≈ 90% orange / 10% blue; Figures 3.4-3.5).

The discriminant function analyses correctly classified 195 of 233 (83.6%) individuals to their region of origin based on leg and flank coloration alone. The number of misclassified individuals per population was very low (mean ± SD = 0.165 ± 0.659; range: 0 - 5). Therefore, these characters are diagnostic for the five regions ($\chi^2_{df=16} = 595.45, P < 0.0001$; Figure 3.4). The largest misclassification occurred between Northwestern and Southwestern CR (52% of all misclassified individuals), reflecting the high similarity in coloration between these two regions (Figures 3.4-3.5).

**MATRIX CORRESPONDENCE TESTS: ALL POPULATIONS**

At the broadest spatial scale, the greatest determinant of the distribution of genetic diversity was the barrier to gene exchange imposed by the Cordillera de Talamanca ($r = 0.513, P = 0.0021$; Table 3.4). Concordant with this result, genetic diversity varied with isolation by geographic distance around the mountains (proximityAR; $r = 0.428, P = 0.0021$; Appendix 3.2) but not ‘across’ them, as measured by the straight-line geographic distance (proximityST; Appendix 3.2). These results emphasize the prominent role of the cordillera in determining probable dispersal routes in this species. Thus, we used proximityAR for subsequent MCT analyses for populations across the range.

Overall, the geographic distribution of coloration was also strongly determined by the Cordillera de Talamanca (flank $r = 0.632, P = 0.0045$; leg $r = 0.306, P = 0.0045$; Table 3.5). In MCT, geographic proximity was associated with both color measures, but those relationships lost statistical significance in pMCT that accounted
for the variation due to the Talamancan mountains (Table 3.5). In all cases, the effect of the Cordillera was much stronger for flank coloration, indicating that the mountains had a bigger effect on flank coloration than leg coloration (Table 3.5).

Genetic and phenotypic diversity patterns were incongruent at the broadest scale; the associations between genetic distance and divergence in leg coloration \((r = 0.138, \ P = 0.0045; \text{Table 3.5})\) and flank coloration \((r = 0.3527, \ P = 0.0045; \text{Table 3.5})\) were attenuated and not statistically significant in a pMCT that accounted for the variation due to isolating effects of the Cordillera de Talamancan (Table 3.5). Thus, the geographic distribution of both genetic and phenotypic diversity at the broadest spatial scale (Pacific versus Caribbean) was driven primarily by the isolating effect of the central mountain range for \(A. \ callidryas\).

**MATRIX CORRESPONDENCE TESTS: CARIBBEAN POPULATIONS ONLY**

Genetic diversification of Caribbean populations was associated with geographic distance \((r = 0.686, \ P = 0.0021; \text{Table 3.4})\) as well isolation due to both of the putative biogeographic barriers, Bocas del Toro \((r = 0.630, \ P = 0.0021)\) and the Caribbean Valley Complex \((r = 0.255, \ P = 0.0021; \text{Table 3.4})\). However, genetic diversity was better explained by geographic distance, and not a barrier across the Caribbean Valley Complex in the pMCT \((r = -0.351, \ P > 0.1; \text{Table 3.4})\). In fact, the relationship between genetic and geographic distance strengthened after removing the variation imposed by the barrier in pMCT (Appendix 3.2). Combined, these results indicate that the Caribbean Valley Complex was not a historical barrier to gene flow for \(A. \ callidryas\).

Flank coloration was associated with a barrier at Bocas del Toro \((r = 0.141, \ P = 0.0350; \text{Table 3.5})\) but did not vary with geographic distance in MCT \((r = 0.0759, \ P = 0.4005; \text{Table 3.5})\), indicating that flank color varies regionally but is not clinal. The
distribution of leg coloration was associated with geographic distance \( (r = 0.6821, P = 0.018; \text{Table 3.5}) \) and both putative Caribbean barriers, the Caribbean Valley Complex \( (r = 0.7712, P = 0.0045) \) and Bocas del Toro \( (r = 0.5257, P = 0.0045; \text{Table 3.5}) \). However, only the barrier across the Caribbean Valley Complex remained a significant predictor for leg coloration in pMCT when accounting for variation due to geographic distance (Table 3.5). Thus, leg coloration varied along a cline among southern Caribbean populations and regionally among northern Caribbean populations.

Patterns of phenotypic and genetic diversity were incongruent among Caribbean populations for MCT of flank \( (r = 0.102, P = 0.135) \) but not leg coloration \( (r = 0.306, P = 0.0045; \text{Table 3.5}) \). However, the association between genetic distance and divergence in leg coloration was weakened in pMCT \( (r = 0.175, P = 0.0135) \) when considering the variation due to the effects of the Caribbean Valley Complex barrier (Table 3.5). Thus, geographic barriers had an overall larger effect on the spatial distribution of leg coloration for Caribbean populations (Table 3.5).

**MATRIX CORRESPONDENCE TESTS: PACIFIC POPULATIONS ONLY**

Genetic diversity on the Pacific versant was structured by geographic factors in MCT: geographic distance \( (r = 0.508, P = 0.0021) \) and isolation due to two barriers,
TABLE 3.4. Matrix and Partial Matrix Correspondence Tests of the determinants of genetic diversity (phylogeny).

<table>
<thead>
<tr>
<th>Region</th>
<th>Parameters</th>
<th>Dependent variable</th>
<th>Independent variable(s)</th>
<th>r</th>
<th>$P_{raw}$</th>
<th>$P_{adj}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caribbean &amp; Pacific</td>
<td>Populations</td>
<td>phylogeny</td>
<td>barrier C</td>
<td>0.5133</td>
<td>0.0001</td>
<td></td>
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<td></td>
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<td>proximityST</td>
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<tr>
<td>Caribbean Populations</td>
<td></td>
<td>phylogeny</td>
<td>barrier D</td>
<td>0.2558</td>
<td>0.0001</td>
<td>0.0021</td>
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<tr>
<td></td>
<td></td>
<td></td>
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<td>0.6303</td>
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<td></td>
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<tr>
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<td></td>
<td></td>
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<td>0.2343</td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
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<td>(proximity)</td>
<td>0.0021</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>barrier D</td>
<td></td>
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<td>21</td>
</tr>
<tr>
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<td></td>
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<td>(proximity)</td>
<td>0.3513</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pacific Populations</td>
<td></td>
<td>phylogeny</td>
<td>barrier A</td>
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<td>0.0001</td>
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</tr>
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<td></td>
<td>barrier B</td>
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</tr>
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<tr>
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<td></td>
<td>(barrier B)</td>
<td></td>
<td>0.2394</td>
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<tr>
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<td></td>
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<td>proximityAR</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>(barrier A)</td>
<td></td>
<td>0.0021</td>
<td></td>
</tr>
</tbody>
</table>

The genetic distance matrix (dependent variable), was estimated as pairwise patristic distances. Patterns of phylogenetic diversity were correlated with two measures of geographic distance (proximityST and proximityAR) and five specific biogeographic barriers at three spatial scales. Specific barriers tested: A (Osa Peninsula), B (Rio Parrita), C (Cordillera de Talamanca), D (Caribbean Valley Complex), E (Bocas del Toro). Significance values were determined by comparing the observed and expected z-statistic, generated by 10000 permutations. The raw p-value for each test ($P_{raw}$) is provided as well as the P value adjusted for multiple tests, using Bonferroni correction ($P_{adj}$), significance indicated in bold. - = test not conducted. Parentheses indicate which factor was controlled for pMCT. See Appendix 2 for full set of analyses.
**TABLE 3.5. Matrix and Partial Matrix Correspondence Tests of the determinants of phenotypic diversity.**

<table>
<thead>
<tr>
<th>dependent variable</th>
<th>independent variable(s)</th>
<th>flank</th>
<th>p&lt;sub&gt;raw&lt;/sub&gt;</th>
<th>p&lt;sub&gt;adj&lt;/sub&gt;</th>
<th>leg</th>
<th>p&lt;sub&gt;raw&lt;/sub&gt;</th>
<th>p&lt;sub&gt;adj&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pacific &amp; Caribbean populations phenotype</td>
<td>barrier C</td>
<td>0.632</td>
<td>0.0001</td>
<td><strong>0.0045</strong></td>
<td>0.3063</td>
<td>0.0001</td>
<td><strong>0.0045</strong></td>
</tr>
<tr>
<td></td>
<td>phylogeny</td>
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<td>0.0001</td>
<td><strong>0.0045</strong></td>
<td>0.1382</td>
<td>0.0001</td>
<td><strong>0.0045</strong></td>
</tr>
<tr>
<td></td>
<td>proximityAR (barrier C)</td>
<td>0.028</td>
<td>0.0444</td>
<td>1.9980</td>
<td>-0.0250</td>
<td>0.8074</td>
<td>36.333</td>
</tr>
<tr>
<td></td>
<td>phylogeny (barrier C)</td>
<td>0.042</td>
<td>0.0083</td>
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<td>0.8291</td>
<td>37.3095</td>
</tr>
<tr>
<td></td>
<td>phenotype</td>
<td>barrier D</td>
<td>-0.018</td>
<td>0.8091</td>
<td>36.4095</td>
<td>0.7712</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>barrier E</td>
<td>0.141</td>
<td>0.0007</td>
<td><strong>0.0315</strong></td>
<td>0.525</td>
<td>0.0001</td>
<td><strong>0.0045</strong></td>
</tr>
<tr>
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<td>phylogeny</td>
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<td>0.0029</td>
<td>0.1305</td>
<td>0.306</td>
<td>0.0001</td>
<td><strong>0.0045</strong></td>
</tr>
<tr>
<td></td>
<td>proximity</td>
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<td>0.0089</td>
<td>0.4005</td>
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<td><strong>0.0045</strong></td>
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<td>Caribbean populations phenotype</td>
<td>barrier D (proximity)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.587</td>
<td>0.0001</td>
<td><strong>0.0045</strong></td>
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<tr>
<td></td>
<td>barrier E (proximity)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.021</td>
<td>0.2167</td>
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</tr>
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<td>phylogeny (barrier D)</td>
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<td>0.0020</td>
<td>0.0900</td>
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<td>0.0003</td>
<td><strong>0.0135</strong></td>
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<td></td>
<td>phylogeny (barrier E)</td>
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<td>11.8125</td>
<td>-0.039</td>
<td>0.8807</td>
<td>39.6315</td>
</tr>
</tbody>
</table>

The dependent matrices are flank and leg coloration. Patterns of phenotypic diversity were correlated with two measures of geographic distance (proximityST and proximityAR) and five specific biogeographic barriers at three spatial scales. Specific barriers tested: A (Osa Peninsula), B (Rio Parrita), C (Cordillera de Talamanca), D (Caribbean Valley Complex), E (Bocas del Toro). Significance values were determined by comparing the observed and expected z-statistic, generated by 10000 permutations. The raw p-value for each test (p<sub>raw</sub>) is provided as well as the P value adjusted for multiple tests, using Bonferonni correction (p<sub>adj</sub>), significance indicated in bold - = test not conducted. See Appendix 3.3 for full set of analyses.
the Río Parrita (\(r = 0.728, P = 0.0021; \) Table 3.4) and the Osa Peninsula (\(r = 0.255, P = 0.0021; \) Table 3.4). We used pMCT to determine that genetic and geographic distance covaried among populations in the southern Pacific region, even after accounting for the effects of the Osa Peninsula (Table 2.4). However, the effects the other barrier (Río Parrita), separating northwestern and southwestern CR, contributed significantly to spatial genetic structure (Table 2.4). Flank, but not leg, coloration varied across the Osa Peninsula (\(r = 0.348, P = 0.0045\)). Otherwise, neither flank nor leg color varied with geographic distance or across the other Pacific barrier (Table 2.5).

We detected incongruent patterns of genetic and phenotypic diversity for populations along the Pacific versant. These results were not surprising for leg coloration (\(r = -0.024; P = 0.8987\)), a trait that exhibits minimal variation across Pacific populations (Figure 3.4). The weak association between flank coloration and genetic diversity was marginally insignificant in MCT (\(r = 0.1669, P = 0.063; \) Appendix 3).

**DISCUSSION**

Our objectives were to examine the geographic determinants of genetic and phenotypic diversity in red-eyed treefrogs and to examine the congruency between these spatial patterns, at multiple geographic scales and across putative biogeographic barriers. Across some regions, our analyses revealed the concordant distribution of mtDNA diversity and coloration, implicating evolutionary history and geological factors as important drivers of spatial diversity patterns. However, we detected multiple departures from this pattern, indicating that restrictions in gene flow alone cannot fully explain regional diversification in coloration. A direct test for selection on color was not the primary objective of our study, nonetheless, our analyses reveal
contexts in which there is opportunity for selection. We discuss the interplay among evolutionary and geographic processes with respect to the three general patterns of diversity detected in this study: co-variation of genetic and phenotypic diversity across historical barriers; phenotypic similarity across genetically isolated groups; and phenotypic differentiation in the presence of gene flow.

HISTORICAL BARRIERS AND COVARIATION OF PHENOTYPIC AND GENETIC DIVERSITY

Genetic and phenotypic diversity patterns that covary across geographic barriers indicate that the same processes have shaped both aspects of population diversity, and that the interruption of gene flow associated with that barrier facilitated the differentiation in the two isolated populations. We found that genetic differentiation was associated with color differences across two biogeographic barriers: the Cordillera de Talamanca and Bocas del Toro.

Matrix correspondence analyses, pairwise $\phi_{ST}$ values, and the multivariate discriminant function analyses based on coloration all indicate population isolation due to the Cordillera de Talamanca (Tables 2,3). The uplift of the Cordillera de Talamanca occurred approximately 3 million years ago (Coates and Obando 1996; Kohlmann et al. 2002) and has limited gene exchange between Pacific and Caribbean populations of terrestrial snakes (Zamudio and Greene 1997), frogs (Crawford 2003), and montane salamanders (García-París et al. 2000). This mountain range extends 400 km along the Central American continental divide and reaches its highest point of 3800 m at Cerro Chirripó (Coates and Obando 1996; Kohlmann et al. 2002). The wet tropical forest typical of lower elevations is replaced by cloud forest and dry Páramo above 3100 meters (Kohlmann et al. 2002). These higher elevation habitats are inhospitable to *A. callidryas* and prohibit movement across the Cordillera de
Talamanca. Nonetheless, the barrier imposed by the Cordillera de Talamanca is not absolute. Our phylogenetic analyses revealed a single Pacific individual with a haplotype nested within Caribbean clade A (cab1; Figure 3.3), suggesting either incomplete lineage sorting or low levels of gene flow, current or historic, connecting Pacific and Caribbean populations. The observation that flank-stripe patterns were mixed between Northwestern and Northeastern Costa Rican populations, a trait that otherwise exhibited regional variation, led to the hypothesis that mountain passes at these lower elevations (ca. 900 m) may have historically facilitated migration through dispersal corridors under more lenient climatic and habitat conditions (Savage and Heyer 1967). Despite this potential connection, our data indicate that phenotypic divergence in flank coloration on either side of the mountain range was due to continued geographic isolation over evolutionary time scales and genetic drift.

Limited gene flow and genetic drift (due to lower effective population sizes) will promote phenotypic divergence of admixed populations at contact zones (Rosenblum et al. 2007). Indeed, the largest regional pairwise $\phi_{ST}$ (0.863) and greatest difference in coloration was found between the two regions separated by the Cordillera de Talamanca, (Northwestern and Northeastern CR) despite close geographic proximity (56 km) and relatively recent time since divergence (Figure 3.3). Leg and flank color are more differentiated across this narrow contact zone than between the two of the most geographically distant sites in our study, Gamboa (Panama) and Pavones (Southwest CR), populations that are 800 km apart (Figure 3.5). Fine scale analyses of genetic structure using microsatellite markers are currently underway to examine contemporary patterns of gene flow across the Cordillera de Talamanca. This will determine whether phenotypic divergence is maintained by geographic isolation and/or by selection in the presence of gene flow (Robertson, in prep).
Flank and leg coloration covaried with divergence in mtDNA across Bocas del Toro (Figure 3.3, Figure 3.4). This concordance is consistent with a history of isolation across this smaller barrier. Prior to the formation of the Cordillera de Talamanca, the region of Bocas del Toro experienced a short-lived uplift, approximately 5-7 million years ago. Multiple colonization events consistent with the uplift have been documented for other taxa (Zeh et al. 2003; Weigt et al. 2005). In general, we detected subtle differences in gene and color divergence compared to those observed across the Cordillera de Talamanca. Our results indicate that the barrier at Bocas del Toro is more permeable to gene flow, as evidenced by mtDNA admixture and less phenotypic differentiation. Admixture of mtDNA could also reflect incomplete lineage sorting, or temporally isolated colonization events in the Bocas del Toro region.

**PHENOTYPIC UNIFORMITY IN STRUCTURED POPULATIONS**

Restrictions in gene flow across two Pacific barriers were not associated with divergence in leg coloration for red-eyed treefrog populations. The pattern of genetically structured yet phenotypically uniform populations alert us to the possible roles of selection and drift in shaping these populations. Pacific populations were genetically structured into two clades (Northwestern and Southwestern CR; Figure 3.2) with reductions in gene flow coincident with a geological barrier. The Rio Parrita 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 regions. The nature of this landscape barrier is unknown for red-eyed treefrogs, but genetic isolation could be due to the effects of these historical geological factors and/or dispersal limitations imposed by a riverine barrier. Independent of the exact nature of the barrier, the MCTs and phylogenetic
analyses corroborate the geographic division between northern and southern populations ($\phi_{ST} = 0.781$). Our analyses identified a potential contact zone centered between ‘plb’ and ‘uvi’: mtDNA clade admixture and the exceptionally large average number of pairwise nucleotide differences among haplotypes sampled at ‘uvi’ (Table 3.3) favors the hypothesis of some migrant gene exchange between historically divergent northern and southern haplotypes. Thus, even low levels of gene flow, possibly coupled with selection favoring a similar phenotype seem to be sufficient to prevent phenotypic differentiation that would otherwise arise in isolation (Wright 1937; Slatkin 1985a).

We detected evidence of a second Pacific barrier, the Osa Peninsula: the phylogenetic tree and MCT indicated that the single population from the Osa Peninsula (cam) forms a deeply divergent monophyletic clade within a larger Southwestern CR clade. 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 off-shore island that drifted into the mainland of Costa Rica approximately 2 million years ago (Kohlmann et al. 2002). Genetic isolation of Osa Peninsula populations has also been detected for other vertebrates, including frogs (Crawford 2003; Crawford et al. 2007) and snakes (Zamudio and Greene 1997) suggesting that Osa Peninsula populations are still isolated, despite the reconnection to the mainland. Despite this isolation, we found little evidence of phenotypic divergence in leg coloration, thus indicating that either selection favors a single phenotype in the Osa and adjacent populations, or that contemporary gene flow maintains phenotypic homogeneity of Pacific populations. In contrast, flank color differed across the Osa Peninsula barrier, indicating that our two measures of phenotype are evolving independently.
PHENOTYPIC DIVERGENCE IN THE PRESENCE OF GENE FLOW

Phenotypic divergence of red-eyed treefrog populations was not associated with restricted gene flow across the Caribbean Valley complex, a series of floodplain valleys of the south Caribbean region, that includes, Valle de la Estrella, Valle de Talamanca and Llanura de Santa Clara (Kohlmann et al. 2002; Kohlmann and Wilkinson, 2004; Figure 3.1). 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) and is significant for red-eyed treefrog populations, as well. Phylogenetic analyses revealed historical gene flow between two phenotypically divergent regions, Northeastern and Southeastern CR (Figures 3.4-3.5), indicating that color differentiation across the Caribbean Valley Complex is determined by strong localized selection sufficient to counteract the homogenizing effects of gene flow. Leg coloration across the barrier ranged from entirely blue legs in the north to a mixed blue/orange coloration in the south (Figures 3.3-3.5). These differences may be exaggerated further by reductions in effective population size due to strong selection against maladapted phenotypes (Nosil and Crespi 2004; Nosil et al. 2005; Rosenblum et al. 2007).

MODE OF SELECTION

Our data indicate that the high degree of phenotypic regionalization in the red-eyed treefrog reflects a number of processes, including: differential fixation (through selection) of variants derived from a widespread, ancestral polymorphic state; stochastic processes after isolation; pleiotropic effects (Schemske and Bierzychudek 2007); and/or linkage to other traits under selection. Independent of the exact mechanism, our analyses revealed that different processes drive color divergence in different parts of this species’ range.
Disentangling the selective environment controlling the evolution of color is difficult, because several different processes could underlie divergent phenotypic expression (Hairston 1979; Endler 1980; West-Eberhard 1983; Hoffman et al. 2006). Natural selection for habitat background matching is the most common form of selection documented to date, including for rodents (Hoekstra et al. 2004; Hoekstra et al. 2005), lizards (Thorpe and Baez 1993; Thorpe 2002; Rosenblum 2006; Rosenblum et al. 2007; Stuart-Fox et al. 2007), frogs (Pyburn 1961; Nevo 1973; Stewart 1974; Hoffman and Blouin 2000; Woolbright and Stewart 2008), insects (Kettlewell and Conn 1977b; Sandoval and Nosil 2005; Nosil et al. 2006) and snakes (King and Lawson 1995). Background matching is unlikely to drive color divergence in *A. callidryas* because there are no observable sharp environmental gradients that coincide with breaks among regions. In addition, the flank and leg color patches measured in this study are not cryptic. At rest, red-eyed treefrogs lie still with their brightly-colored flanks and limbs tucked underneath their body; in this position the frog perfectly matches the leaf it sits on (Schwalm et al. 1977; Emerson et al. 1990). In contrast, while active at dusk and throughout the night they sit upright and expose their limbs and flanks. The conspicuous nature of this display suggests that color pattern is used as a visual signal to conspecifics and/or predators during the night when this species is active.

Natural and sexual selection have contributed to color evolution in other species of anurans (Summers and Clough 2001; Siddiqi et al. 2004; Reynolds and Fitzpatrick 2007; Rudh et al. 2007). Selection for brighter, contrasting coloration is an effective, aposematic signal to deter predators for species in the highly toxic poison dart frogs of the family Dendrobatidae (Summers and Clough 2001; Siddiqi et al. 2004). Natural selection for conspicuous coloration could be driven by predator-avoidance success if the contrasting coloration of *A. callidryas* warns predators of the
noxious skin peptides common in this and other phyllomedusine frogs (Sazima 1974; Mignogna et al. 1997; Conlon et al. 2007). Geographic variation in skin peptide composition co-varies with differences in coloration in the Australian treefrog, *Litoria rubella* (Steinborner et al. 1996), therefore color differences could reflect geographic regionalization in skin peptide profiles in *A. callidryas* as well.

Sexual selection can rapidly drive population divergence through female mate choice (West-Eberhard 1983; Masta and Maddison 2002; Summers et al. 2003; Maan et al. 2004; Siddiqi et al. 2004; Summers et al. 2004). Directional selection favoring a large and a highly contrasted color pattern has been observed in the hylid frog, *Hyla squirelli* (Buchanan 1994). In *Dendrobates pumillo*, mate choice experiments revealed that intraspecific population divergence in coloration has also evolved through sexual selection (Summers et al. 1999; Reynolds and Fitzpatrick 2007). Female choice can vary under different environmental conditions, which in turn will promote population divergence (Maan et al. 2004; Maan et al. 2006a; Gray and McKinnon 2007).

For divergent sexual selection to drive color differences among *A. callidryas* populations, divergent hues must be sufficiently distinguishable under the low-ambient light conditions when this crepuscular species is active (Lythgoe and Patridge 1991; Endler 1992; Endler et al. 2005). In addition, *A. callidryas* must possess the visual system for discriminating hues (or differences in luminosity of hues) that are obvious to human observers (Endler et al. 2005). We determined that these two criteria are met for *A. callidryas*, based on microspectrophotometry to characterize the visual pigments in *A. callidryas* and tests of the contrast luminosity of the colored flank and legs against a natural background (Robertson 2008). In addition, we examined color pattern among phyllomedusine frogs using phylogenetic and community-based statistical analyses and found corroborating support for the hypothesis that conspicuous coloration in *A. callidryas* is (at least, in part) a social signal used for
species recognition (Robertson 2008). If dendrobatid frogs serve as a generalized model of color evolution in aposematic colored frogs, then we would predict that bright, contrasting coloration evolved as a response to natural predators in *A. callidryas*, but that sexual selection through female choice has driven the divergence among populations and that the fixation of these regional differences have been facilitated by geographic isolation. While the effects of genetic drift should not be underestimated (Hoffman et al. 2006), sexual selection provides the most compelling initial hypothesis of selection to be addressed with future behavioral studies.

**COLOR EVOLUTION IN RED-EYED TREEFROGS**

The genetic control of coloration is poorly understood in most anurans. However, our analyses lend support to the hypothesis that regional variation in *A. callidryas* is due to differential fixation of pre-existing color morphs rather than the evolution of novel coloration. Our phylogenetic analyses revealed that the Southwest CR region diverged first and is sister to all other regions. This clade contained individuals with predominantly orange legs and orange flanks; however, the two southernmost populations in this region (‘pav’ and ‘cam’) were the most variable of all Pacific populations (Figure 3.4). The presence (albeit limited) of blue coloration in ‘pav’ and ‘cam’ informs us that the full range of coloration occurred deep in the phylogeny and is present across most populations, including populations that are historically isolated from other populations.

**CONCLUSIONS**

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

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LITERATURE CITED


CHAPTER FOUR

VARIABLE ROLES OF BARRIERS ON GENE FLOW AND COLOR PATTERN IN A POLYTPIC ANURAN (AGALYCHNIS CALLIDRYAS)

ABSTRACT

Differences in the relative strength of gene flow, genetic drift, and natural selection across a species’ range can produce markedly different evolutionary outcomes in local populations. Here, I focused on the contemporary evolutionary processes operating at the geographic boundaries of mtDNA clades to understand mechanisms driving phenotypic divergence between some regions while promoting homogeneity between others. The red-eyed treefrog, Agalychnis callidryas, is a common and widespread Neotropical frog that exhibits strong regional differentiation in coloration, color pattern and body size. I used nuclear microsatellite loci to determine the probable number of genetic demes and estimate gene flow within and among 5 mtDNA clades, representing 5 biogeographic regions in Costa Rica and Panama. Within biogeographic regions, I detected genetic isolation with distance without co-varying differences in coloration. Investigation of gene flow across these mtDNA boundaries revealed that two of five putative geographic barriers tested in this study, demarcated differences in color pattern while only one barrier effectively limited gene flow. I determined three general patterns that characterize diversity across clades: genetic isolation by distance with no concordant variation in coloration; genetic isolation with concordant divergence in color pattern; differentiation in color pattern in the presence of gene flow. These results indicate that even across the range
of a single species, gene flow, genetic drift and selection interact in both complementary and contrasting ways to influence phenotypic differences.¹

INTRODUCTION

Intraspecific populations across a species’ range may not evolve at the same rate or be subject to the same diversifying forces (Hendry and Taylor 2004; Chaves et al. 2007). Thus, the degree of phenotypic divergence can vary markedly within a species if local selective constraints counteract the homogenizing effects of gene flow, or if reduced gene flow promotes divergence due to genetic drift in small populations (Endler 1973; Endler 1977; Slatkin 1985). The interplay between gene flow, selection and drift across landscape barriers can therefore result in a mosaic of phenotypically differentiated populations (Sandoval 1994); some may be connected by migrants while others may evolve in relative isolation. The long term consequences of these spatially-varying processes can include divergent ecological adaptations and eventual speciation (Gray and McKinnon 2007).

There are several ways to study the relative effects of gene flow, drift, and selection (Endler 1973; Spitze 1993; McKay and Latta 2002). One approach is to test whether the spatial distribution of polymorphisms in genetic or phenotypic characters is predicted by the degree of connectivity among populations (Shaw 1996a; Richmond and Reeder 2002; Hoekstra et al. 2004; Nosil and Crespi 2004; Richmond 2006; Schemske and Bierzychudek 2007). Under a model of neutral (non-selective) evolution, genetic divergence increases with the geographic distance separating populations (Wright 1937). Color pattern polymorphisms that vary in this same way indicate an underlying role gene flow and drift in determining phenotype (Spitze 1993;

¹ KEYWORDS: Agalychnis callidryas, anuran, Central America, divergent selection, polymorphism, balancing selection
McKay and Latta 2002). In contrast, when the spatial distributions of genetic and phenotypic diversity are incongruent, selection is often invoked as a possible mechanism driving this discordance.

In this study, I examined the relative roles of contemporary gene flow, geographic barriers, selection and drift within and across genetic demes. The red-eyed treefrog, *Agalychnis callidryas*, is a common Neotropical frog broadly distributed from Central Mexico to Colombia (Savage 2002). It is a generalist species that occurs from sea level to approximately 1900 m elevation and can be found associated with most types of standing water (Savage 2002). *Agalychnis callidryas* exhibits striking, regional phenotypic differentiation (Savage and Heyer 1967; Savage 2002; Robertson and Robertson 2008). Despite striking regional differences in color, there are no obvious environmental differences to explain this phenotypic diversity; sites vary in habitat, elevation, and visual environment. It is unlikely that color variation among regions results from differences in localized ecological selection, as observed for many polytypic species that vary along stark environmental gradients (Kettlewell and Conn 1977; Hoekstra et al. 2005; Rosenblum 2006).

Interest in phenotypic diversity and mode of selection on color patterns stems from the assumption that different phenotypes confer differences in survival and fitness, thus informing us of processes leading to differentiation and speciation (Kettlewell and Conn 1977; Endler 1980; Gray and McKinnon 2007; Kingsolver and Pfennig 2007). Color pattern polymorphisms in amphibians (including cryptic and aposematic coloration) are known to evolve through natural and sexual selection processes (Nevo 1973; Milstead et al. 1974; Hoffman and Blouin 2000; Summers and Clough 2001; Summers et al. 2003). I previously discussed the possible modes of selection underlying color pattern in red-eyed treefrog populations (Robertson and Zamudio, in review) and here I focus specifically on the possibility that phenotypic
diversity is maintained due to differential mating success, and thus evolved by sexual selection. Color pattern as a visual signal in mate recognition and choice is shown to evolve through sexual selection in brightly colored diurnal frogs (Summers et al. 1999; Siddiqi et al. 2004; Reynolds and Fitzpatrick 2007) as well as crepuscular/nocturnal frogs (Buchanan 1994), indicating that the visual system of many anurans adequately discriminates color pattern, even in low light environments (Hailman and Jaeger 1974). I chose to examine regional diversification in color pattern as this trait is related to reproductive isolation in many anurans, and likely to evolve through divergent selection in the face of reduced gene flow.

Previous study of the phylogenetic relationship among red-eyed treefrog (*Agalychnis callidryas*) populations in Costa Rica and Panama uncovered five mitochondrial DNA clades, with some admixture among neighboring lineages (Robertson and Zamudio, in review). While mitochondrial DNA is useful for detecting the historical processes that shape population diversity, there are limitations to inferences based on mtDNA alone, including the relatively slower rate of evolution and exclusively maternal inheritance of the marker (Avise 1999). In addition, mitochondrial DNA may underestimate contemporary genetic structure, especially for taxa exhibiting sex-biased dispersal. This study extends previous work by focusing at finer geographic scales and using multiple, unlinked nuclear markers, which elucidate contemporary population dynamics. Specifically, the objective of this study was to examine the underlying roles of gene flow and migration in maintaining relative phenotypic similarity within regions yet promoting diversification across regions. I compared levels of genetic divergence to spatial patterns of phenotypic diversity and tested the null hypothesis that phenotypic divergence is explained by limits to gene flow. Concordant spatial patterns of gene flow and phenotypic diversity supports the hypothesis that evolutionary history, migration, and geographic factors primarily
determine geographic variation. Departures from this pattern would indicate that coloration is adaptive and could evolve through divergent selection and/or genetic drift.

MATERIAL AND METHODS

Field Sampling

Previous research identified populations of *A. callidryas* in Panama and Costa Rica as belonging to five genetic regions (Robertson and Zamudio, in review). Three of these regions are located on the Caribbean versant of Isthmus of Central America, east of the central Cordillera de Talamanca (Northeastern CR, Southeastern CR/PA, and Central PA; Figure 4.1, Appendix 4.1). The other two regions are located on the Pacific versant, west of the Talamancas (Northwestern CR and Southwestern CR; Figure 4.1, Appendix 4.1). I detailed the population and regional differences in phenotypic characters in previous analyses (Robertson and Robertson 2008). Thus, in this study I focus on phenotypic changes with gene flow and migration patterns within and across five mtDNA clades that occupy unique biogeographic regions. I tested the isolating effect of putative barriers across each of five zones (Zones A – E; Figure 4.1).

I conducted field surveys during the breeding seasons (May – August) of 2003, 2004, and 2005. At each site, I captured 10 – 26 individuals and collected data on body size (snout to vent length and mass) and coloration. I documented coloration by taking digital photographs of every individual using a Nikon Coolpix 5700 against a background black-white-grey card for color standardization. For each individual, I photographed three areas of the body to capture the full range of coloration and pattern: 1) posterior surface of the thighs, 2) left flank and 3) right flank of the body.
The Cordillera de Talamanca isolates the Pacific and Caribbean versants on Costa Rica and Panama. The shading of topological relief corresponds to elevation: dark grey (>1300 m), light grey (300 – 1299), white (0 – 300). Elevation higher than 1300 represents unsuitable habitat that exceeds the physiological tolerance of *A. callidryas*. Dry forest habitat between Southwestern CR and Central Panama restricts red-eyed treefrogs dispersal along the Pacific coast of Panama. Five zones of interest shown A – E, population numbers correspond to Appendix 4.1.

**FIGURE 4.1.** Sampling localities for 20 populations of *Agalychnis callidryas* in five biogeographic regions.
At each sampled population, I also collected tissue samples for genetic analyses (Appendix 4.1).

Up to three individuals from eight of the sampled populations were preserved as vouchers and deposited at the Cornell University Museum of Vertebrates (CUMV: 14093, 14206-13, 14228, 14230-35) and the University of Costa Rica, San José (UCR accession numbers: 19100-101, 19213). Photographs have also been archived at the CUMV.

**Quantifying Color Pattern and Differentiation**

The methodology implemented for measuring and quantifying color pattern is described in detail in Robertson and Robertson (2008). Briefly, light intensity was standardized for photographs in Adobe Photoshop with reference to a grey-black-white card present in the background of photographs. The color-corrected photographs of the dorsal aspect of the leg were imported in ImageJ for analyses in the Hue, Saturation, Brightness (HSB) realm. ImageJ generates a frequency histogram of Hue of every pixel along the 0 – 256 color spectrum. I transformed these data into the more conventional 360 degree color spectrum and consolidated the data into 8 bins (each 45 degrees). I calculated the percent and hue (degree) of each bin. Color bins were named according the central hue of that bin (red, orange, yellow, green, light blue, dark blue, purple, violet). For example, the red bin encompasses the range between +/- 22.5 degrees, which subjectively includes the range from violet-red through pure red to red-orange and the orange bin is centered on + 45 degrees and includes shades of orange.

Previous analyses determined that leg coloration of red-eyed treefrogs can be defined broadly as ‘red’ and ‘blue’ encompassing morphs that are entirely red to part red/part blue to entirely blue (Robertson and Robertson 2008). For these analyses, I considered ‘red’ as including the violet, red and orange bins and ‘blue’ as including...
the light blue, dark blue, and purple bins. Yellow and green are excluded because those colors contribute minimally to color differentiation among individuals and regions (Robertson and Robertson 2008).

For each individual, I calculated three color parameters: composite red hue, composite blue hue, and the percentage of leg that was red. Composite measures were the average of three red and three blue bins weighted by the percent contribution of each. To determine if the three color parameters varied within and across zones, I conducted ANOVAs for each parameter separately. The probability of a Type 1 error was adjusted for multiple comparisons using Tukey HSD.

**Microsatellite characterization and genotyping**

I constructed a partial genomic library to isolate microsatellite repeat motifs for *A. callidryas*. I used QIAGEN DNeasy kit to extract DNA from three individuals for microsatellite construction. DNA was digested with two restriction enzymes, Alu I and Hae III, and ligated to a double-stranded SNX linker. I probed DNA fragments with di-, tri-, and tetra biotinylated oligonucleotides and captured with streptavidin-coated magnetic beads, followed by polymerase chain reaction (PCR) amplification using the SNX primer and Vent exo-polymerase for 35 cycles under the following conditions: 95°C for 50 sec; 60°C for 60 sec; 72°C for 90 sec. The product was electrophoresed on a 1% Agarose gel and purified using Qiaquick PCR purification kit. The PCR product was then digested with Nhe 1 and ligated to pUC 19 cloning vector for transformation in Epicurian Coli XL1-Blue MRF’ supercompetent cells. I sequenced colonies containing microsatellites with M13 forward and reverse primers using Big-Dye Terminator-Cycle Sequencing Kit (Applied Biosystems) on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, California). I tested 34 microsatellite
primer pairs, of these 6 were polymorphic and amplified across all populations in the study (Appendix 4.2).

I digested toe-clips in proteinase K (20 µg/ml) and extracted genomic DNA using 5% Chelex solution (cite), incubated for 120 min at 55°C and followed by denaturation at 90°C for 10 min. Six microsatellite loci were amplified in touchdown PCR reactions using a MJ Research DNA Engine Thermocycler. PCR conditions consisted of an initial 90 °C denaturation for 2 minutes, followed by 35 amplification cycles of denaturation at 94 °C for 50 sec, annealing (ranging from 64 – 56°C or 65 to 54°C; Appendix 4.1) for 60 sec, extension at 72 °C for 60 sec, and a final 5 minute extension at 72 °C. The annealing stage in the touchdown program decreased by 2°C until it reached 56°C/54°C (6 cycles) and completed the remaining 29 cycles at the lowest annealing temperature. I performed PCR in 10µl reaction volumes containing: 1X Roche reaction buffer without MgCl₂, 1 mM MgCl₂ (0.75 mM for ACA127 and ACA29), 0.2µM of each PCR primer, 0.2 µM dNTPs, 2.5U Roche Taq (except ACA 36 which contained 2.5U Platinum Taq), and ~ 50 ng of DNA. The forward primer was 5’ labeled with a fluorescent dye (NED, 6-FAM, PET, VIC; Appendix 4.2) and amplicons were multiplexed in two groups and electrophoresed on an Applied BioSystems 3730xl DNA Analyzer. I assigned fragment sizes by comparison with a LIZ 500bp ladder and binned alleles into discrete size categories according to microsatellite repeat motif using Genemapper v3.5 software (Applied Biosystems).

Genetic diversity and differentiation

I calculated allelic diversity and observed and expected heterozygosities for each locus in FSTAT (Version 2.1), and tested for significant deviation from Hardy-Weinberg Equilibrium (HWE) accounting for unequal sample sizes with 2520 permutations of the data across loci. Likewise, I tested for linkage disequilibrium (LD)
based on 300 permutations. Significance for both HWE and LD was determined at \( \alpha = 0.05 \) after Bonferonni correction. I used standard population genetic analyses to characterize population structure. Pairwise F\(_{ST}\) estimates between all populations were estimated in FSTAT (following Weir and Cockerham 1984). I conducted an analysis of molecular variance (AMOVA) to determine the genetic variation among and within groups, in Arlequin v.2 (Schneider et al. 2000). I tested for a significantly positive correlation between genetic and geographic distance (isolation by distance: IBD) correcting for a correlation among points with a Mantel test in Alleles in Space (Miller 2005; Miller et al. 2006). Geographic distances were derived from UTM global positioning system coordinates.

I used Bayesian Inference implemented in Structure 2.0 (Pritchard et al. 2000) to estimate the number of genetic demes represented by sampled individuals and to evaluate the degree of admixture among them. Structure utilizes a Markov chain Monte Carlo (MCMC) algorithm to find the posterior probability that individuals belong to each of \( K \) clusters assuming linkage equilibrium and HWE across multiple, unlinked loci. I applied an admixture model with correlated allele frequencies, alpha max = 10.0 for three datasets: Caribbean only, Pacific only, and the Caribbean/Pacific populations located on the continental divide (across Zone C). Each run consisted of 3 million generations, following a burn in of 1 million generations. The average maximum likelihood values, for each of 25 runs (\( K=1 \) to \( K=15 \): Caribbean; \( K=1 \) to \( K=9 \): Pacific; and \( K=1 \) to \( K=9 \): Continental Divide) were plotted to visually determine the plateau in likelihood scores. I also calculated \( \Delta K \) to identify the greatest rate of change between each subsequent \( K \) (Evanno et al. 2000). Based on these two methods, I chose the most likely values of \( K \) for each dataset and plotted the assignment score for all individuals for a range of most probable \( K \) demes. I used these methods to inform the most probable number of demes and whether individuals within each deme
are a randomly mating population. Assignment profiles for 25 runs were coalesced in Clumpp (Jakobsson and Rosenberg 2007) for the final deme assignment graph.

**Role of geographic barriers**

I tested the isolating effects of five barriers to dispersal previously shown to reduce gene flow among regions (Robertson and Zamudio, in review). Geographic barriers can interrupt gene flow because of physical limitations to dispersal or divergent selective environments that limit survival and/or reproductive success of migrants (Nosil et al. 2005). Thus, in general, barriers can be physical landscape features (e.g., mountains, bodies of water) or climatic and/or environmental characteristics (Grinnell 1914, 1924; Holdridge 1947; Kohlmann et al. 2002). In Central America, the Cordillera de Talamanca, the large mountain range extending 200 km along the continental divide isolates Pacific and Caribbean populations in a number of vertebrate and invertebrate taxa (Zamudio and Greene 1997; García-París et al. 2000; Kohlmann et al. 2002; Crawford 2003). The non-continuous distribution and presence of wet or dry tropical forest has also been implicated in isolating Central American populations (Crawford et al. 2007). To test whether biogeographic regions structured phenotypic and/or genetic diversity patterns on contemporary time scales, I used Matrix Correspondence Tests. The hypotheses regarding the nature of each barrier are discussed in previous papers (Crawford et al. 2007; Robertson and Robertson 2008).

Matrix Correspondence Tests (MCT) and partial MCT (pMCT) can test for associations between genetic and phenotypic divergence by using repeated randomization and permutation to test for the correlation between two distance matrices. The randomized values provide a null distribution with which to test the hypothesis of no association. Significance values were determined by comparing the
observed and expected Z-statistic, generated by 10,000 permutations in the program R ver 1.8-5 using the package Vegan (Oksanen et al. 2007). I conducted pMCT when more than one independent variable was significant ($P < 0.05$) in the individual MCT. Partial MCT tests measure the association between two matrices, while ‘removing’ the variation due to a third variable. As with many partial regression analyses, pMCT can be biased when dependent variables are correlated (Raufaste and Rousset 2001; Castellano and Balletto 2002; Rousset 2002). I therefore compared the pairwise regression and partial regression analyses and interpreted our results in light of this possible limitation.

I conducted MCT and pMCT at two geographic scales: within regions and across putative barriers (Zones A – E). For all analyses, I first determined the effects of geographic distance and barriers on genetic diversity. Then, I tested the association between coloration (independent variable) and geographic distance, genetic distance, and possible barriers. Partial analyses were computed so that the association between coloration and gene flow could be assessed while taking into account the variation due to geographic factors.

I computed the pairwise matrix Euclidean distance of color for individuals in R based on the color parameters discussed above. I used pairwise estimates of $F_{ST}$ for measures of genetic divergence among populations (represented by a matrix of linearized $F_{ST}$ values). Because migration rates may be unequal, I implemented asymmetrical MCT for this comparison. Geographic distance was represented by a matrix of linear distances among populations based on UTM coordinates. A dissimilarity matrix for each biogeographic barrier was represented by a binary matrix with 0 representing populations on the same side of the barrier and 1 for populations located on the opposite sides of the barrier.
The MCT and pMCT allow for specific tests of the contributions of gene flow, geographic distance and landscape barriers under a general framework of genetic IBD. Positive correlations between color pattern, gene flow and geographic distance provide evidence that gene flow/genetic drift equilibrium processes largely drive coloration. Alternatively, selection for an optimal trait is inferred in cases where populations are phenotypically similar but gene flow is restricted. Finally, phenotypic divergence in the presence of gene flow implicates the role of regional selection.

RESULTS

Phenotypic Divergence

I detected color differentiation across three of the five contact zones analyzed (C, A, D; Table 4.1) and no differentiation across two zones (B and E; Table 4.1, Figure 4.3). Zones C, A, and D correspond to the Cordillera de Tilarán, Golfo Dulce, and Talamanca Valley, respectively; the characteristics and significance of each barrier are discussed later. Zone C exhibited maximal divergence: individuals from population 6 were entirely red while individuals from population 9 were entirely blue (Figure 4.3). Zones A and D contained individuals that exhibited both red and blue coloration, but differed in the percentage and average red and blue hue (Table 4.1, Figure 4.3).

Gene Flow Within And Between Regions

Allelic diversity per locus ranged from 7 – 53 and averaged 30.3 across loci (Appendix 4.2). Mean heterozygosity was 0.487 with locus-specific estimates ranging from 0.194 to 0.672 (Appendix 4.2). Overall, I detected no consistent deviation in HWE or LD following Bonferonni correction.
Each zone contrasts two populations (1 vs 2), each located on either side of a putative barrier. Three color parameters examined were: percent of leg colored red (% RED), weighted average blue hue (HUE blue) in degrees (std error), and weighted average red hue (HUE red) in degrees (std error). The LSmeans (std error) compared color differences between the two populations for each parameter. The maximal divergence possible for % red was 100 and the maximal possible HUE differences were 134 degrees. If both populations did not contain blue coloration, then tests were not conducted (-). The probability of a Type 1 error was adjusted for multiple comparisons using Tukey HSD.

I detected significant population genetic structure both among populations and among study regions: in AMOVA, population explained 8% of genetic variance and region explained 22% of genetic variability. The distance-based estimate of genetic divergence (F_{ST}) corroborated the findings based on AMOVA. I detected significant restrictions in gene flow in 204 of 210 population pairwise comparisons (Appendix 4.3). Overall, significant pairwise F_{ST} values were lowest within regions (0.018 – 0.0189) and highest among regions (0.027 – 0.497; Table 4.3). I detected genetic
isolation with geographic distance at all three spatial scales in the study: across all populations \( r = 0.3436, P = 0.0009 \); among Pacific populations \( r = .0981, P = 0.0009 \); and Caribbean populations \( r = 0.3922, P = 0.0009 \).

At the broadest spatial scale, Bayesian clustering revealed strong differentiation between Pacific and Caribbean populations with the exception of localities across Zone C. *Structure* divides five sampling localities into three genetic clusters, but with some admixture.

*Structure* analyses of Pacific populations revealed three demes (Figure 4.2). Along the Pacific, three demes showed a generalized divide between Northwestern and Southwestern populations, with some admixture between populations 8 and 9. The single population sampled from the Nicoya Peninsula (7) was genetically isolated from all other populations, consistent with \( F_{ST} \) estimates. I detected significant admixture at the centrally located population (4), with membership of individuals to both Northwestern and Southwestern demes.

Caribbean populations belonged to five demes (Figure 4.3). Among Northeastern CR populations, I detected a north-south clinal distribution in the membership frequency of two demes. The northern-most population (8) contained individuals that almost exclusively were assigned to a single deme, whereas neighboring populations (9 and 10) contained individuals of mixed ancestry.
Upper panel. Structure plots of Pacific and Caribbean populations revealed K number of demes uncovered for Pacific populations (K = 3) and Caribbean populations (K = 5) and shows admixture among regions. Bracketed arrows indicate populations examined across Zones (A – E). Lower panel. Skeletal phylogram based on Bayesian phylogenetic analyses of the mtDNA gene, NADH1, shows five regional clades with admixture among most neighboring lineages (Robertson and Zamudio, in prep). This study examined the isolating effects of geographic barriers and gene flow in determining phenotype across these admixed zones, indicated above each clade (Zones A–E).

FIGURE 4.2. (Upper panel) Structure plots of Pacific and Caribbean populations. (Lower panel) Skeletal phylogram based on Bayesian phylogenetic analyses of the mtDNA gene, NADH1.
Three parameters of coloration were measured in this study: percent of red, observed as the LS means ± std error (open circles); average weighted blue hue (blue squares); and average weighted red hue (red squares). Each of five zones (A – E) cross genetic demes and are shown in brackets. Color profiles for all individuals contained in the two populations in closest geographic proximity across each zone are shown as a pie chart of the percentage of color in each of eight bins. Dashed lines divide the population pairs according to genetic deme. Photograph of the dorsal aspect of the legs for one representative individual illustrates the range of color variation observed for *Agalychnis callidryas*.

**FIGURE 4.3. The geographic distribution of phenotypic variation across all populations.**
Southeastern CR populations also exhibited a clinal distribution in membership frequency of two primary demes. These two Caribbean CR demes exhibited limited admixture into neighboring northern and southern lineages. Panama was divided into two demes: one deme almost exclusively contained the southernmost population (21) and showed high admixture with Southeastern CR; the other deme contained three Panamanian populations with near-equal membership frequency.

**Association Among Genes, Color Pattern And Geographic Factors: Matrix Correspondence Tests**

Within regions, estimates of genetic differentiation were positively correlated with geographic distance, indicating that populations are genetically structured and in drift-migration equilibrium (Table 4.2). Color pattern was relatively homogenous within regions and did not correlate with estimates genetic or geographic distance for four of five regions (Table 4.2). The exception occurred in Southwestern CR where color varied with geographic distance in pMCT (Table 4.2).

I detected genetic isolation by distance across all five zones as well as an isolating effect of a barrier across Zone C (Table 4.3). For Zone C color differences were associated with restrictions to gene flow due to a barrier, rather than to geographic distance (Table 4.3). The determinants of color across the other four zones varied, and included geographic distance (Zone A), a barrier (Zone D), genetic distance (Zone B), and ‘none’ of the variables tested in this study (Zone E; Table 4.3).

Graphical representation of the correlations estimated in MCT reflects the magnitude of genetic and phenotypic divergence for each zone comparison. For four zones, estimates of gene flow did not deviate significantly from a null model of genetic IBD generated from pairwise comparisons within regions (Figure 4.4), exemplifying the conclusion that barriers did not impede gene flow across these zones.
TABLE 4.2. Matrix and Partial Matrix Correspondence Tests of the determinants of genetic diversity and color pattern within five genetic demes.

<table>
<thead>
<tr>
<th>Independ. variable</th>
<th>Depend. variable</th>
<th>WITHIN REGIONS</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Northeast CR</td>
<td>Southeast CR</td>
<td>Panama</td>
<td>Northwest CR</td>
</tr>
<tr>
<td>$F_{ST}$</td>
<td>KM</td>
<td>0.470 (0.0001)</td>
<td>0.851 (0.0001)</td>
<td>0.778 (0.0001)</td>
<td>0.996 (0.0001)</td>
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<tr>
<td>color</td>
<td>$F_{ST}$</td>
<td>0.047 (0.0920)</td>
<td>0.036 (0.0910)</td>
<td>0.045 (0.1168)</td>
<td>0.0808 (0.0290)</td>
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<td></td>
<td>KM</td>
<td>0.042 (0.0610)</td>
<td>-0.023 (0.6830)</td>
<td>0.098 (0.0229)</td>
<td>0.0525 (0.0750)</td>
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<tr>
<td>Partial</td>
<td>color</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$F_{ST}$ (KM)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>KM ($F_{ST}$)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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Significance values were determined by comparing the observed and expected $z$-statistic, generated by 10000 permutations. Parentheses indicate which factor was controlled for pMCT. Alpha for individual tests were adjusted for multiple comparisons using Bonferonni procedure (adjusted $\alpha = 0.008$).
TABLE 4.3. Matrix and Partial Matrix Correspondence Tests of the determinants of genetic diversity and color pattern across zones.

<table>
<thead>
<tr>
<th>Independent Variable</th>
<th>Dependent Variable</th>
<th>ZONE A</th>
<th>ZONE B</th>
<th>ZONE C</th>
<th>ZONE D</th>
<th>ZONE E</th>
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<tr>
<td></td>
<td>F&lt;sub&gt;ST&lt;/sub&gt;</td>
<td>0.724</td>
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<td>0.931</td>
<td>0.940</td>
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<td></td>
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<td>(0.0001)</td>
<td>(0.0001)</td>
</tr>
<tr>
<td></td>
<td>barrier</td>
<td>0.598</td>
<td>0.08 (0.133)</td>
<td>0.903</td>
<td>0.866</td>
<td>0.958</td>
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<tr>
<td></td>
<td>(0.0001)</td>
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<td>(0.0001)</td>
<td>(0.0001)</td>
<td>(0.0001)</td>
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<tr>
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<td>Color</td>
<td>F&lt;sub&gt;ST&lt;/sub&gt;</td>
<td>0.207</td>
<td>0.351</td>
<td>0.647</td>
<td>0.515</td>
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<td></td>
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<td></td>
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<td>0.592</td>
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<td></td>
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<td>(0.0001)</td>
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<td></td>
<td>Partial</td>
<td>F&lt;sub&gt;ST&lt;/sub&gt;</td>
<td>KM (barrier)</td>
<td>0.597</td>
<td>0.835</td>
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<td></td>
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<td>0.763</td>
<td>-0.722</td>
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<td>(0.0001)</td>
<td>(1.000)</td>
<td>(1.00)</td>
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<td></td>
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<td>F&lt;sub&gt;ST&lt;/sub&gt; (KM)</td>
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<td>0.418 (0.01)</td>
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<td></td>
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<td>(0.0001)</td>
<td>(1.000)</td>
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<td></td>
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<td>(0.001)</td>
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<tr>
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<td>Barrier (F&lt;sub&gt;ST&lt;/sub&gt;)</td>
<td>0.210</td>
<td>0.395</td>
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<tr>
<td></td>
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<td>(0.0001)</td>
<td>(0.0001)</td>
<td>(0.001)</td>
<td>(0.001)</td>
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<tr>
<td></td>
<td>KM (F&lt;sub&gt;ST&lt;/sub&gt;)</td>
<td>0.293</td>
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<td>-0.132</td>
<td>0.431 (0.001)</td>
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<td>(1.000)</td>
<td>(1.000)</td>
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<tr>
<td></td>
<td>KM (barrier)</td>
<td>0.236</td>
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<td>(0.0001)</td>
<td>(0.0001)</td>
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<tr>
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<td>F&lt;sub&gt;ST&lt;/sub&gt;, barrier</td>
<td>barrier</td>
<td>None</td>
<td></td>
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</table>

Significance values were determined by comparing the observed and expected z-statistic, generated by 10000 permutations. Parentheses indicate which factor was controlled for pMCT. The factor(s) determining color differences across zones indicated for each zone. Alpha for individual tests were adjusted for multiple comparisons using Bonferroni procedure (adjusted α = 0.0023).
Linear regression of within-region comparisons (black squares) shows a positive relationship with $F_{ST}$ and geographic distance (top panel) but with no relationship between phenotype and geographic distance (bottom panel). **Top panel.** Pairwise comparisons across zones (grey squares) show larger than expected $F_{ST}$ for only Zone C. **Bottom panel.** Population pairwise comparison of phenotypic divergence within regions (black squares) with standard error (dashed lines) shows that three zones exhibit high phenotypic divergence from expectations based on within-region comparisons (Zones A, C, D).

**FIGURE 4.4.** Relationship between genetic, phenotypic and geographic distance within regions and across zones.
Zone C is the single exception: $F_{ST}$ was higher than predicted under a model of IBD for the geographic distance between sites (Figure 4.4). I did detected significant deviation in the magnitude of phenotypic differentiation across three zones.

**DISCUSSION**

I examined genetic and color pattern differentiation within and across regions to test the hypothesis that restrictions to gene flow due to geographic factors (geographic distance and barriers) underlie spatial patterns of phenotypic diversity. Inferences of fine scale gene flow patterns using multiple, unlinked genomic markers revealed that red-eyed treefrog populations are in genetic equilibrium within biogeographic regions and that gene flow across admixed mtDNA clades was restricted due to effects of a one of five barriers tested in this study. Within regions, we detected genetic IBD but with no concordant change in phenotype indicating that 1) processes other than gene flow and drift favor phenotypic homogeneity within regions 2) regional differentiation is due primarily to processes acting at deme boundaries.

Evaluation of contemporary patterns of gene flow in light of the historical barriers that determine population structure allows us to discern the effects of dispersal, lineage sorting, and recent introgression on the distribution of genetic and phenotypic diversity. The inferred patterns of genetic structure based on nuclear and mitochondrial DNA differed across red-eyed treefrog populations, underscoring the importance of looking across evolutionary time scales. Mitochondrial DNA is useful for detecting the historical processes that shape populations (Avise 1999), but can underestimate contemporary genetic structure when populations have become genetically isolated over recent time scales. Alternatively, isolating barriers to historical gene flow can be broken down with changes in climate and/or landscape
features. The inferences based on mtDNA and ncDNA revealed several instances of discordance consistent with both of these scenarios.

In one case, I detected mtDNA haplotype sharing among geographic regions that were found to be genetically isolated using ncDNA estimates of gene flow. The Northwestern CR region is one of several biogeographic zones known for its high species endemicity due geographic isolation imposed by dry forest (Savage 2002). Northwestern CR population 7 was contained in the same mtDNA clade as other Northwestern CR and Northeastern CR populations (Robertson and Zamudio, in review; Figure 4.2). However, analyses using microsatellite loci revealed this population is genetically isolated from all other sample localities (Appendix 4.3, Figure 4.2). Shared mtDNA haplotypes and the incomplete lineage sorting of Northwestern CR population 7 most likely reflect historical rather than contemporary dispersal patterns. Persistent isolation over time can lead to differentiation in a suite of behavioral, genetic, and phenotypic traits (Gillespie et al. 1994; Shaw 1996b, a; Jordan et al. 2003); thus the geographic isolation detected for the Northwestern CR population 7 has important implications for potential incipient speciation.

In two cases, I detected discordance between ncDNA and mtDNA characterized by mtDNA divergence but high ncDNA gene flow, indicating that historical geographic barriers that resulted in early divergence have likely been broken down, permitting recent gene flow. For instance, Osa Peninsula individuals (Southwestern CR population 2) formed a well-supported mtDNA clade distinct from other Southwestern CR populations (Robertson and Zamudio, in review), yet this population was not well-differentiated based on microsatellite data (Figure 4.2; Appendix 4.3). In a second case, the phylogenetic reconstruction of mtDNA haplotypes supported a north-south divide among Pacific groups, with limited admixture between the two most proximate populations (Northwest 5 and Southwest
Analyses of microsatellite data, however, indicated that high gene flow (no effects of a barrier) currently connects these two populations (Figure 4.3, Table 4.3).

In light of the historical mechanisms responsible for genetic structure at broad spatial scales, we can approach finer-scale questions through focus on the covariation of phenotypic and genetic diversity with respect to putative geographic barriers to gene flow. Phenotypes of *A. callidryas* are patchily distributed across this species’ range and just as several phylogeographic scenarios underlie the discordance between mtDNA and ncDNA, multiple evolutionary mechanisms are likely responsible for high levels of phenotypic diversity. I examined phenotypic/genotypic covariance under the theoretical expectation that covariation implicates a lack of strong sexual and/or natural selection. This framework, tested at multiple spatial scales and across multiple putative barriers, allows us to examine the extent to which the relative roles gene flow, selection and drift varies across populations.

Within regions, genetic divergence varied with geographic distance. Individuals within Caribbean and Pacific versants exhibited genetic isolation by distance (Table 4.4) and a clinal change in deme membership estimated by the assignments in the *Structure* analyses (Figure 4.3). This indicated a stepping stone pattern of migration within regions. In contrast, color pattern was relatively homogenous within regions: that is, I observed no change in phenotype with either genetic or geographic distance. These results indicate that color pattern does not evolve through gene flow processes alone. Overall, phenotypic similarity within regions points towards the maintenance of a single phenotype through stabilizing selection and/or through the homogenizing effects of gene flow that counter any localized divergent selection. The exception occurred in Southwest CR where I observed an association between restrictions in gene flow and color differentiation,
indicating the evolutionary processes driving phenotypic diversity within the Southwestern CR region is distinct from the other regions in the study.

The discontinuous array of phenotypes across study sites was not solely due to the isolating effect of barriers nor the result of a similar evolutionary process. Instead the geographic structure of genetic and phenotypic variation indicates heterogeneity in the relative effects of barriers, gene flow, selection, and genetic drift across red-eyed treefrog populations; a pattern observed in other studies of morphological and genetic isolation across ecological zones (Chaves et al. 2007). These data support three mechanisms of genetic and phenotypic differentiation across the five zones I examined.

Restricted gene flow: concordant genetic and phenotypic divergence is due to the isolating effects of a geographic barrier.

Phenotypic differentiation across geographic boundaries at small spatial scales has been observed in conspecific populations of plants (Geber and Eckhart 2005; Schemske and Bierzychudek 2007), insects (Nosil et al. 2003; Nosil et al. 2006), and vertebrates (Endler 1980; Hoekstra et al. 2004; Crispo et al. 2006) and in some cases divergence can lead to rapid ecological speciation (Hairston et al. 2005; Nosil et al. 2005; Hendry et al. 2007). *Agalychnis callidryas* populations spanning Zone C exhibited high genetic divergence and extreme color differentiation over this relatively small spatial scale (56 km): multiple microsatellite loci exhibited non-overlapping alleles across this physical boundary and individuals from Pacific localities had orange legs and those from the Caribbean localities had blue legs. The geographic distance between the Northwest 6 and Northeast 9 (56 km) was comparable to other population pairs elsewhere in the study area that exhibited much lower estimates of genetic ($F_{ST}$) and phenotypic distance (Figure 4.4), further reinforcing the conclusion that both
genetic and phenotypic isolation is due to the presence of a barrier (Table 4.3). Zone C lies at the junction of two volcanic mountains, the Cordillera de Tilarán and Cordillera de Talamanca, that separate Caribbean and Pacific populations. Throughout most of Costa Rica and Panama, the Cordillera de Talamanca reaches an elevation outside the physiological capacity of the species (> 3100 m) and contains inhospitable habitat to A. callidryas (dry Paramo) and thus explains the effectiveness of this mountain range as a barrier to transcontinental migration. However, Zone C lies at the northern edge of the Cordillera de Talamanca where the elevation is lower (970 m; Appendix 4.1) and the habitat ideal to support A. callidryas, thus indicating that dispersal between the Caribbean and Pacific could be possible. The effectiveness of this barrier, however, has remained robust and intensive population sampling across this putative contact zone might reveal the evolutionary processes underlying the maintenance of this barrier.

Moderate gene flow: phenotypic divergence in the presence of gene flow (no barrier).

For A. callidryas populations across Zone D, differences in color were not associated with isolation due to a geographic barrier, nor genetic isolation by distance. Instead, phenotypic divergence was greater than expected based on the geographic and genetic distance of populations sampled across the zone (Figure 4.4). Because geographic and genetic factors cannot solely explain observed levels of phenotypic differentiation, other non-neutral processes such as spatially varying (or divergent) selection are invoked as mechanisms contributing to color pattern divergence across Zone D. Zone D is centered among Caribbean populations; it is possible that the coloration characteristic of the Southeastern CR region (blue and orange legs) could be an ‘intermediate’ phenotype resulting from hybridization of Northeastern CR (blue) and Central Panama (primarily orange) forms. In this case, the discontinuous
distribution of phenotypes along Caribbean populations would be due to the combined effects of geographic factors (both distance and barriers), genetic isolation by distance, and spatially varying selection operating primarily at the boundaries of distinct biogeographic regions.

Populations across Zone A (Southwestern CR 1 and 3) exhibited a similar pattern of substantial phenotypic differentiation despite moderate levels of gene flow (Appendix 4.3; Figures 3.3-3.4). I found no evidence that color pattern differences were due to a barrier or restricted gene flow, and thus raises the possibility of divergent selective environments. Alternatively, this pattern could have arisen if populations were historically isolated and experienced only recent contact. The phylogenetic reconstruction of populations, however, shows limited mtDNA divergence between these populations spanning Zone A, thus does not support this hypothesis (Robertson and Zamudio, in review).

*High gene flow: phenotypic similarity due to gene flow (no barrier).*

Finally, some populations of red-eyed treefrogs, such as those across Zones B and E, exhibited little to no genetic or phenotypic differentiation across potential barriers. Virtually no color differences were found within or between populations spanning Zone B over a moderate spatial scale (86 km): frog legs were orange-red in all populations. I detected low genetic divergence among these Pacific populations suggesting that phenotypic similarity across this zone is maintained by ongoing gene flow without strong, spatially variable selection for color.

Likewise, despite spanning large geographic and elevational distances across Zone E (124 km and 700 m elevation), most individuals exhibited primarily orange coloration in the leg but with a low percentage of blue. These populations contained multiple phenotypes that varied in the percent of blue from 0 – 29%, (Figure 4.3). The
putative barrier was ineffective at preventing gene flow or in accumulating differences in color pattern. Thus, gene flow among populations likely underlies phenotypic similarity across this zone. The greater inter-individual variation across Zone E compared to Zone B could be due to the higher overall phenotypic diversity exhibited by Caribbean populations relative to Pacific populations. In addition, demographic differences, such as higher effective populations sizes might contribute to the maintenance of within-population polymorphism.

CONCLUSIONS

Spatial patterns of phenotypic diversity reflect a balance between gene flow and the diversifying effects of selection and genetic drift. Estimates of gene flow between populations based on nuclear data combined with spatial patterns of phenotypic diversity revealed that the interplay of these evolutionary processes varies across the species range. Barriers can impede gene flow and lead to phenotypic divergence, such as the barrier across Zone C that isolated Caribbean and Pacific versants, but this effect was not universal. In other instances, phenotypic diversity across barriers was maintained in the presence of some genetic connectivity, while in other instances there was neither genetic nor phenotypic differentiation across a barrier. Thus, spatially varying selection combined with the isolating effects of geographic factors has resulted in a patchy distribution of phenotypes across Costa Rican and Panamanian populations. Disentangling the evolutionary processes acting across regions is essential to understanding the maintenance of diversification in a polytypic species and underscores the importance of evaluating spatial diversity patterns across historical and contemporary time scales and across multiple, putative barriers.
ACKNOWLEDGMENTS

I thank the Smithsonian Tropical Research Institute (STRI) and the Organization for Tropical Studies (O.T.S.) for logistical support during this project. The Ministry of the Environment (ANAM) in Panama and the Ministry of the Environment and Energy (MINAE) in Costa Rica granted research, collecting, and export permits. La Selva Biological Station and the Cabo Blanco Nature Preserve gave permission to conduct research. C. Dardia (Cornell University Museum of Vertebrates) and G. Chaves (Museo de Zoología, Universidad de Costa Rica, San José) assisted with curation of voucher specimens. Microsatellite library construction was conducted in the Evolutionary Genetics Core Facility at Cornell University and I thank S. Bogdanowicz for his assistance. I am especially grateful to L. Davis, A. Vega, C. Vriesendorp, and M. Williams for field assistance; G. Hooker, J. Richmond, A. Robertson, F. Vermeylen and V. Zayas for statistical help; and K. Jenkins and J. Sigala for assistance with the map. This work benefited from resources of the Computational Biology Service Unit at Cornell University, which is partially funded by Microsoft Corporation. For discussion that significantly improved this manuscript I thank A. Coulon, L. Chan, K. Galbreath, H. Greene, J. Richmond, A. Vega, C. Vriesendorp, and K. Wagner. This study was funded by an NSF Doctoral Dissertation Enhancement Grant (DEB-0506043) and awards from OTS, STRI, Tinker Foundation, Einaudi Center, and the Department of Ecology and Evolutionary Biology at Cornell University to JR, and a National Science Foundation Population Biology Grant (DEB-0542848) to KZ.
APPENDIX 4.1. Sampling populations of *Agalychnis callidryas* from five regions in Costa Rica and Panama.

<table>
<thead>
<tr>
<th>Region</th>
<th>No.</th>
<th>Province</th>
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<th>GIS (Lat, Long, El)</th>
<th>color</th>
<th>DNA</th>
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<td>Puntarenas, CR</td>
<td>Pavones</td>
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<td>26</td>
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<td>Sierpe</td>
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<td>19</td>
<td>22</td>
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<td>4</td>
<td>Puntarenas, CR</td>
<td>Uvita</td>
<td>9.123, -83.701, 26</td>
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<td>32</td>
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<td>3</td>
<td>Puntarenas, CR</td>
<td>Campo</td>
<td>8.6909, -83.5013, 35</td>
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<td>19</td>
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<td>Pacific</td>
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Geographic coordinates (GIS) are Latitude (Lat), Longitude (Long), elevation (El, m). Sampling sizes for color pattern (color) and microsatellite analyses (DNA) provided. Population number (No.) indicates position on Figure 4.1.
APPENDIX 4.2. Polymorphic di- and tetranucleotide microsatellite loci characterized for *Agalychnis callidryas*.

<table>
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<th>Locus</th>
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T_a is the annealing temperature used in touchdown PCRs. The product range size for each locus (bp), the number of alleles (A), proportion of observed (H_o) and expected (H_e) heterozygosities, and estimates of Weir and Cockerham F_{IT} and F_{IS} averaged overall populations.

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Significance tested following Bonferonni correction set at 0.000238 and indicated in bold. Estimates based on 4200 permutations in FSTAT. Geographic distance in Kilometers (below diagonal) between each site was calculated as the distance around the Cordillera de Talamanca. Population numbers according to Appendix 4.1.


CHAPTER FIVE

DIVERGENCE IN HISTORICAL AND CONTEMPORARY ESTIMATES OF GENE FLOW AND COLOR PATTERN IN TWO SYMPATRIC AND WIDESPREAD TREEFROGS

ABSTRACT

Comparative phylogeographic studies of co-distributed taxa test the degree to which historical processes have shaped contemporary population structure. Concordant patterns of lineage divergence among taxa suggest that shared processes across landscape features result in similar evolutionary outcomes. 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 co-distributed and wide-ranging Neotropical frogs (*Agalychnis callidryas* and *Dendropsophus ebraccatus*) that share many ecological characteristics yet exhibit different spatial patterns of phenotypic diversity. We detected significant differences in the phylogenetic history, the degree of admixture among clades, and limits to gene flow due to specific geographic barriers. Due to low concordance of historical diversification processes in our two focal species, we compared the relative effects of different microevolutionary processes to explain the unique patterns of diversification in each taxon. Differences in the levels of gene flow, patterns of genetic isolation by distance (IBD), and selective mechanisms underlying phenotypic diversity in our study taxa underscore the significance of species-specific ecological and life history traits in determining the phylogeographic and fine scale structure of phenotypic and genetic diversity. Our comparative approach allows us to test the generality of biogeographic structure and
the evolutionary processes that determine spatial patterns of diversification in lower Central America.

**INTRODUCTION**

Concordant phylogeographic patterns are an indication that landscape features are a predominant determinant of lineage diversification of co-distributed taxa. The dynamics of natural populations, however, are often more complex with different organisms responding to common historical processes in differing ways. Mitochondrial DNA is commonly used to infer the evolutionary history of populations and to test for the generality of population structure in co-distributed taxa (Avise et al. 1987). In contrast, multi-locus genomic nuclear DNA markers (e.g., microsatellites) are typically used to test contemporary gene flow patterns at relatively fine spatial and temporal scales. Combining estimates derived from both genetic markers with a phenotypic marker is a hallmark of comparative taxon studies (Avise 1986; Avise et al. 1987; Avise 1989; Bermingham and Lessios 1993; Knowles 2004). Such studies permit a comprehensive investigation of historical and contemporary processes that drive spatial patterns of diversity. Further, they disentangle the generalities of dominant landscape features with species-specific processes to explain lineage diversification.

Due to its complex and dynamic geologic and climatic history, lower Central America is an ideal setting for the study of the biogeographic and ecological processes underlying species distribution patterns (Coates and Obando 1996; Campbell 1999; Kohlmann et al. 2002; Savage 2002). Plate tectonics have played an important role in the geologic history of Central America, in particular in Costa Rica and Panamá (Campbell 1999; Kohlmann et al. 2002; Savage 2002). In recent geologic time (5 mya) volcanic formations such as the Cordillera de Talamanca, the mountain range
extending along the Central American continental divide (Campbell 1999; Coates and Obando 1996) have effectively altered the climate experienced by the Caribbean, Pacific and Central regions (Campbell 1999; Holdridge 1947). Biotic regions in Costa Rica and Panama vary from dry, lowland, deciduous forest (Pacific) to cloud forest (along the divide) to hot and wet lowland rainforest (Caribbean): the Isthmus of Central America is marked by high floral and faunal diversity and endemcity, in part due to this biome diversity (Holdridge 1947; Savage 2002). In addition, the major geographic features, such as the Cordillera de Talamanda, contribute to concordant diversification patterns across many Central American taxa (Zamudio and Greene 1997; Garcia-París et al. 2000; Kohlmann et al. 2002; Crawford 2003). We tested whether specific landscape features were reliable predictors of genetic structuring and adaptive color pattern variation for co-distributed Isthmusian anuran taxa in Costa Rica and Panama. If these barriers significantly restrict migration, they could potentially create opportunities for divergent local selection and genetic drift to cause diversification among lineages.

To test the generality of the effects of biogeographic landscape features on distribution patterns we investigated historical and contemporary patterns of gene flow in two highly polymorphic species, the red-eyed treefrog (Phyllomedusinae: *Agalychnis callidryas*) and the hourglass treefrog (Dendropsophini tribe: *Dendropsophus ebraccatus* (Faivovich et al. 2005). These 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 of water, and color pattern polymorphisms (Savage 2002). Despite these life history similarities, *A. callidryas* and *D. ebraccatus* show marked differences in phenotypic distribution patterns. *Agalychnis callidryas* exhibits striking, regional differentiation in body size, flank-stripe pattern, and leg and flank coloration (Savage and Heyer 1967; Savage
2002; Robertson and Robertson 2008). In contrast, *D. ebraccatus*, also exhibits multiple dorsal color pattern types, but the polymorphisms are distributed within, rather than among, populations (Duellman 2001).

Animal color pattern (CP) evolves through natural selection (favoring conspicuous and/or crypsis) to avoid/deter predators and sexual selection favoring conspicuousness to aid in conspecific recognition. It is probable that natural selection has favored bright coloration as an aposematic, warning signal to predators of the noxious skin peptides common in phyllomedusine frogs (Sazima 1974; Mignogna et al. 1997). However, community and phylogenetic-based analyses of character displacement in Central American phyllomedusine frogs support the hypothesis that color pattern serves (at least, in part) a species recognition function. For this color signal to evolve through sexual selection, the taxon must have the visual system to detect the signal under ambient conditions (Cott 1940; Endler 1990; Lythgoe and Patridge 1991; Summers et al. 2003). We used microspectrophotometry to characterize the visual pigments of the red-eyed treefrog and confirmed that this species possesses the visual system to discriminate among hues that represent the full range of color pattern exhibited across its range.

In contrast, our analyses of Central American Dendropsophinii frogs revealed that color pattern polymorphisms likely evolved through natural selection pressures. The Convergent Niche Hypothesis (Grinnell 1924) predicts that multiple, syntopic species will exhibit similar characteristics (color pattern) if those characters maximize survival in a particular visual environment (Grinnell 1924; Warburg 1965; Rand and Williams 1970; Stewart 1974; Endler 1982; Harmon et al. 2005). In this case, color pattern serves as an effective signal for predator avoidance, indicating a role of the environment in the evolution of CP (Endler 1982). Thus, if the geographic distribution of color pattern in *D. ebraccatus* could not explained by patterns of gene flow, then
localized balancing selection favoring multiple phenotypes would be the best initial hypothesis to explain population-level heterogeneity (Endler 1973; Slatkin 1985; Sandoval 1994; Lenormand 2002). Testing the adaptive significance of coloration was not the objective of this study. However, quantification of the nature and geographic distribution of color pattern within and among populations is the requisite first step for interpreting the mechanisms underlying phenotypic diversification and for subsequent hypothesis testing on the mode of selection on maintaining color pattern (Endler 1982; Hoffman et al. 2006; Gray and McKinnon 2007).

Within natural populations, genetic drift, migration and selection contribute to the maintenance of CP (Endler 1973, 1980; Gray and McKinnon 2007). Concordance in genetic and phenotypic diversity indicates that populations experiencing gene flow are also similar in color pattern (Wright 1937; Ritchie et al. 2007; Roberts et al. 2007). However, discordance between these two distance measures departs from this null model, indicating a role of localized selection, drift or both (Endler 1982; Lenormand 2002; Saint-Laurent et al. 2003; Price 2006; Harper and Pfennig 2008). Thus, the relative strength of the homogenizing effects of gene flow and the diversifying effects of selection has consequences for the geographic distribution of phenotypic diversity.

If gene flow is highly restricted among populations, then the maintenance of CP polymorphisms will reflect population-level processes, and thus be largely driven by localized selection and drift.

Our main objective was to elucidate and compare the processes underlying genetic and phenotypic variation in two common and widespread Neotropical frogs. To achieve this, we had four primary aims: (1) recover the historical biogeography and contemporary population genetic structure using mtDNA sequences and polymorphic microsatellites markers; (2) quantify the geographic distribution of phenotypic diversity within and among regions; (3) examine the associations among gene
diversity, color pattern and geography; and (4) compare patterns and processes of diversification between our two focal taxa. Quantification of regional phenotypic and genetic diversity (mtDNA and microsatellite genotyping) for *D. ebraccatus* was conducted and analyzed in this study; while data from prior studies of *A. callidryas* color pattern (Robertson and Robertson 2008), phylogenetic history and microsatellite genotyping were combined and analyzed here for comparative purposes.

The strength of this study lies in its comparative nature and geographical coverage, both factors that allow us to disentangle historical and species-specific traits in shaping populations. Together, the relative effects of geographic barriers, gene flow patterns and the strength of localized selection results in a mosaic of differentiated populations. The processes of diversification, therefore, may not be universal across populations within species, or across species (Sandoval 1994; Shaw 1996; Quesada et al. 2007). We demonstrate how diversity is maintained in space and time in two species that differ markedly in their geographic distribution of phenotypic diversity, despite occupying nearly equivalent ranges, living in similar habitats, and sharing similar reproductive biologies.

**MATERIALS AND METHODS**

*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 (Savage 2002). These five regions structured the genetic and phenotypic distribution of diversity in *A. callidryas* (Figure 5.3) and were used in this study as an initial hypothesis for the diversification in *D. ebraccatus*. We sampled fewer sites for *D. ebraccatus* (n = 15) than *A. callidryas* (n = 21), although 14 of those sites were sampled for both species (Appendix 5.1). 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, measured body size (snout-vent-length; SVL and mass) and collected data on dorsal color pattern (Appendix 5.1). We sampled all individuals we encountered at each population so as to not bias our measures of color pattern frequencies. We documented color pattern by taking digital photographs of every individual using a Nikon Coolpix 5700. Tissue samples (1 – 2 toe clips) were collected in the field and stored in 100% ethanol for use in genetic analyses (Appendix 1 for sample sizes).

Two individuals of D. ebraccatus and three individuals of A. callidryas were preserved as vouchers and deposited at the Cornell University Museum of Vertebrates, CUMV (accession #: CU 14029, 14093,14206-13,14228,14230-35) and the University of Costa Rica, San José (UCR; accession #: 19102, 19100-101, 19213). All photographs have been archived at the CUMV. All other individuals were released at sites of capture.

QUANTIFYING PHENOTYPIC DIVERSITY

Dendropsophus ebraccatus

Yellow, gold and brown blotches and spots characterize the dorsal pattern of D. ebraccatus, but the dominant dorsal pattern in most populations resembles an hourglass shape (Patterns 1 and 2, Figure 5.1 (Duellman 2001). Previous analyses of the geographic distribution of color pattern revealed widespread polymorphisms (Duellman 2001); however, this study addressed the extent to which dorsal pattern frequencies vary within and among populations at fine spatial scales (Figures 5.2-5.3).
Agalychnis callidryas

We detected significant co-varying regionalization in coloration and flank-stripe patterns in a previous study of A. callidryas populations (Robertson and Robertson, 2008). In this study, we focused our analyses on flank-stripe pattern because this trait provides an appropriate comparison to type of color pattern exhibited by D. ebraccatus. Agalychnis callidryas has bright, contrasting flank-stripes, usually white to pale yellow, overlaying the background color. We implemented the scoring system designed by Savage and Heyer (1967) to assign individuals to one of five flank-stripe pattern types: A, AB, B, BC, and C (Figure 5.1). In 2005, we discovered frogs with two novel combinations of the three basic types; for these individuals, we modified the Savage and Heyer protocol to include categories AC and ABC (Figure 5.1). We analyzed only the left side of the body for each frog to avoid any bias that might occur due to lateral asymmetry (Savage and Heyer 1967(Robertson and Robertson 2008)). For both species, we conducted contingency analyses to determine whether the frequency distribution of color-pattern types varied among populations. Significance of Chi-Square test was conducted using Fisher’s exact test to control for small sample sizes.
Color pattern in *Dendropsophus ebraccatus* was characterized into four categories: (1) hourglass with spots (2) hourglass shape with no spots (3) spots, (4) and plain (Duellman 2001). Flank pattern variation in *Agalychnis callidryas*. Pattern A individuals have a horizontal line connecting all vertical stripes, in pattern B, the vertical stripes are disconnected, and each is ‘T’ shaped, and 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.

**FIGURE 5.1. Color pattern polymorphisms for *Dendropsophus ebraccatus* and *Agalychnis callidryas*.**
The frequency of each dorsal pattern (Figure 5.1) shown for each sampled population. Population number indicated in the upper left hand corner of each frequency histogram chart. The Cordillera de Talamanca isolates the Pacific and Caribbean versants on Costa Rica and Panama. The shading of topological relief corresponds to elevation: dark grey (>1300 m), light grey (300 – 1299), white (0 – 300). Dry forest habitat between Southwestern CR and Central Panama restricts red-eyed treefrogs dispersal along the Pacific coast of Panama. Five zones of interest shown A – E, population numbers correspond to Appendix 5.1.

FIGURE 5.2. Sampling localities for 16 populations *Dendropsophus ebraccatus* in five biogeographic regions.
The frequency of each of seven flank stripe pattern (Figure 5.1) shown as pie graphs for each sampled populations. Flank pattern C is shown for each population in grey to illustrate the disjunct distribution of Pacific and Caribbean populations. Population number indicated in the upper left hand corner of each frequency histogram chart. The Cordillera de Talamanca isolates the Pacific and Caribbean versants on Costa Rica and Panama. The shading of topological relief corresponds to elevation: dark grey (>1300 m), light grey (300 – 1299), white (0 – 300). Dry forest habitat between Southwestern CR and Central Panama restricts red-eyed treefrogs dispersal along the Pacific coast of Panama. Five zones of interest shown A – E, population numbers correspond to Appendix 5.1.

FIGURE 5.3. Sampling localities for 20 populations Agalychnis callidryas in five biogeographic regions.
MOLECULAR BIOGEOGRAPHY AND POPULATION GENETIC STRUCTURE

Phylogenetic Analyses

We extracted DNA from 79 *D. ebraccatus* individuals and two outgroup taxa *Pseudohyla puma* and *Dendropsophus microcephalus*. Tissues (toe-clips) collected in the field were digested in standard lysis buffer with Proteinase K using the Qiagen DNeasy Tissue Kit (QIAGEN, Valencia, California) following manufacturer’s protocols. 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 (5’TGGGGTATGGGCCAACAGCT3’; Wiens *et al.* 2005 and 16S-frog (5’-TTACCCTTGGGATAACAGCGCAA-3’; Reeder, personal communication). PCR reactions were performed in a total volume of 25 µl, each containing 100 ng template DNA, 1X PCR Buffer, 0.75 mM dNTPs, 1.5 mM MgCl₂, 1 µM primer, and 0.625 units of Taq polymerase. PCR amplification conditions were: 95 ºC initial denaturation for 5 minutes, 35 cycles of denaturation at 94 ºC for 1 min, annealing at 50 ºC for 1 min, extension at 72 ºC for 1 min, and a final 5 minute extension at 72 ºC. We used Exonuclease (10 units) and SAP (1 unit) to remove unincorporated oligonucleotides and dNTPs. For each successful amplification, we performed cycle sequencing reactions with Big Dye terminator sequencing kits according to manufacturer’s protocol, using the same primers used for fragment amplification (Applied Biosystems, Perkin Elmer, Foster City, CA).

Cycle sequencing reaction conditions were 25 cycles of 96 ºC (30 sec), 50 ºC (15 sec), and 60 ºC (4 min). We sequenced the gene in both directions to avoid base-calling ambiguities. Products were column purified to remove non-incorporated
terminator dye using Sephadex™ G-50 and products were electrophoresed on an ABI 3100 Genetic Analyzer. Electropherograms were checked by eye and contigs were created using Sequencher ver.4.1 (GeneCodes, Michigan).

We partitioned the sequences into two datasets, ND1 and partial 16S + tRNA gene sequences for alignment ClustalW (Thompson et al. 1994) in the MegAlign ver. 6.1.2 program of the Lasergene sequence analysis software (DNASTAR, Inc., Madison, Wisconsin). The initial guide tree was aligned using Gap Length Penalty = 6.66, Gap Extension Penalty = 0.05, Delay Divergence Sequences = 30%, and Transitions = .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 across this range were excluded as characters in phylogenetic analyses.

We conducted phylogenetic analyses in PAUP* ver. 4.0 b10 (Swofford 2001) using maximum-likelihood (ML) and Bayesian analyses in MrBayes 3.0b4. For ML and Bayesian analyses, we used Modeltest ver. 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 (range = 0.05 – 5.66 %; μ = 2.61%; σ = 1.65%) was selected as the preferred model. We used unequal base frequencies according to the model (A=0.3076, C=0.22190, G=0.11182, T=0.35230), pinvar of 0.340837 and gamma shape parameter 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 in Mr. Bayes with the exception of the alpha shape parameter (exponential, mean = 1.0) and branch lengths (exponential, mean = 0.1). Standard measures of haplotype and nucleotide diversities within each
population were calculated in Arlequin ver. 3.01. Pairwise $F_{ST}$ estimates between all populations were estimated in FSTAT, and we implemented an analysis of molecular variance (AMOVA), to determine the genetic variation among and within groups, in Arlequin ver. 3.01 (Schneider et al. 2000).

**Microsatellite characterization and genotyping**

We constructed an enriched partial genomic library to isolate and characterize microsatellite loci for *D. ebraccatus*. We extracted genomic DNA from three individuals using a QIAGEN DNeasy kit. DNA was digested with two restriction enzymes, Alu I and Hae III, and ligated to a double-stranded SNX linker. We probed DNA fragments with di-, tri-, and tetra biotinylated oligonucleotides and captured them with streptavidin-coated magnetic beads, followed by polymerase chain reaction (PCR) amplification using the SNX primer and Vent exo-polymerase for 35 cycles under the following conditions: 95ºC for 50 sec; 60º C for 60 sec; 72º C for 90 sec. The product was electrophoresed on a 1% Agarose gel and purified using Qiaquick PCR purification kit. We then digested PCR product with Nhe 1 and ligated to pUC 19 cloning vector for transformation in Epicurian Coli XL1-Blue MRF’ supercompetent cells. 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 (Applied Biosystems, Foster City, California). We tested 54 microsatellite primer pairs, of these 6 were polymorphic and amplified across all populations in the study (Table 5.1).
TABLE 5.1. Polymorphic di- and tetranucleotide microsatellite loci characterized for *Dendropsophus ebraccatus*.

<table>
<thead>
<tr>
<th>MP</th>
<th>Locus</th>
<th>Primer sequences 5' – 3'</th>
<th>F 5' Label</th>
<th>Repeat Structures</th>
<th>T&lt;sub&gt;a&lt;/sub&gt;</th>
<th>A</th>
<th>(bp)</th>
<th>H&lt;sub&gt;O&lt;/sub&gt;</th>
<th>H&lt;sub&gt;T&lt;/sub&gt;</th>
<th>F&lt;sub&gt;IT&lt;/sub&gt;</th>
<th>F&lt;sub&gt;IS&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HEB 165</td>
<td>U5' GTG GGT AGC CAT GTA TTC AGA GAT 3' L5' ACA CCC CAA CAC CGC TCA CA 3'</td>
<td>NED</td>
<td>(GT)&lt;sub&gt;10&lt;/sub&gt;AT(GT)&lt;sub&gt;3&lt;/sub&gt;G(GT)&lt;sub&gt;10&lt;/sub&gt;</td>
<td>55.6</td>
<td>48</td>
<td>278</td>
<td>0.463</td>
<td>0.815</td>
<td>0.410</td>
<td>0.293</td>
</tr>
<tr>
<td>1</td>
<td>HEB 231</td>
<td>U5' GCA CTG CCC GGG AAT AAA G 3' L5' AAT GAG GAG AGG GTT GGG GAA AAA 3'</td>
<td>6 FAM</td>
<td>(TG)&lt;sub&gt;10&lt;/sub&gt;</td>
<td>58.6</td>
<td>51</td>
<td>152</td>
<td>0.727</td>
<td>0.944</td>
<td>0.343</td>
<td>0.285</td>
</tr>
<tr>
<td>1</td>
<td>HEB 310</td>
<td>U5' TCC CCT GCA TAA AGT AAG AGT GAG 3' L5' ACC CCT CTG TCC CCT TCA GAC 3'</td>
<td>PET</td>
<td>(TG)&lt;sub&gt;15&lt;/sub&gt; (N)&lt;sub&gt;9&lt;/sub&gt;(TG)&lt;sub&gt;6&lt;/sub&gt;</td>
<td>55.0</td>
<td>30</td>
<td>98</td>
<td>0.539</td>
<td>0.868</td>
<td>0.347</td>
<td>0.292</td>
</tr>
<tr>
<td>2</td>
<td>HEB 161</td>
<td>U5'TCA CAT GAC GTC CRG AGC CAA TC 3' L5'CAG CCA CCC ATG AGC ACT AA 3'</td>
<td>PET</td>
<td>(TG)&lt;sub&gt;15&lt;/sub&gt;(N)&lt;sub&gt;10&lt;/sub&gt;(TG)&lt;sub&gt;6&lt;/sub&gt;</td>
<td>67.6</td>
<td>24</td>
<td>50</td>
<td>0.550</td>
<td>0.900</td>
<td>0.414</td>
<td>0.313</td>
</tr>
<tr>
<td>2</td>
<td>HEB 337</td>
<td>U5' GCA CTG CTA CGC ATA TAC ATG TG'3 L5' GAG TGC TGG GTT CTT TCT ATG C 3'</td>
<td>NED</td>
<td>(CA)&lt;sub&gt;14&lt;/sub&gt;</td>
<td>55.0</td>
<td>27</td>
<td>70</td>
<td>0.734</td>
<td>0.921</td>
<td>0.172</td>
<td>0.059</td>
</tr>
<tr>
<td>2</td>
<td>HEB 226</td>
<td>U5' TGG GAT GTG GGT CAC GGT TTG A3' L5' ATT ATG ACA CTT ATT GTG GAA AAT 3'</td>
<td>6 FAM</td>
<td>(TG)&lt;sub&gt;13&lt;/sub&gt;</td>
<td>55.0</td>
<td>30</td>
<td>88</td>
<td>0.604</td>
<td>0.937</td>
<td>0.449</td>
<td>0.407</td>
</tr>
</tbody>
</table>

Mean values are also provided. Annealing temperature (T<sub>a</sub>) °C used PCR reactions. The number of alleles (A), product range size (bp), proportion of observed (H<sub>O</sub>) and expected (H<sub>T</sub>) heterozygosities, are listed for each locus averaged across all populations sampled. Estimates of Weir and Cockerham F<sub>IT</sub> and F<sub>IS</sub> were calculated in FSTAT.
We digested tissues (approximately 1 mm³) from all populations in 150 μL of a 5% Chelex solution (Chelex-100, BioRad) incubated with 19 μg Proteinase K for 120 min at 55 °C followed by denaturation at 90 °C for 10 min. We amplified six microsatellite loci using a Hybaid Gradient Thermocycler. PCR conditions consisted of an initial 90 °C denaturation for 2 minutes, followed by 35 amplification cycles of denaturation at 94 °C for 50 sec, annealing (ranging from 55 - 67.6 °C; Table 5.2) for 60 sec, extension at 72 °C for 60 sec, and a final 5 minute extension at 72 °C. We performed PCR in 10μl reaction volumes containing: 1X Roche reaction buffer without MgCl₂, 1 mM MgCl₂, 0.2μM of each PCR primer, 0.2 μM dNTPs, 2.5U Roche Taq and ~ 50 ng of template DNA. In each case, the forward primer was 5’ labeled with a fluorescent dye (NED, 6-FAM, PET, VIC; Table 5.1) and amplicons were multiplexed in two groups and electrophoresed on an Applied BioSystems 3730xl DNA Analyzer. We assigned fragment sizes by comparison with a LIZ 500bp ladder and binned alleles into discrete size categories according to microsatellite repeat motif using Genemapper ver.3.5 software (Applied Biosystems).

**Estimating Gene Flow**

All microsatellite analyses of *A. callidryas* were conducted previously and matched those of *D. ebraccatus*. For *D. ebraccatus*, we calculated allelic diversity and observed and expected heterozygosities for each locus in FSTAT (ver. 2.1), and tested 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 was determined at α = 0.05 after Bonferonni correction. We used standard population genetic analyses to characterize population structure. Pairwise *F*<sub>ST</sub> estimates between all populations were estimated in FSTAT (following Weir and
TABLE 5.2. Summary of within-population diversity of ND1 and 16S sequences for *D. ebraccatus*.

<table>
<thead>
<tr>
<th>Region</th>
<th>Population</th>
<th>N</th>
<th>H</th>
<th>Po</th>
<th>II</th>
<th>Pi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Southwest CR</td>
<td>1</td>
<td>4</td>
<td>0.75</td>
<td>20</td>
<td>0.0053 (0.0037)</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5</td>
<td>0.84</td>
<td>13</td>
<td>0.0038 (0.0025)</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>5</td>
<td>0.80</td>
<td>10</td>
<td>0.0042 (0.0024)</td>
<td>6.2</td>
</tr>
<tr>
<td>Northwest CR</td>
<td>5</td>
<td>5</td>
<td>0.84</td>
<td>12</td>
<td>0.0067 (0.0043)</td>
<td>3.00</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>5</td>
<td>0.86</td>
<td>16</td>
<td>0.0078 (0.0048)</td>
<td>5.00</td>
</tr>
<tr>
<td>Northeast CR</td>
<td>10</td>
<td>6</td>
<td>0.75</td>
<td>5</td>
<td>0.0008 (0.0006)</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>6</td>
<td>0.50</td>
<td>7</td>
<td>0.0009 (0.0005)</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>5</td>
<td>0.50</td>
<td>6</td>
<td>0.0009 (0.0007)</td>
<td>1.8</td>
</tr>
<tr>
<td>Southeast CR/PA</td>
<td>14a</td>
<td>4</td>
<td>0.75</td>
<td>5</td>
<td>0.0013 (0.0001)</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>14b</td>
<td>5</td>
<td>0.84</td>
<td>3</td>
<td>0.0007 (0.0063)</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>5</td>
<td>0.84</td>
<td>15</td>
<td>0.0033 (0.0022)</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>5</td>
<td>0.84</td>
<td>2</td>
<td>0.0005 (0.0004)</td>
<td>1</td>
</tr>
<tr>
<td>Central PA</td>
<td>20</td>
<td>6</td>
<td>0.56</td>
<td>2</td>
<td>0.0004 (0.0004)</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>5</td>
<td>0.48</td>
<td>1</td>
<td>0.0003 (0.0002)</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>5</td>
<td>0.84</td>
<td>9</td>
<td>0.0020 (0.0014)</td>
<td>3.8</td>
</tr>
</tbody>
</table>

The number of individuals sampled per population (N), Heterozygosity (H), number of polymorphic sites (Po), nucleotide diversity (π with standard error), mean number of pairwise differences (Pi).

Cockerham 1984). We conducted an analysis of molecular variance (AMOVA) to determine the genetic variation among and within groups, in Arlequin ver. 3.1 (Schneider et al. 2000).

We used Bayesian Inference assignment tests in Structure ver. 2.0 (Pritchard et al. 2000) to estimate the number of genetic demes represented by sampled individuals and to evaluate the degree of admixture among them. Structure utilizes a Markov chain Monte Carlo (MCMC) 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, 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 (K=1 to K = 15) were plotted to visually determine the plateau in likelihood scores. We also calculated ΔK to identify the greatest rate of change between each subsequent K (Evanno et al. 2000). Based on these two methods, we chose the most likely values of K for each dataset and plotted the assignment score for all individuals for a range of most probable K demes. Assignment profiles for 25 runs were coalesced in CLUMPP (Jakobsson and Rosenberg 2007) for the final deme assignment graph.

**ISOLATION BY DISTANCE: COMPARISON BETWEEN SPECIES**

We tested for a pattern of genetic isolation by distance (IBD) by comparing the relationship among pairwise values of the natural logarithm of geographic distance and genetic distance, as $F_{ST}/(1 - F_{ST})$ (Rousset, 1997). Estimates of genetic divergence based on allele frequencies are constrained by the spatial distribution of populations and by the heterozygosity of the genetic markers (Hedrick, 2005; Meirmans, 2006). Therefore, to compare patterns of IBD between species, we computed a standardized measure of genetic differentiation ($F'_{ST}$; Meirmans 2006). We transformed our datasets in GenoDive ver 2.0b9 and assigned each population a unique set of alleles (Meirmans, 2006). We used FSTAT to calculate the maximum population pairwise genetic differentiation ($F_{ST_{max}}$). Standardized measures of genetic differentiation ($F'_{ST}$) were computed by dividing the true pairwise values of $F_{ST}$ by $F_{ST_{max}}$. We used linear regression to examine the relationship between pairwise natural logarithm of geographic distance and the standardized ($F_{ST} - 1 - F_{ST}$) and performed 10,000 permutations of the Mantel test (Mantel, 1967) implemented in R ver. 1.11-4 (Oksanen et al. 2007) to determine whether the slope of each regression was significantly greater than zero. We used Fisher’s $r$-to-$Z$ transformation to test the difference between correlation coefficients for IBD along Caribbean and Pacific populations for both species, adjusting for unequal sample sizes (Preacher 2002).
MATRIX CORRESPONDENCE TESTS: ASSOCIATIONS OF GENE FLOW, PHENOTYPE, AND GEOGRAPHY

We conducted Matrix Correspondence Tests (MCT) to test whether population frequencies of color pattern varied with respect to gene flow and/or geographic factors. Matrix Correspondence Tests use repeated randomization and permutation to test for the correlation between two distance matrices by comparing the individual pairwise distance for each parameter. Randomized values provide a null distribution with which to test the hypothesis of no association. Significance values were determined by comparing the observed and expected Z-statistic, generated by 10,000 permutations in the program R ver. 2.7.0 (Team 2005).

We constructed pairwise dissimilarity matrices and conducted MCT and pMCT in R. For color pattern, we constructed a matrix of the pairwise Euclidian distances based on the frequency distribution of each color pattern within populations of *D. ebraccatus* and *A. callidryas*. Pairwise gene flow estimates among populations were represented by a matrix of linearized F<sub>ST</sub> values. We represented geographic distance as the linear distance between populations based on UTM coordinates taken from a handheld GPS unit, but calculated as the distance around the Cordillera de Talamanca because dispersal is restricted across the cordillera, likely due to physiological constraints of high elevation mountain passes. We compared the isolating effect of five barriers on gene flow and color pattern: the Golfo Dulce (Zone A), Rio Tárcoles (Zone B), Cordillera de Talamanca/ Cordillera de Tilarán (Zone C), Caribbean Valley Complex (Zone D), and Bocas del Toro (Zone E; Figures 2-3). A matrix for each biogeographic barrier was constructed using a binary system: 0 representing populations on the same side of a barrier and 1 for populations located on opposite sides of the barrier.
RESULTS
Phenotypic Diversity

*Dendropsophus ebraccatus*

We sampled, on average 26 individuals (range 8 – 50) from 12 populations for a total of 312 individuals (Appendix 5.1). The frequency distribution of dorsal patterns differed among all populations (p = 0.001) and differed among populations within all regions except for Northwestern CR (Northwestern CR: p = 0.2882; Northeastern CR: p < 0.001; Southeastern CR: p = 0.013; Panama: p = 0.003; and Southwestern CR: p = 0.002; Figure 5.1). Overall, the two hourglass patterns (patterns 1 and 2; Figure 5.1) accounted for 68% of the total variation of all individuals while the plain type was least common and observed in only 11.5% of individuals. Not all populations contained all pattern types: we did not observe any individuals with the plain type in three populations (populations 4, 7, 13), two of these populations (populations 7 and 13; Figure 5.2) also lacked the spotted type. Only 2 populations exhibited a balanced frequency of all types (populations 14 and 16).

*Agalychnis callidryas*

We sampled, on average 23 individuals (range = 13- 48) from 17 populations for a total of 392 individuals (Appendix 5.1). The frequency distribution of flank stripe pattern differed over all populations (p < 0.001) and differed among populations within all regions except for Northwestern CR (Northwestern CR: p = 0.554; Northeastern CR: p = 0.0005; Southeastern CR: p = 0.002; Panama: p = 0.040; and Southwestern CR: p = 0.059). Flank patterns were more variable in the Caribbean; Caribbean populations contained individuals with all pattern types, although flank pattern A was rare and observed in only a single population (Figure 5.3). In contrast, Pacific populations were nearly monomorphic for flank pattern C (92%), with only a
few individuals of pattern type BC, one of the dominant pattern types among Caribbean populations (31.7%).

**Genetic Diversity Patterns**

*Molecular Phylogeny of Dendropsophus ebraccatus*

We amplified 79 *D. ebraccatus* and 2 outgroup taxa, *D. microcephalus* and *Hyla pseudopuma*, at the ND1 and partial 16S mtDNA gene fragment, resulting in sequences 1898 nucleotides in length (Figure 5.4). This fragment contained 685 variable sites, of those 217 were parsimony informative; no insertions/deletions were detected. Haplotypic (*h*) and nucleotide (*π*) diversity varied among populations with high *h* for most populations and relatively low *π* (Table 5.2). We found high genetic divergence among individuals. Significant historical genetic structure was evident both within and among five regions based on mtDNA: AMOVA estimated that 19.52% of genetic variance was partitioned among populations and 18.40% among regions. Historical gene flow estimates (pairwise F*ST*) revealed high differentiation among regions (Appendix 5.2).

The Bayesian topology for 16 populations suggested a pattern of high regional differentiation; all biogeographic regions formed highly supported, reciprocally monophyletic clades (Figure 5.4). Populations from Panama diverged earliest from all other regions. Following that, the consensus phylogeny revealed two major clades 1) Southwestern CR clade and 2) Southeastern-Northeastern-Northwestern clade. The Southwestern CR clade diverged second from a widespread clade, indicating that this species had an ancestral widespread distribution and that Southwestern CR populations became isolated from the remaining Isthmusian populations. The phylogenetic reconstruction infers colonization of Southeastern CR from southern
FIGURE 5.4. Left panel. Bayesian consensus phylogram for *Dendropsophus ebraccatus* based on 1907 basepairs of the 16S Ribosomal subunit + NADH1 mitochondrial DNA gene fragment. Values above branches are posterior probabilities. Phylogram is rooted with the outgroup taxon, *Agalychnis saltator*. Right panel. Bayesian consensus phylogram for *Agalychnis callidryas* based on 1149 basepairs of the NADH1 mitochondrial DNA gene fragment. Values above branches are posterior probabilities. Phylogram is rooted with the outgroup taxon, *Agalychnis saltator*. Bars are coded by geographic regions in Figure 5.1.
populations, with dispersal into Northeastern and Northwestern CR clades (Figure 5.4).

For *A. callidryas*, the Bayesian analyses and resulting topology for 20 populations is reported elsewhere. The consensus topology revealed five regional clades with admixture among most neighboring lineages. We detected an early divergence of the Southwestern CR populations relative to the other four regions (Figure 4). Populations from the remaining regions fell within three clades of admixed geographic regions united at their base by a polytomy: Northwestern and Southwestern CR; Northwestern CR- Northeastern CR-Southeastern CR-Central Panama; and Central Panama (Figure 5.4).

**Gene flow within and between regions**

For *D. ebraccatus*, the number of alleles per locus ranged from 24 - 51 and averaged 30.3 across all loci (Table 5.2). Mean heterozygosity was 0.898 with specific loci ranging from 0.815 – 0.944 (Table 5.2). Overall, we detected no consistent deviation in HWE or LD among populations.

Significant population genetic structure was evident both within and among five regions at microsatellite loci. The AMOVA estimate that 13% of genetic variance was detected among populations within regions and 11% among regions. We detected significant restriction in gene flow in 92 of 105 population pairwise $F_{ST}$ comparisons (Appendix 5.2). Overall, pairwise $F_{ST}$ values were lowest within regions (Appendix 5.2). We detected genetic isolation with geographic distance at all three spatial scales in the study: across all populations ($r = 0.1971$, $P = 0.035$); Caribbean populations ($r = 0.331$, $P = 0.026$); and Pacific populations ($r = .784$, $P = 0.037$).

Bayesian analyses revealed six demes (four Caribbean demes and 2 Pacific demes) with no detectable admixture between Pacific and Caribbean populations.
Solid lines labeled A – E indicate the position of geographic barriers tested in this study.

**FIGURE 5.5. Structure plot of all *Dendropsophus ebraccatus* populations revealed six demes, including 2 Pacific demes and 4 Caribbean demes with admixture among regions.**

(Figure 5.5). Among Caribbean populations, the Northeast populations comprised two demes, one of which contained a single isolated population (population 9; Figure 5.5). The other two Caribbean demes corresponded to Southeastern CR and Panamanian populations, with limited admixture detected at the geographic borders of these regions. Structure analyses of Pacific populations only revealed $K = 2$ demes (Figure 5.5) with admixture between the demes: one deme was comprised of the Northwestern CR populations + 1 Southwestern CR population (population 4) and the other deme contained the two southern-most populations from Southwestern CR (Figure 5.5).

For *A. callidryas*, at the broadest spatial scale, Bayesian clustering revealed strong differentiation between Pacific and Caribbean populations. Caribbean populations belonged to five demes. Among Caribbean populations, we detected a north-south clinal distribution in the membership frequency of demes. Among three Pacific demes, we detected significant admixture at the centrally located population (4), with membership of individuals to both Northwestern and Southwestern demes.
**Isolation by Distance**

We calculated $F_{ST}'$ to compare the relationship between genetic and geographic distance (IBD) for *D. ebraccatus* and *A. callidryas*. Both species exhibited IBD along Caribbean populations (*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* $r = 0.872$, $p = 0.0011$; Figure 5.6). Test of equal variance of the predictor variable (migration estimates) indicated that the variance in migration estimates were unequal between Caribbean and Pacific populations of *A. callidryas* (stat), populations of *A. callidryas* and *D. ebraccatus* along the Caribbean (stat) and Pacific (stat), but were equal for Caribbean and Pacific populations of *D. ebraccatus* (stat). Therefore, we could only implement the Fishers $r$-to-$Z$ transformation test of the difference between two correlation coefficients for a comparison between Caribbean and Pacific populations of *D. ebraccatus*. The correlation coefficients for IBD did not differ between Pacific and Caribbean populations of *D. ebraccatus* ($z$-score = -1.06, $p = 0.10$). All other comparisons were not conducted because of the violation of the assumption of equal variance in the predictor variable (migration estimates).

**Matrix Correspondence Tests: gene flow, phenotype, geographic factors**

*Geographic barriers to gene flow*

We used Matrix Correspondence Tests to test for the association between gene flow and geographic factors (geographic distance and barriers) within and across five regions for *D. ebraccatus*. We examined these factors using both historical genetic distance (mtDNA) and contemporary gene flow estimates (ncDNA). Within regions,
Correlation coefficient from Mantel tests based on 10,000 permutations.

**FIGURE 5.6.** Genetic isolation by distance (IBD) expressed as the relationship between \( \frac{F_{ST}}{1 - F_{ST}} \) and natural log transformed kilometers (km), based on Standardized \( F_{ST} \) for *Agalychnis callidryas* (black squares) and *Dendropsophus ebraccatus* (grey squares) populations across Caribbean (top) and Pacific (bottom) populations.
we detected a positive relationship between mtDNA and geographic distance for three regions but no association between ncDNA and geographic distance for any region (Table 5.3). We detected the role of three barriers in restricting gene flow across three zones (B, D, E) using mtDNA estimates of genetic divergence (Table 5.3). Gene flow estimates derived from ncDNA were associated with a barrier across zones C and E, but these relationships were nullified in pMCT when geographic distance was taken into account (Table 5.3).

For *A. callidryas*, genetic divergence among regions inferred from mtDNA varied with geographic distance and was structured by to the isolating effects of four biogeographic barriers (A, B, C, E; Table 5.4). Using contemporary estimates of gene flow (ncDNA), we found that only one of these barriers (Zone C; Table 5.4) was effective at restricting gene flow.

**Determinants of color pattern**

For *D. ebraccatus*, the frequency distribution of color pattern at the population level was random with respect to nuclear gene flow ($r = 0.026$, $p = 0.454$) and geographic distance ($r = 0.0009$, $p = 0.47$). For *A. callidryas*, the frequency distribution of flank stripe pattern was associated with nuclear gene flow ($r = 0.668$, $p < 0.001$) but not geographic distance in a partial MCT that accounted for the variation due to gene flow ($r = -0.7411$, $p = 0.743$). Comparison of color pattern between the two taxa revealed that the dorsal pattern frequency of *D. ebraccatus* was not
TABLE 5.3. Matrix and Partial Matrix Correspondence Tests of the determinants of genetic diversity (mtDNA and nc DNA) across five genetic demes.

<table>
<thead>
<tr>
<th>Independent Variable</th>
<th>Dependent Variable</th>
<th>WITHIN REGIONS</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SW</td>
<td>NW</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Distance (KM)</td>
<td>0.2816 (0.014)</td>
<td>-0.0556 (0.672)</td>
</tr>
<tr>
<td>ncDNA</td>
<td>Distance (KM)</td>
<td>0.3538 (0.676)</td>
<td>na</td>
</tr>
</tbody>
</table>

|                      |                    | ZONE A        | ZONE B              | ZONE C              | ZONE D              | ZONE E              |
|                      |                    | ZONE B        | ZONE C              | ZONE D              | ZONE E              |                      |
|                      | mtDNA              | Barrier       |                      |                      |                      |                      |
|                      | Distance (KM)      | 0.8221 (0.001)| 0.8221 (0.001)      | 0.8245 (0.001)      | 0.798 (0.001)       | 0.9552 (0.001)      |
|                      | Barrier             | 0.0387 (0.188)| 0.9026 (0.001)      | 0.0110 (0.366)      | 0.5451 (0.001)      | 0.9982 (0.001)      |
|                      | ncDNA              | Barrier       |                      |                      |                      |                      |
|                      | Distance (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)      | 0.1992 (0.032)      | 0.1729 (0.121)      | 0.8351 (0.0293)     |

Significance values were determined by comparing the observed and expected z-statistic, generated by 10000 permutations. Partial MCT indicated in italics. Bonferroni adjusted $P$-value for multiple tests is $\alpha = 0.006$ (a tablewide value of 0.05). Some tests not conducted due to an insufficient number of populations (<3).
### TABLE 5.4. Comparison of historical biogeography, population genetic structure and color pattern in *D. ebraccatus* and *A. callidryas*.

<table>
<thead>
<tr>
<th>COLOR PATTERN</th>
<th>POPULATION STRUCTURE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spatial Divergence</td>
</tr>
<tr>
<td><em>A. callidryas</em></td>
<td>Among populations</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><em>D. ebraccatus</em></td>
<td>Among populations</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>BIOGEOGRAPHY</th>
<th>OVERALL CONCLUSIONS</th>
</tr>
</thead>
</table>
| *A. callidryas* | mtDNA clades 5 admixed clades  
-Southwest diverged first  
-Colonization of Pacific, then Caribbean  
-Relatively greater dispersal  
-Gene flow drives color pattern distribution  
-Selection contributes to diversification in color pattern |
| *D. ebraccatus* | 5 monophyletic clades  
-Panama diverged first  
-Southwest diverged, but remained isolated  
-Colonization of Caribbean, then Pacific  
-Relatively weaker dispersal  
-Balancing selection maintains polymorphisms within populations |

<table>
<thead>
<tr>
<th>Zone A</th>
<th>Barrier</th>
<th><em>A. callidryas</em> mtDNA</th>
<th><em>D. ebraccatus</em> mtDNA</th>
<th>Studies showing barrier isolates populations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Golfo Dulce</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>(Crawford et al. 2007)</td>
</tr>
<tr>
<td>Zone B</td>
<td>Rio Tarcoles</td>
<td>✓</td>
<td>✓</td>
<td>(Kohlmann et al. 2002)</td>
</tr>
<tr>
<td>Zone C</td>
<td>Cordillera de Talamanca/Cordillera de Tilarán</td>
<td>✓</td>
<td>✓</td>
<td>(Demastes et al. 1996; Zamudio and Greene 1997; Garcia-Paris et al. 2000; Crawford 2003)</td>
</tr>
<tr>
<td>Zone D</td>
<td>Caribbean Valley Complex</td>
<td>✓</td>
<td>✓</td>
<td>(Kohlmann et al. 2002)</td>
</tr>
<tr>
<td>Zone E</td>
<td>Bocas del Toro</td>
<td>✓</td>
<td>✓</td>
<td>(Zeh et al. 2003; Weigt et al. 2005)</td>
</tr>
</tbody>
</table>
associated with the frequency distribution of flank stripe pattern in *A. callidryas* across 11 populations \((r = -0.0053, p =0.471)\), indicating biogeographic history does not underlie expression of color pattern in these two taxa, in the same way.

**DISCUSSION**

We examined patterns of genetic and color pattern differentiation in two co-distributed species and found limited evidence to support the hypothesis that common historical processes have resulted in concordant patterns of diversification. Our findings of strong discordance underscore the importance of interpreting comparative phylogeographic studies in light of specific ecological and life history traits. The two study taxa exhibited differences that highlight the complex and dynamic processes underlying population structure, including the effects of landscape features, gene flow patterns, localized selection and drift. We discuss these differences in four sections. First, we highlight the unequal effects of biogeographic barriers in isolating populations between species and across temporal scales. Next, we address species differences in the colonization history of Isthmus populations. Third, we evaluate differences in the patterns of genetic isolation by distance across regions and between species. Finally, we evaluate the implications for the selective mode on color pattern polymorphisms on the evolution of populations.

*Geographic isolation due to biogeographic barriers varies at temporal scales*

Through the combined use of mitochondrial and nuclear markers, we discerned processes occurring at deeper historical and relatively recent contemporary time scales. Because of differences in recombination, inheritance, and mutation between these two markers, we directly compare gene flow estimates or use mtDNA inferences to comment on female dispersal. Instead, we compare estimates derived from these
markers to gain insight into historical and contemporary processes of population diversification. We found that three biogeographic barriers were effective in historical isolation of populations that are now connected through contemporary gene flow. Across two other barriers, the isolating effects were unequal between the two study taxa (Table 5.4). The effects of a geographic barrier are likely to be more pronounced for a continuously distributed, vagile species, such as *A. callidryas*. Whereas, the isolating effect of that same barrier for a less vagile and philopatric species might be undetectable because the genetic variance between populations is accounted for at smaller geographic scales (Irwin 2002). Indeed we detected significant restrictions in gene flow due to three geographic barriers for *A. callidryas* at ncDNA markers and none for *D. ebraccatus* (Table 5.4). The greater overall restrictions in nuclear gene flow at small spatial scales for *D. ebraccatus* supports the conclusion that the spatial scale at which we tested barriers exceeded the dispersal capacity of *D. ebraccatus*.

Among the five putative barriers tested in this study, two barriers limited historical gene flow among both *A. callidryas* and *D. ebraccatus* populations (Zones B and E). For both taxa, our phylogenetic reconstructions revealed historical isolation of the Southwestern CR clade. The unique biogeographic history of Southwestern CR (Zone B) has resulted in exceptional faunal endemism. This region was isolated from Northwestern CR and Caribbean lowland forest following the uplift of the Cordillera de Talamanca, approximately 2.5 MYA (Kohlmann et al. 2002; Savage 2002). The second barrier, located within the Caribbean region of Bocas del Toro (Zone E) is located in Southeastern CR/Western Panama (Figures 5.2-5.3). This region has a complex vicariant and dispersal history, marked by multiple dispersal events of South American fauna over the last 5 MY (Coates and Obando 1996; Kohlmann et al. 2002). Lowland Caribbean forest has been isolated by fluctuations in sea levels and continual volcanic uplift since the Miocene. Multiple pulses of dispersal from South America
during periods of low sea levels with subsequent isolation could account for the early divergence of Central Panama populations of *D. ebraccatus* and the relative isolation of *A. callidryas* populations in that same region. However, nuclear gene flow across these barriers (B and E) was unrestricted for both species. Thus, contemporary gene flow is acting to eliminate the footprint of historical isolation and, thus any historical local adaptations are likely to attenuate over time with the homogenizing effects of migration.

Differences between the focal taxa in response to landscape features were evident across two barriers, Zones C and D. For *A. callidryas*, both historical and contemporary estimates of gene flow were shaped by the isolation due to the central cordillera range (Zone C). Zone C marks the intersection of Caribbean and Pacific populations at the northwestern edge of the Cordillera de Talamanca, a barrier known to structure the distribution of many terrestrial organisms in Costa Rica (see Table 5.4). This barrier is also associated with high phenotypic divergence in *A. callidryas* in both leg and flank coloration and flank-stripe pattern, providing substantial evidence that color pattern diversification has been largely driven by genetic isolation. We detected no evidence of restricted gene flow across the Cordillera for populations of *D. ebraccatus*, nor did we detect phenotypic differentiation coincident with this landscape feature. However, highly restricted gene flow among populations within regions bordered by the Cordillera de Talamanca provides support for our previous conclusion the spatial scale spanning this geographic barrier exceeds the dispersal capacity of the species; that is, the barrier itself is unlikely to effect migration patterns. For *D. ebraccatus*, finer spatial sampling between populations (e.g., 5 km) is required to resolve this issue.

Historical gene flow was reduced across the Caribbean Valley Complex (Zone D) for *D. ebraccatus* only. This zone is characterized by three valleys at the foothills
of the Cordillera de Talamanca (Valle de Talamanca, Valle de Estrella, Llanura de Santa Clara) situated between Northeastern and Southeastern CR and coincident with the geographic range limits of several taxa (Kohlmann et al. 2002). Fossil records dating to the Miocene (6 MYA) indicated a land connection between North and South America along this Caribbean coastline that has been interrupted repeatedly by marine introgressions (Kohlmann et al. 2002). Despite historical limitations to migration, our estimates of contemporary gene flow patterns revealed no obvious restrictions in gene flow due to this barrier. In contrast, we did not detect an effect of this barrier on gene flow at either historical or contemporary time scale for *A. callidryas*. Yet, this barrier demarcates significant phenotypic divergence between Northeastern and Southeastern populations of red-eyed treefrogs, providing an example of phenotypic differentiation in the presence of gene flow, likely due to the contributing effects of drift, selection or both.

*Colonization history of Central American Isthmus*

Historical colonization of Isthmus populations, inferred from mtDNA phylogenetic reconstruction, revealed differences between the species in both the dispersal routes and degree of regional admixture. For *A. callidryas*, we detected five regional mtDNA clades with admixture among neighboring lineages (and no regional monophyly). The phylogenetic relationships among populations indicated that the Southwestern CR clade diverged first and was sister to all other Costa Rican and Panamanian clades. Dispersal likely occurred northward through Northwestern CR, followed by transcontinental migration into Caribbean populations. This interpretation is supported by a broad-scale phylogeographic study across the range of *A. callidryas* (Crawford et al., in preparation).
In contrast, for *D. ebraccatus*, we detected five, deeply divergent, reciprocally monophyletic regional clades with no admixture among lineages (Figure 5.4). The biogeographic history, inferred from mtDNA haplotypes, indicated an initial divergence of Panamanian populations, followed by the isolation of Southwestern CR. Thus, for *D. ebraccatus*, a simple vicariance model does not explain the sister relationship between Caribbean and Pacific populations, a finding for other populations of Isthmus frogs (Crawford 2003). Our reconstruction revealed dispersal into Caribbean populations from Panama with transcontinental migration from the Caribbean (Northeastern CR) into Pacific (Northwestern CR) populations. Our two taxa differ significantly in their response to major landscape features, as well as dispersal and colonization history of Central American Isthmus populations.

*The effects of landscape complexity on gene flow and isolation by distance patterns*

*Agalychnis callidryas* exhibited clinal variation in genetic membership coefficients for individuals as well as strong associations between genetic and geographic distance across both Pacific and Caribbean demes (Figure 5.6), indicating a stepping-stone model of migration. In contrast, *D. ebraccatus* populations exhibited very restricted gene flow, reduced admixture among the four Caribbean demes (Figure 5.5) and no association between genetic and geographic distance among populations within each of the five regions. Overall, greater restrictions in gene flow among *D. ebraccatus* populations could result from inherent limitations in dispersal, the presence of habitat barriers not included in this study, as well as differences in demographic and life history traits (e.g., small body size, lower tolerance to desiccation, and higher philopatry). This extreme population differentiation was also observed for small, specialized leaf liter frogs in the genus *Eleutherodactylus* (Crawford 2003).
Agalychnis *callidryas* is 2–4 times larger than *D. ebraccatus* and large body size could account for differences in dispersal capacity.

We could not directly compare IBD relationships between our two focal taxa or between Caribbean and Pacific populations of *A. callidryas*, due to violation of the assumptions of equal variance in our migration estimates. However, the unequal variance between Caribbean and Pacific populations revealed that geographic distance is a poor predictor of genetic distance at large spatial scales. Thus, illustrating the effects of a heterogeneous landscape on dispersal patterns. Because of the high variance in migration estimates among Caribbean population relative to the Pacific, we conclude that landscape complexity and geographic barriers (untested in this study) corroborate our findings of higher overall genetic and phenotypic diversity.

**Determinants of color pattern**

Color pattern varied among populations of *A. callidryas* and *D. ebraccatus*, but the mechanisms driving phenotypic diversity within and among populations differed between taxa. *A. callidryas* exhibited clinal change in color pattern that was associated with gene flow and geographic distance, whereas color pattern of *D. ebraccatus* populations were random with respect to both genetic and geographic distance.

The adaptive significance of color pattern in *A. callidryas* is discussed in detail elsewhere; briefly we favor the hypothesis that CP is (in part) a social signal used for species recognition. Thus, this trait may have evolved and differentiated among populations through combined forces of genetic isolation, sexual selection and drift. Pacific populations of *A. callidryas* were nearly monomorphic for flank pattern C, a pattern observed in low frequency in Caribbean populations of Panama and Southeastern CR. The high frequency and widespread distribution of pattern C along
Pacific populations is associated with higher estimates of gene flow and lower levels of genetic diversity. The sharpest distinction in flank pattern occurred at the divide between Caribbean and Pacific populations, corroborating the strong barrier to gene flow observed at this site on both historical and contemporary time scales (Zone C). This is consistent with divergence in flank coloration: Pacific populations contain individuals with orange flanks, while individuals in the Caribbean exhibit blue flanks. Along Caribbean populations, we observed a clinal change in the frequency distribution of flank stripe (this study) and leg coloration coinciding with a stepping-stone model of migration and genetic IBD. While associations between flank-stripe pattern and gene flow indicated that color pattern evolved through gene flow-drift equilibrium, the directional diversification among populations could be reinforced by sexual selection. This is observed for other frogs known to use visual signals for mate discrimination (Buchanan 1994; Siddiqi et al. 2004; Rudh et al. 2007).

For *D. ebraccatus* the frequency distribution of color pattern varied among almost all populations (within and among regions) but was not determined by genetic or geographic factors. In this species, restrictions in gene flow reduced the likelihood that gene flow patterns underlie color pattern at fine spatial scales and instead, underscores the potentially important role of within-population processes in structuring color pattern. The occasional loss of a color morph in three disjunct populations across the study region implicates genetic drift as one of these factors (Figure 5.2). The large sample size for NE 10 (N = 50) justifies our position that losses were due to stochastic population-level processes rather than observational bias due to insufficient sampling. While our research goal was not to examine the adaptive significance of dorsal color pattern, it is useful to evaluate the possible selection pressures driving balanced polymorphisms. Within-population polymorphisms are common in anurans and often result from frequency-dependent selection (Hoffman
and Blouin 2000), non-assortative mating, life history characteristics (Summers et al. 1997) or natural selection favoring crypsis (refs). Blotches and spots, characteristic of *D. ebraccatus*, are a common form of disruptive coloration, making individuals cryptic against a heterogeneous background (Cott 1940; Endler et al. 2005). Our community-based analyses of character displacement supported the hypothesis that color pattern polymorphisms in this frog are not used for species recognition, but indicated a role of the environment in the maintaining CP. Predator pressures could favor population polymorphisms if visual predators more readily identify common morphs as potential prey items, thus allowing rarer forms to persist (Cott 1940). Our best initial hypothesis of the maintenance of CP in *D. ebraccatus* is the interaction between stochastic and frequency-dependent processes, and is an area for future behavioral studies.

**CONCLUSIONS**

Although many co-distributed taxa experience common historical processes, this study revealed how species-specific life history and demographic characteristics can result in very different evolutionary outcomes. We detected differences in the biogeographic history, population genetic structure and dispersal biology of *A. callidryas* and *D. ebraccatus*. In addition, the diversification and distribution of color pattern and genetic diversity have proceeded through different evolutionary and geographic mechanisms in each taxon. Our study species have two of the broadest geographic ranges of Middle American treefrogs, indicating their relative success in dispersal and colonization, yet our results indicate that these species have achieved this broad distribution in different ways.
ACKNOWLEDGMENTS

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APPENDIX 5.1. Sampling populations of *Agalychnis callidryas* from five regions in Costa Rica and Panama.

<table>
<thead>
<tr>
<th>Region</th>
<th>Province, CR</th>
<th>No.</th>
<th>Population</th>
<th>GIS (Lat, Long, El)</th>
<th>ACA</th>
<th>HEB</th>
<th>HEB micro</th>
<th>HEB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Southwestern</td>
<td>Puntarenas, CR</td>
<td>1</td>
<td>Pavones</td>
<td>8.4204, -83.1069, 37</td>
<td>20</td>
<td>39</td>
<td>30</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Puntarenas, CR</td>
<td>2</td>
<td>Sierpe</td>
<td>8.8892, -83.477, 17</td>
<td>19</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Puntarenas, CR</td>
<td>4</td>
<td>Uvita</td>
<td>9.123, -83.701, 26</td>
<td>24</td>
<td>25</td>
<td>28</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Puntarenas, CR</td>
<td>3</td>
<td>Campo</td>
<td>8.6909, -83.5013, 35</td>
<td>16</td>
<td>26</td>
<td>25</td>
<td>5</td>
</tr>
<tr>
<td>Northwestern</td>
<td>Puntarenas, CR</td>
<td>5</td>
<td>Pl. Bandera</td>
<td>9.5188, -84.3774, 23</td>
<td>19</td>
<td>21</td>
<td>28</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>San Jose, CR</td>
<td>6</td>
<td>Carara</td>
<td>9.725, -84.531, 385</td>
<td>25</td>
<td>24</td>
<td>31</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Guanacaste, CR</td>
<td>7</td>
<td>Cabo Blanco</td>
<td>9.580, -85.124, 166</td>
<td>18</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Northeastern</td>
<td>Guanacaste, CR</td>
<td>8</td>
<td>Tilarán</td>
<td>10.5162, -84.9601, 637</td>
<td>22</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Alajuela, CR</td>
<td>9</td>
<td>San Ramon</td>
<td>10.2335, -84.5287, 638</td>
<td>14</td>
<td>22</td>
<td>21</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Heredia, CR</td>
<td>10</td>
<td>La Selva Biological Station</td>
<td>10.4327, -84.0080, 37</td>
<td>20</td>
<td>50</td>
<td>98</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Limón, CR</td>
<td>11</td>
<td>University of EARTH</td>
<td>10.2318, -83.5, 44</td>
<td>0</td>
<td>0</td>
<td>29</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Limón, CR</td>
<td>12</td>
<td>Siquirres</td>
<td>10.0134, -83.565, 667</td>
<td>0</td>
<td>0</td>
<td>26</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Limón, CR</td>
<td>13</td>
<td>Guayacan</td>
<td>10.0134, -83.557, 680</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
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<td>Southeastern</td>
<td>Limón, CR</td>
<td>14</td>
<td>Cahuita</td>
<td>9.718, -82.814, 16</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Limón, CR</td>
<td>15</td>
<td>Manzanillo</td>
<td>9.6332, -82.6556, 2</td>
<td>26</td>
<td>15</td>
<td>14</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Bocas del Toro, PA</td>
<td>16</td>
<td>Chiriqui Grande</td>
<td>8.9460, -82.1571, 21</td>
<td>21</td>
<td>8</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Bocas del Toro, PA</td>
<td>17</td>
<td>Almirante</td>
<td>9.1980, -82.3445, 13</td>
<td>10</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>Central</td>
<td>Veraguas, PA</td>
<td>18</td>
<td>Santa Fe</td>
<td>8.529, -81.139, 714</td>
<td>17</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Panama</td>
<td>Cocle, PA</td>
<td>19</td>
<td>El Cope</td>
<td>8.6681, -80.592, 792</td>
<td>22</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Cocle, PA</td>
<td>20</td>
<td>El Valle</td>
<td>8.6299, -80.1159, 866</td>
<td>21</td>
<td>24</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Panamá, PA</td>
<td>21</td>
<td>Gamboa</td>
<td>9.1231, -79.6930, 51</td>
<td>22</td>
<td>31</td>
<td>109</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Panamá, PA</td>
<td>22</td>
<td>Cerra Azul</td>
<td>9.1671, -79.419, 638</td>
<td>-</td>
<td>27</td>
<td>31</td>
<td>5</td>
</tr>
</tbody>
</table>

Geographic coordinates (GIS) are Latitude (Lat), Longitude (Long), elevation (El, m). Sampling sizes for color pattern (color), microsatellite analyses (micros), and mtDNA sequencing (seq) provided. Population number (No.) indicates position on Figure 5.1.
APPENDIX 5.2. Pairwise FST estimates based on mtDNA (below diagonal) and six microsatellite loci (above diagonal) for 15 populations of *D. ebraccatus*.

<table>
<thead>
<tr>
<th></th>
<th>SW CR</th>
<th>NW CR</th>
<th>NE CR</th>
<th>SE CR</th>
<th>PA</th>
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<td>6</td>
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<tr>
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<td>0.92</td>
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<td>0.07</td>
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<td>-0.01</td>
<td>-0.06</td>
</tr>
<tr>
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<td>0.91</td>
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<td>0.94</td>
<td>0.52</td>
<td>0.92</td>
<td>0.98</td>
</tr>
</tbody>
</table>

FST significance corrected for multiple comparisons at 0.05 in bold. Negative FST values are not different from zero. Population number correspond to Figure 5.1.


Warburg, M. R. 1965. Evolutionary significance of ecological niche. Oikos 16:205-


