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Host Defense Skin Peptides Vary with Color Pattern in the Highly Polymorphic Red-Eyed Treefrog

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Patterns of phenotypic variation across a geographic range provide important insights into evolutionary processes underlying diversification and speciation. Most evolutionary studies use putatively neutral markers to examine evolutionary diversification. However, functional phenotypes such as gene-encoded host-defense polypeptides (HDPs) could provide key insights into the processes of population differentiation, yet they are rarely included in population analyses. The red-eyed treefrog, *Agalychnis callidryas* Cope, 1862, exhibits regional variation in multiple traits, including color pattern and body size across a narrow geographic range. This treefrog produces bioactive peptides exuded onto the skin surface, presumably for pathogen and predator defense. However, the geographic patterns of variation in peptides and the factors that mediate intraspecific peptide variation across the range of this species remain untested. Here, we examine the roles of phylogenetic history, geographic barriers, geographic distance, and color-pattern variation as determinants of skin peptide diversity in 54 individuals among 11 populations across Costa Rica and Panama. Each of the five distinct *Agalychnis* color morphs are represented in our sample. We performed peptide mass fingerprinting and compared mass spectral data from skin peptide secretions to quantify divergence in peptide profiles among individuals, both within and among regions. We used two metrics to estimate genetic variation: Genetic distance estimated from microsatellites and patristic distance estimated from mtDNA haplotype diversity. Matrix correspondence tests revealed that skin peptide variation is best predicted by differences in leg color pattern across all regions. In addition, we found that flank color pattern and phylogeny also explain differences in peptide diversity. Patterns of peptide differentiation and phylogenetic topology were incongruent in two regions, indicating a possible role of localized selection on peptide variation. Skin peptide profiles are useful in population differentiation studies of

a polymorphic species as well as studies of selection and phenotype co-variation among closely related species. Our results highlight the use of skin peptides as characters for future studies of population differentiation and contribute to our understanding of biogeography in Central America.

Keywords: MALDI mass spectrometry, color pattern variation, *Agalychnis callidryas*, AMPs

INTRODUCTION

Patterns of phenotypic variation across a geographic range can provide insight into the evolutionary processes of population diversification and speciation. Traditionally, evolutionary biologists have used neutral genetic markers to infer patterns and processes of population differentiation (Pröhl et al., 2006; Conlon, 2011b). However, functional phenotypes such as aposematic warning colors and host defensive secretions provide a perspective for how selection changes over a landscape. The tremendous diversity of bioactive proteins in skin secretions, when examined in the context of fine-scale sampling among populations, can enhance our understanding of determinants of variation at the intraspecific level and increase our knowledge of functional diversity across geographic ranges.

Gene-encoded host-defense polypeptides (HDPs), more commonly referred to as antimicrobial peptides (AMPs; Harder and Schröder, 2005; Conlon, 2011b; Mansour et al., 2014), represent one such class of biochemical molecules rarely included in studies of population differentiation. HDPs are evolutionarily conserved molecules that are endogenous to the defensive tissues of hosts including bacteria, plants, insects, and vertebrates (Hancock and Diamond, 2000). An extraordinary suite of HDPs are synthesized and stored in the granular glands of many amphibian species (Severini et al., 2000), secreted onto the skin surface in response to stressful stimuli (Simmaco et al., 1998; Conlon et al., 2004). Mounting this swift-acting innate immune response provides a broad defensive spectrum against microbial pathogens and potential macro predators (Rollins-Smith et al., 2002; Nicolas et al., 2003; Zasloff, 2006). Amphibian peptides are highly variable, accounting for more than half of the 2636 peptides described in the Antimicrobial Peptide Database to date (Wang et al., 2009). The underlying function of this biochemical hyper variability in amphibians is unclear but HDPs likely have specific roles, given the known specificity of some for particular classes of microbes (Apponyi et al., 2004). However, not all amphibian species synthesize dermal peptides. For instance, several arboreal species of the Hylinae sub-family in North America do not, whereas hylids of the Phyllomedusinae sub-family of Central and South American and the Pelodryadinae sub-family of Australia contain rich stores of HDPs (Conlon, 2011a).

HDPs are excellent markers for studying population-level variation and higher-level taxonomy because of their heritability and remarkable functional diversity (Vanhoye et al., 2003; Tennessen, 2005). On a broad evolutionary scale, HDPs have taxonomic applications (Cei et al., 1967, 1968). Variation in amphibian defense chemicals (e.g., alkaloids and skin peptides) have been included in the phylogenetic reconstruction of dendrobatids (Grant et al., 2006), bufonids (Wittliff, 1964; Pollard et al., 1973), ranids (Conlon, 2008), hylids (Wabnitz et al., 1999), and species in the genus *Pelophylax* (Daum et al., 2012). At finer spatial scales, the Australian tree frog *Litoria rubella,* displays a clinal north-south distribution in peptide profiles that correlates with pigmentation (Steinborner et al., 1996; Apponyi et al., 2004; Pukala et al., 2006). In another example, peptide variation in *Litoria caerulea* reflects phylogenetic relationships among populations across regions (Donnellan et al., 2000). Combined, these studies demonstrate the relevance of a fine-scale characterization of peptide profiles in the context of genetic and phenotypic diversification.

The red-eyed treefrog, *Agalychnis callidryas,* presents an opportunity to examine factors contributing to peptide diversity in a highly polymorphic frog. The red-eyed treefrog is a common Neotropical frog with a broad distribution from southern Mexico to Colombia (Savage, 2002). This species exhibits high phenotypic diversity in color pattern and body size across the southern extent of its range in Costa Rica and Panama, such that five distinct color-pattern morphs have been identified among 24 populations (Robertson and Robertson, 2008; Robertson et al., 2009). Population-level analyses have revealed concordance in color pattern and genetic variation across some regions, but color pattern differentiation in the presence of genetic exchange across other regions, suggests that selection could shape phenotypic differences among populations (Robertson and Zamudio, 2009).

Numerous HDPs have been described for *A. callidryas*, yet the geographic variation of peptides among populations has not been studied and their functional diversity has yet to be fully appreciated. Quantification of peptide variation in *A. callidryas* has thus far been based on few individuals ($n = 3$–6) collected from a single population in Costa Rica (Mignogna et al., 1997), or from the commercial animal trade (Wang et al., 2008, 2015; Ge et al., 2014; Jiang et al., 2014). The regional diversification in other heritable phenotypic characters (body size, color pattern) and variable levels of genetic connectivity among red-eyed treefrog populations (Robertson and Robertson, 2008; Robertson et al., 2009; Robertson and Vega, 2011) sets the stage for examining the determinants of geographic variation in skin peptides in this polymorphic species.

Here, we compare skin peptide diversity among 11 populations of *A. callidryas* across Costa Rica and Panama, representing five regional color morphs. Our objectives were to characterize intraspecific peptide variation and examine whether the expression of gene-encoded HDPs in skin exudates co-varies with genetic and phenotypic divergence among populations of *A. callidryas.* Specifically, we determined the extent to which peptide variation varies with (1) flank and leg color pattern, (2) geographic distance, (3) genetic distance, and (4) biogeographic

barriers. Patterns of peptide diversification among populations with phenotypic and genetic characters can potentially elucidate the history and evolution of this functional trait.

METHODS

Population Sampling

In June–July of 2005, we sampled skin peptides and genetic material from 53 adult males and one female *A. callidryas* from 11 populations in Costa Rica and Panama (Table S1), representing five biographic regions: Northeastern (NE) Costa Rica, southeastern (SE) Costa Rica, northwestern Costa Rica, southwestern (Robertson et al., 2009) Costa Rica, and Central Panama. Unlike the green dorsum of Phyllomedusines, the flank and leg color pattern measured in the present study does not change with light intensity or in response to stress (Duellman, 2001). At the time of capture, we photographed individuals to quantify color differences against a standard black–gray–white card and measured color (Robertson and Robertson, 2008). Two adult male frogs from the closely related congener species, *A. saltator* were sampled from La Selva, in Northeast Costa Rica, for use as an outgroup.

We sampled and processed all frogs from each location during the same night they were collected, with the exception of two populations (Santa Fé and Playa Bandera), where individuals were processed the following morning. Three individuals per population were preserved as voucher specimens and deposited at the Cornell University Museum of Vertebrates (CUMV 14093, 14206–13, 14228, 14230–35) and the University of Costa Rica, San Jose (UCR 19100–10,119,213). All other individuals were released at the site of capture. Skin exudates were collected using a transcutaneous amphibian stimulator (TAS; Grant and Land, 2002), a peptide extraction method that provides a mild electrical stimulus to stimulate skin secretions. Each frog was held by its forelimbs and hindlimbs in a stretched position and gently massaged in a circular manner with the TAS as in Steinborner et al. (1996) along the dorsum until skin secretions became either visible or detectable by a distinct resin-like odor. We collected skin secretions from each individual by rubbing a sterile cotton swab across the dorsal, ventral, and inguinal regions of each frog following electrical stimulation. Cotton swabs were immediately submerged into a glass vial containing 5 ml of 90% HPLC-grade methanol to reduce proteolytic degradation of the peptide repertoire (Samgina et al., 2016). The vials containing the preserved cotton swab were stored in a portable electric cooler during transport until permanent storage at 8°C at Cornell University. All amphibian collection methods were approved by Cornell University IACUC protocol 2003-0049.

Peptide Profile Characterization

We analyzed the 54 skin peptide samples using matrix-assisted laser-desorption ionization time of flight (MALDI-TOF) mass spectrometry (Voyager DE-STR, Applied Biosystems, Foster City, CA). Refrigerated crude peptide samples were concentrated in a Sorvall Speedvac manifold to a final volume of 200–250 μl. Aliquots of concentrated samples were applied to a MALDI target plate via the dried droplet method in a sample-to-matrix ratio of 1:1 for each matrix. We used two matrices consisting of either α-cyano-4-hydroxycinnamic acid (α-cyano) at a final concentration of 6.6 μg/ml in 70% acetonitrile, 0.1% TFA or 2,5 dihydroxybenzoic acid (DHB) at a final concentration of 10 mg/ml in 1% phosphoric acid, 50% acetonitrile. Mass spectra were manually acquired in reflectron mode with a mass range of 400–3000 Da, a range selected based on known peptide masses for *A. callidryas* in the literature (Mignogna et al., 1997; Apponyi et al., 2004) at the time of analysis. Each spectrum represents an average of 300 laser shots. Masses from spectra were annotated with a resolution of 6000 and a signal-to-noise ratio (s/n) = 2. In addition, we used mass over charge (m/z) and signal intensity as parameters for cluster analyses, with a ±1 Da error for each m/z. Singletons lacking evidence of an isotope envelope on the spectra were manually removed and not included in the analysis.

Because the efficiency of peptide ionization depends on the matrix used, we included peaks from both the DHB and α-cyano analyses for full coverage of the peptide profile. For each frog, the top 50 mass peaks sorted by intensity level from both the DHB and α-cyano MALDI-TOF mass spectra were selected and merged into a single data set (Laugesen and Roepstorff, 2003; Kouvonen et al., 2010). Duplicate peaks, defined as two that are <0.5 m/z apart, were removed so as to be represented only once in the final peptide profile. Merged profiles for each frog were then used to create a pairwise distance matrix. To calculate the distance between profiles, we used a Dice coefficient, defined as:

$$D\left(x, y\right) = 1 - \frac{2Ns}{(Nx + Ny)} \quad (1)$$

where *Ns* is the number of shared peaks within a distance of 0.5 m/z and *Nx* and *Ny* are numbers of peaks in sample x and y, respectively. Regional differences in peptide profiles were visualized with non-metric Multidimensional Scaling constructed with PAST version 3.09 (Hammer et al., 2001) based on Bray–Curtis dissimilarity matrices and an analysis of similarity (ANOSIM) to confirm significant difference among the multivariate profiles. To identify peptides most significantly contributing to regional differences, we used similarity percentage (SIMPER) analysis to identify peptides contributing most significantly to differences between the five regions (Clarke, 1993).

Genetic Variation—Microsatellite and Phylogenetic Reconstruction

We used two metrics of genetic variation for analyses: Genetic distance estimated from microsatellites and patristic distance estimated from mtDNA haplotype diversity. We used pairwise F_{ST} based on six microsatellite loci amplified previously for each of the sampling locations (Robertson et al., 2009). For estimates of phylogenetic diversity, we extracted genomic DNA from 36 of the 54 individuals sampled for HDPs and used previously sampled frogs from the same populations as proxies for the remaining 18 individuals (see Table S3). Toe clips collected in the field were digested in standard lysis buffer with proteinase K followed by purification using DNeasy blood and tissue kits (QIAGEN). We amplified the mitochondrial gene

fragments NADH dehydrogenase subunit 1 (ND1), as described in Robertson and Zamudio (2009). We aligned ND1 sequences using ClustalW (Thompson et al., 1994) in the MegAlign v. 6.1.2 program of the Lasergene sequence analysis software (DNASTAR, Madison, WI). We conducted multiple alignments using the slow/accurate option. The initial guide tree was aligned using a 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 using the "slow/accurate" alignment option (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 our phylogenetic analyses.

Phylogenetic analyses were performed in PAUP∗ version 4.0 b10 (Swofford, 2003) using maximum-likelihood (Kohlmann et al., 2002). Bayesian analyses were performed in MrBayes 3.04. For ML and Bayesian analyses, we used Modeltest v. 3.04 (Posada and 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 was selected as the preferred model. We used unequal base frequencies (A = 0.3105, C = 0.2216, G = 0.1150, T = 0.3529), pinvar of 0.7103 and gamma shape parameter of 0.5 in a heuristic maximum likelihood search. We applied default prior distributions in MrBayes with the exception of the alpha shape parameter (exponential, mean = 1.0) and branch lengths (exponential, mean = 0.1). Bayesian analyses were run using the following conditions: Four chains (one cold and three heated) for 10 million generations, sampling every 1000th iteration. We determined the appropriate number of burn-in samples and tested for stationarity of parameter values using trace plots in the software package TRACER (Drummond et al., 2005). Removal of 10% of the initial samples provided ample burn-in in both analyses.

Color Variation

We quantified color from digital photographs taken in the field with a Nikon Coolpix 5700 (all photographs are archived at the CUMV). We photographed three areas of the body of each individual to characterize color pattern, including the posterior surface of the thighs and the left and right flanks, which contain differentiated colors. We imported photographs of each individual into Adobe Photoshop CS v. 8 (Redwood City, CA) to correct for ambient light intensity and color by reference to a black–white–gray standard (QPcard 101) in the background of every photograph (as in Robertson and Robertson, 2008). Color-corrected photographs were imported into ImageJ v. 10.2 (NIH, Washington, DC) for analyses. We quantified color as "hue" in the HSB domain (hue, saturation, and brightness) because our prior study confirmed that hue accurately represents variation when saturation values are >30% (Robertson and Zamudio, 2009).

Dominant leg colors of *A. callidryas* vary regionally (Robertson and Robertson, 2008); individuals from some populations are 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). Because of the broad range of leg colors in red-eyed treefrogs, we divided the 360° color spectrum into eight equal color bins, each spanning 45° (Robertson and Robertson, 2008). To avoid a sampling bias, we selected the entire posterior leg surface of the leg in ImageJ (as opposed to focal subsampling) to acquire a frequency histogram of the number of pixels for each hue corresponding to 8-bit hue values of 360 (Robertson and Robertson, 2008). This provided a weighted average of hue across the eight color bins for subsequent analyses. We measured flank color on the left side, using the same protocol as in Robertson and Robertson (2008).

Tests of Character Correlation

We used matrix multiple regression analysis to investigate the association between peptide diversity and (1) color pattern variation in leg and flank, (2) geographic distance, and (3) two estimates of genetic distance (pairwise F_{ST} estimated from microsatellite analyses and phylogenetic distance). Multiple matrix regression allows for significance testing of multiple distance matrices (described in Legendre and Legendre, 2012). In step 1, we conducted pairwise Mantel tests between the Y-matrix (peptide diversity) and each X-matrix (flank color, geographic distance, two estimates of genetic distance, leg color). All X-matrices that significantly varied with the Y-matrix were included in step 2. In step 2, we conducted partial Mantel tests. The variable with the highest *r* value from step 1 (i.e., leg color) was held constant in step 2. In step 3, we performed multiple matrix regression to incorporate multiple variables in the model. In step 4, we performed multiple matrix regression for a second time, removing all variables that were non-significant in step 3. Each test used Pearson's method of correlation and 999 permutations. All tests were implemented in R version 3.2.1 using the vegan package (R Core Team) and adjusted for the risk of Type 1 error using Bonferroni corrections. R script available in Supplementary Material (see Data Sheet 1).

For our matrix multiple regression analyses, we constructed distance matrices for each variable. We evaluated the relationship between geographic distance and peptide variation. Isolation by distance models predict that populations in close proximity are more similar than geographically distant populations. Findings that depart from this expectation suggest that other factors underlie peptide variation. Geographic proximity was measured as the straight-line distance between sites for populations on the same side of the Talamanca Mountains. However, the Talamanca Mountains reach an elevation outside the physiological range of the red-eyed treefrog, effectively isolating most Caribbean and Pacific populations (Savage, 2002; Robertson and Zamudio, 2009). Therefore, measuring the shortest geographic distance between two sites on either side of the mountain range is not biologically relevant. To compare the geographic distance for sites spanning the Talamanca Mountains, we measured the shortest distance around the mountains in ArcGIS as in (Robertson and Zamudio, 2009).

For the genetic distance matrices we generated two distance matrices: The first used patristic distance, based on branch lengths among individuals in the Bayesian consensus tree, and the second used pairwise population F_{ST} estimates based on six microsatellite loci (Robertson et al., 2009). We conducted separate analyses for leg and flank color. Color matrices were

generated using Euclidian distances (R v. 3.2.1), as in (Robertson et al., 2009). As color was the strongest predictor for peptide variation, we further explored the role of each hue with canonical correspondence analysis performed in PAST version 3.09 (Hammer et al., 2001).

Canonical Correspondence Analysis

With leg color pattern identified as the strongest predictor for peptide variation based on matrix correspondence tests, we further assessed the relationship between peptide diversity, color hue, and regionalization with Canonical Correspondence Analysis (CCA). A Bray Curtis dissimilarity matrix was generated for peptide profiles of frogs in PAST (Hammer et al., 2001). To visualize the relationship of peptide variation as a function color in the CCA (Ter Braak, 1986), we treated the eight color-bin hues (red, yellow-orange, yellow, green, violet-red, violet, dark blue, and light blue), as the constraining (environmental variables) in PAST (Hammer et al., 2001).

Barrier Analysis

We used Mantel and partial Mantel tests to investigate peptide variation among biogeographic regions by testing for discontinuities in peptide variation across the following biogeographic barriers: (1) Cordillera de Talamanca, an ∼3 Myr old mountain range extending the length of southern Costa Rica into western Panama, divides a large portion of *Agalychnis* populations bordering on the Caribbean and Pacific versants of the mountain range, (2) two rivers (Rio Naranjo and Rio Savegre), located in SW Costa Rica and isolating *Agalychnis* populations in SW Costa Rica from those in NW Costa Rica (Kohlmann et al., 2002), and (3) Limón, which is an important biogeographic barrier for other taxa (Kohlmann et al., 2002, 2007) and could serve to isolate *Agalychnis* populations in NE Costa Rica and those in SE Costa Rica/NW Panama. Barrier matrices were generated such that populations on one side of the barrier were assigned a value of 1 and those on the opposite side were assigned a value of 0. Barrier analysis was only applied to the relevant populations for each barrier (i e., those populations divided by the barrier).

Matrices of genetic distance, peptide diversity and leg color pattern were generated as described above in the multiple matrix regression model. For each barrier, we performed a pairwise Mantel test to assess the correlation between peptide variation (Y) and biogeographic region (X). We then performed partial Mantel tests to test this correlation while accounting for both estimates of genetic diversity and variation in leg color pattern, (color pattern showed the strongest correlation with peptide diversity in our multiple matrix regression analysis). Each test used Pearson's method of correlation and performed 999 permutations. All tests were performed in R version 3.2.1 using the vegan package, and adjusted for the risk of Type 1 error using Bonferroni corrections for stepwise multiple regression analyses (Mundrom et al., 2006).

RESULTS

Peptide Mass Spectrometry

Using MALDI-TOF mass spectrometry, we detected 832 non-redundant peptides (as based on their m/z value and assignment of these molecules as peptides based on their isotopic envelopes) across all individuals of *A. callidryas* with high HDP diversity: We observed a mean of 90 unique peptides per individual with as many as 234 unique peptides detected per individual. Of the 832 peptides identified, only one peptide, 1538 m/z, was present in all 54 individuals of *A. callidryas* (**Table 1**). This peptide was absent from the *A. saltator* outgroup frogs sampled. Other peptides, identified by SIMPER analysis, responsible for major differences across regions, include six small peptides (in the 600 m/z range) detected only in individuals from the La Selva population (**Table 1**).

Regional Variation in Peptide Variation

We detected regional structuring of peptide variation among populations and among regions (**Figure 2**). Overall, skin peptide profiles among each of the five regions are differentiated, as evident in the nMDS plot (**Figure 3**; ANOSIM: Global $R = 0.52$; $P \leq 0.001$). There is limited overlap between Caribbean and Pacific populations and between populations between SW Costa Rica and NW CR. A strong separation across the barrier at Limón, CR is visualized between NE Costa Rica from SE Costa Rica/NW Panama.

Molecular Phylogeny

We performed molecular phylogeny analysis on 52 *A. callidryas* and one outgroup taxon, *A. saltator*, amplifying the complete ND1-tRNA methylene gene consisting of 1149 nucleotides (**Figure 4**; GenBank accession numbers: FJ489259–334). This fragment contains 243 sites; no insertions or deletions were detected. The Bayesian topology for 11 populations suggests a strong pattern of regional differentiation with some, limited historical admixture. Our topology shows three well-supported clades united by a basal polytomy. The two Pacific clades form two distinct clades (SW Costa Rica and NW Costa Rica), although a single isolated population represents the SW Costa Rica clade. On the Caribbean side, we found historical admixture of populations sampled from NE and SE Costa Rica/Panama individuals. Populations in central Panama (Santa Fé, El Valle, Gamboa) showed some admixture with other Caribbean regions; two individuals sampled from Santa Fé, which were united with the larger Caribbean clade.

Tests of Character Correlation

Multiple matrix regression analyses using patristic distance between individuals as a measure of genetic distance confirmed that peptide diversity varies significantly with flank color, leg color, and genetic distance (**Table 2A**). However, geographic distance was not significantly correlated with peptide variation (**Table 2A**). Our analysis determined that leg coloration had the strongest correlation with peptide variation (slope = 0.2). Genetic distance had the next highest correlation with peptide diversity (slope = 0.1), followed by flank coloration (slope = 0.08). When accounting for variation in leg coloration, genetic distance ($r = 0.24$), geographic distance ($r = 0.20$), and flank coloration ($r = 0.18$) were all correlated with peptide variation

TABLE 1 | Prevalence (%) of select *Agalychnis callidryas* peptides detected within a population, grouped by biogeographic region in Costa Rica and Panama.

Peptide	Percentage prevalence by population, region										
Monoisotopic mass (Da)	NE CR		SE CR			NW CR		SW CR	Panama		
	%lse	%til	%man	%alm	%chi	%car	%pba	%pav	%sfe	%elv	%gam
444	100	29	–	–	–	–	–	13	–	–	–
649	100	-	–	–	–	–	–	–	–	–	–
649	100	–	–	–	–	–	–	–	–	–	–
655	100	–	–	–	–	–	–	–	–	–	–
665	100	–	–	–	–	–	–	–	–	–	–
1089	–	–	33	–	–	–	–	–	–	–	–
1287	–	14	–	–	–	–	–	–	–	–	–
1288	33	29	33	–	–	–	–	13	–	–	–
1303	50	–	33	–	–	–	–	–	–	–	–
1304	33	29	33	–	–	–	–	63	–	–	–
1348	67	57	100	100	100	–	–	–	–	–	–
1538	100	100	100	100	100	100	100	100	100	100	100
1811	–	–	–	–	–	–	–	50	20	13	
1827	33	–	–	–	–	–	–	–	20	13	–
2477	–	15	–	–	–	–	–	90	–	–	–
2542	100	–	–	–	–	–	–	–	–	–	–
2655	100	–	–	–	–	–	–	–	–	–	–

Peptides in bold, identified by SIMPER analysis, make the most significant contributions to dissimilarity in a peptide profile across the five regions.

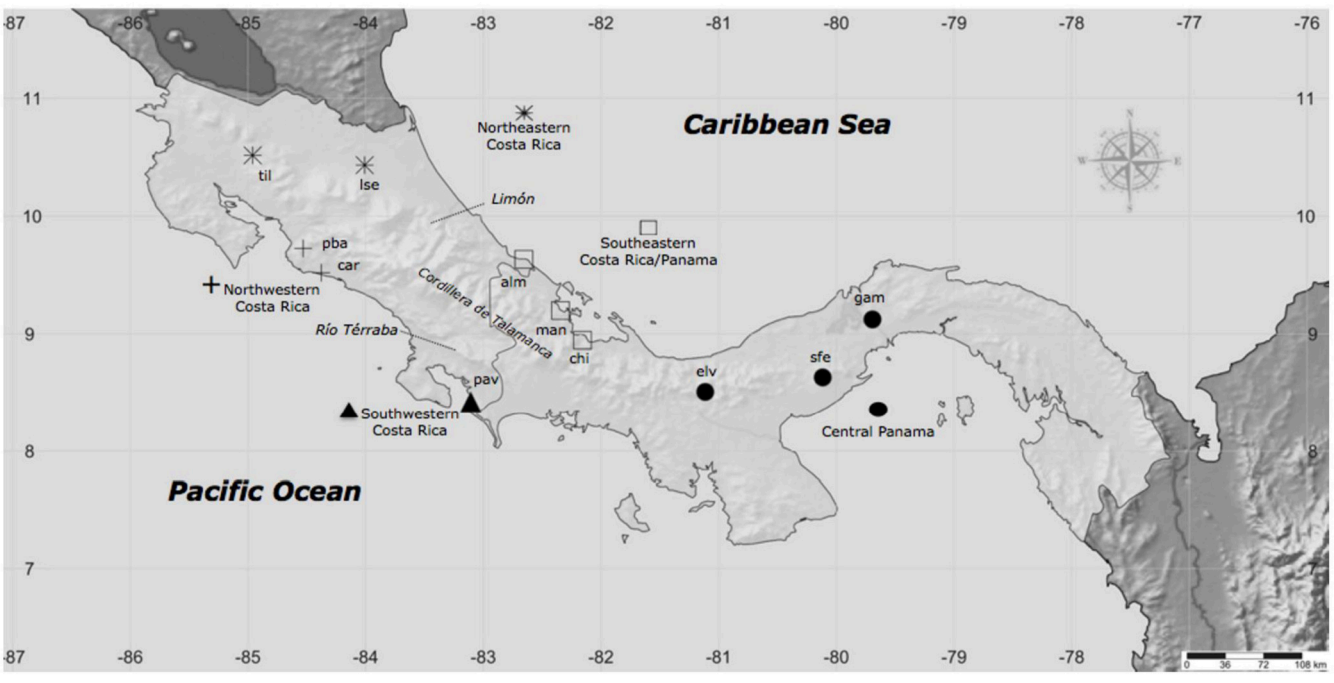

FIGURE 1 | Collection localities of *A. callidryas* skin secretion samples from 11 populations representing five biogeographic regions. At each locality we also sampled genetic tissue and took digital photographs for color analysis. See Table S1 for sample sizes.

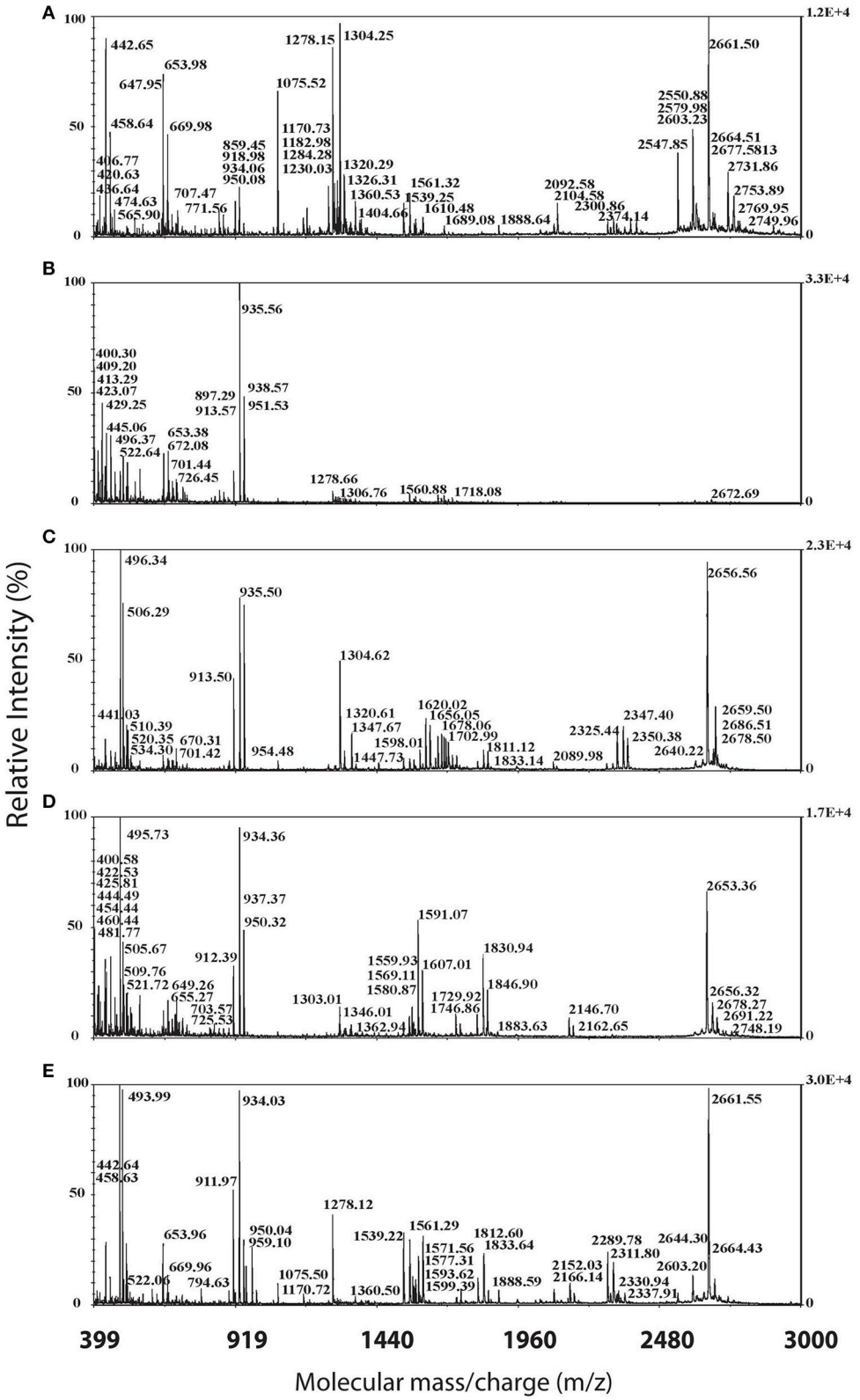

FIGURE 2 | Sample MALDI-TOF spectra of α-cyano peptide profiles for representative individuals of the five sampled regions. Spectra, from top to bottom represent: **(A)** SW Costa Rica (Pavones, Sample 2183), **(B)** SE Costa Rica and Panama (Playa Bandera, Sample 2011), **(C)** NW Costa Rica (Tilaron Sample 2381), **(D)** NE Costa Rica (Manzanillo, Sample 2374), and **(E)** Central Panama (Santa Fé, Sample 2130).

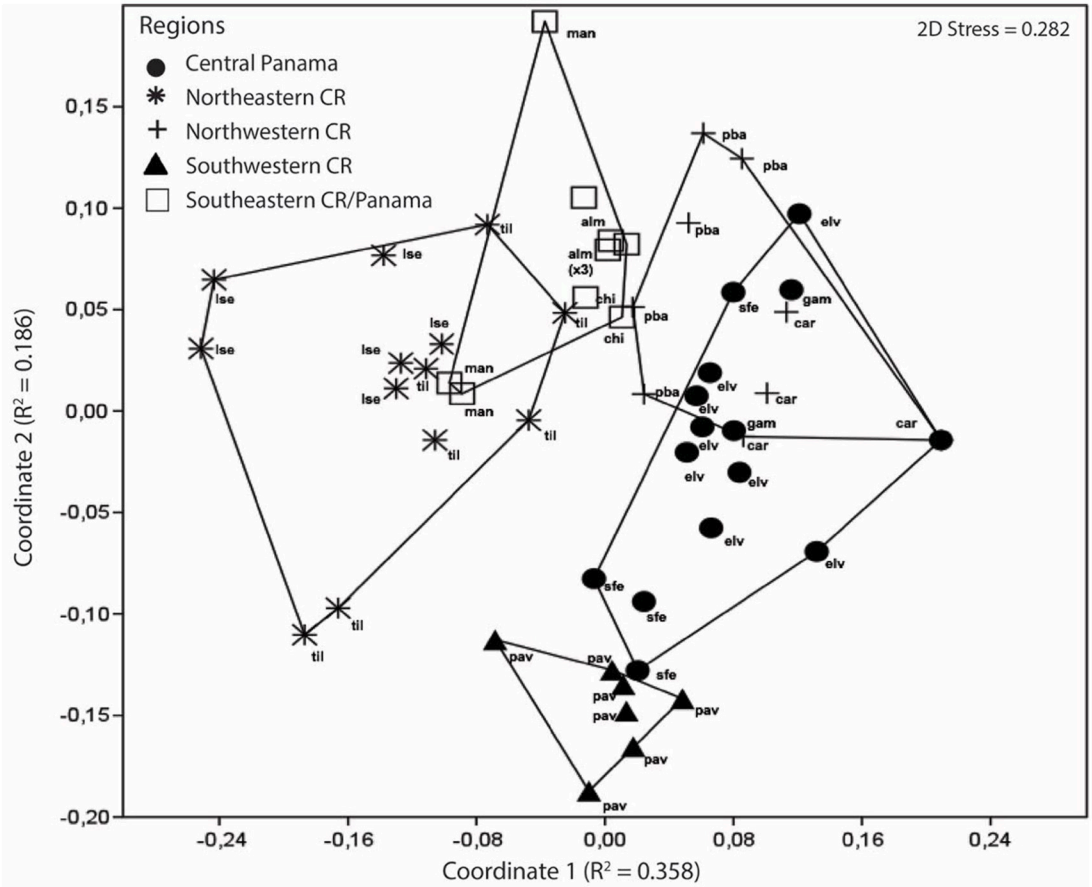

FIGURE 3 | Multidimensional scaling plot of peptide variation in *A. callidryas* from five phenotypically distinct biogeographical regions in Costa Rica and Panama. Each symbol represents an individual frog from a specific geographic region and the corresponding population of origin is annotated. The distance between symbols reflects the difference in peptide variation. On the basis of an analysis of similarity (ANOSIM), peptide variation is significantly different amongst each of the five regions (Global $R = 0.52$; $P \leq 0.001$).

in partial Mantel tests, though geographic distance was later excluded in our multiple matrix regression analysis (**Table 2A**).

These results were largely consistent with results from multiple matrix regression analysis utilizing population pairwise F_{ST} values as a measure of genetic distance, which also confirmed that peptide diversity varies significantly with leg color (slope = 0.21) and genetic distance (slope = 2.07; **Table 2B**). However, geographic distance (slope = 0.08) and flank coloration (slope = 0.09) were not significantly correlated with peptide variation when using population pairwise F_{ST} estimates (**Table 2B**).

Canonical Correspondence Analysis

The CCA biplot (**Figure 5**) complements regionalization patterns visualized in the nMDS (**Figure 3**) with Axis 1 (40.72%) and axis 2 (14.99%) together explaining 55.71% of the total variability in the peptide profiles (**Figure 5**, Table S2). Lines representing color hues pointing in the same direction depict a positive correlation with peptide profiles and lines in opposite directions are uncorrelated. Color divides populations in Northeastern Costa Rica bearing dark blue, light blue, or violet: Colors mostly absent in frogs from other regions (with the exception of two frogs from Manzanillo). Similarly, frogs from Pavones, in Southwestern Costa Rica form a separate cluster, as they exhibit a red-violet and green color pattern only found in one individual from El Valle. Individuals from Central Panama and Northwestern Costa Rica overlap in peptide profile similarity (**Figure 5**).

Barrier Analysis

Mantel and partial Mantel tests confirmed discontinuities in peptide variation across all three biogeographic barriers tested, although only the Talamanca Mountains ($R = 0.191$; **Table 3**) and Río Naranjo/Río Savegre ($R = 0.442$; **Table 3**) remained significantly associated with peptide variation after controlling for variation in leg coloration in partial Mantel tests. However, after controlling for genetic distance (measured both in terms of individual patristic distance and population pairwise F_{ST}), no barrier was associated with discontinuities in peptide variation across both measures of genetic distance ($R = 0.045$ and $R = -0.059$ for Talmanca Mountains; $R = -0.043$ and $R = -0.175$ for Río Naranjo/Río Savegre; $R = 0.198$ and $R = 0.186$ for Limón; **Table 3**).

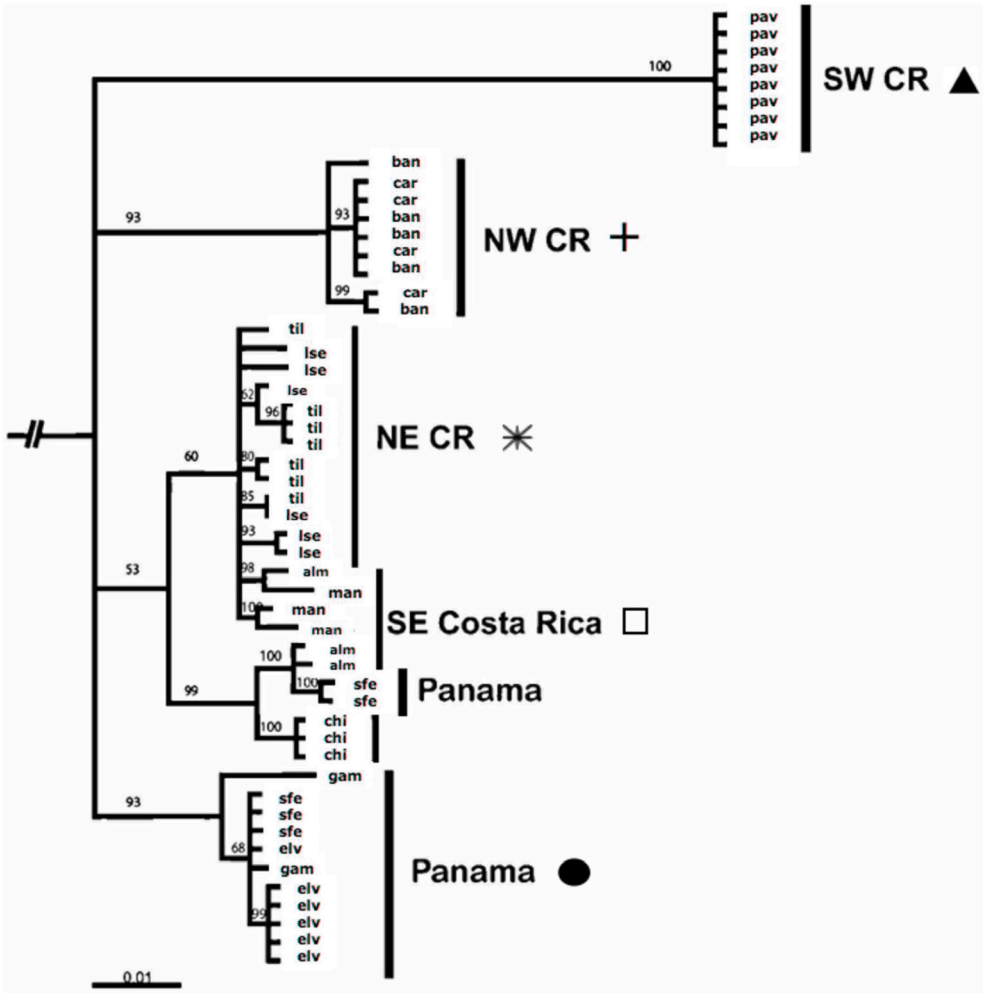

FIGURE 4 | Bayesian consensus phylogram for mitochondrial ND1 gene for 53 *A. callidryas* throughout their range in Costa Rica and Panama and the outgroup, *A. saltator*.

DISCUSSION

We uncovered a pattern of strong clustering of peptide profiles at both population and regional scales. Patterns of peptide diversity in the red-eyed tree frog *A. callidryas* are most strongly correlated with differences in leg color pattern, but are also explained by phylogenetic and population genetic distance. The fine-scale differentiation of HDPs in this species is consistent with patterns of differentiation for other phenotypic characters, such as leg color, flank pattern, and body size (Robertson and Robertson, 2008; Robertson and Zamudio, 2009). Here, we discuss the relevance of peptide profile diversity across populations as a functionally important trait that could evolve through natural selection.

Evolutionary History and Diversification

Generally, the evolutionary diversification of populations predicts peptide diversity. Both peptide diversity (nMDS, **Figure 3**) and phylogenetic topology (**Figure 5**) show patterns of admixture among populations sampled in the Caribbean (NE Costa Rica, SE Costa Rica, and Panama; **Figures 3**, **5**). We also detected incongruences between population diversification and peptide differentiation. The strongest disparity between phylogenetic topology and peptide variation occurs between the northwestern Costa Rica and Panama. For example, sites in northwestern Costa Rica (Carara [car] & Playa Bandera [ban]) are genetically and geographically isolated from Panama sites (Santa Fe [sfe], El Valle [val], and Gamboa [gam]), yet their peptide profiles are indistinguishable (**Figure 3**). We observed the opposite pattern at a finer geographic scale in the Pacific. The HDPs sampled from Carara (car) are very dissimilar to neighboring Playa Bandera (Jiang et al., 2014) despite genetic similarity (same mtDNA clade) and close proximity (25 km apart). The incongruence between HDP diversity and geographic and genetic distance in both scenarios could indicate the roles of genetic drift and/or strong localized selection and should be examined in future experimental studies.

TABLE 2 | Matrix multiple regression results of factors that correlate with peptide variation in *Agalychnis callidryas* across five regions of Costa Rica and Panama.

A								
x-matrix (Determinants of peptide variation)	**Flank color pattern**		**Geographic dist.**		**Genetic dist. (pairwise patristic distance)**		**Leg color pattern***	
y-matrix	***r***	***p***	***r***	***p***	***r***	***p***	***r***	***p***
Step 1: Mantel	0.2024	**0.001**	0.1782	**0.001**	0.2171	**0.001**	0.3084	**0.001**
Step 2: Partial Mantel	0.1772	**0.001**	0.1992	**0.001**	0.2403	**0.001**	N/A	N/A
	Slope	***p***	**Slope**	***p***	**Slope**	***p***	**Slope**	***p***
Step 3: MultiMantel	0.0747	**0.007**	0.0797	0.018 **(NS)**	0.1105	**0.004**	0.2369	**0.001**
Step 4: MultiMantel removing NS variables	0.0839	**0.005**	N/A	N/A	0.1496	**0.001**	0.2344	**0.001**
B								
x-matrix (Determinants of peptide variation)	**Flank color pattern**		**Geographic dist.**		**Genetic dist. (population pairwise F_{ST}—microsatellites)**		**Leg color pattern***	
y-matrix	***r***	***p***	***r***	***p***	***r***	***p***	***r***	***p***
Step 1: Mantel	0.2024	**0.001**	0.1782	**0.001**	0.2937	**0.001**	0.3084	**0.001**
Step 2: Partial Mantel	0.1772	**0.001**	0.1992	**0.001**	0.2591	**0.001**	N/A	N/A
	Slope	***p***	**Slope**	***p***	**Slope**	***p***	**Slope**	***p***
Step 3: MultiMantel	0.0869	0.011	0.0848	0.088 **(NS)**	1.0318	**0.001**	0.2233	**0.001**
Step 4: MultiMantel removing NS variables	0.0863	0.019	N/A	N/A	2.0719	**0.001**	0.2118	**0.001**

Step 1 shows the pairwise Mantel tests between the Y-matrix (peptide variation) and each X-matrix (flank color pattern, geographic distance, genetic distance, and leg color pattern). The X-matrices that significantly contributed to peptide variation were included in step 2. Step 2 used partial Mantel tests which accounted for leg color pattern, the X-matrix with the highest correlation to peptide variation in Step 1. Step 3 used multiple matrix regression to include multiple variables in the model. Step 4 used multiple matrix regression after exclusion of all non-significant variables from step 3. ***Table 2A*** *displays results of multiple matrix regression using the genetic distance matrix based on patristic distance. Flank coloration, leg coloration, and genetic distance were significantly correlated with peptide variation. Geographic distance was not significantly correlated with peptide variation. Significance values were determined based on 999 permutations.* ***Table 2B*** *displays results of multiple matrix regression using the genetic distance matrix based on population pairwise F_{ST}.Genetic distance and leg coloration were significantly correlated with peptide variation. Geographic distance and flank coloration were not significantly correlated with peptide variation. Significance values were determined based on 999 permutations. P-values that were significant after Bonferroni correction ($p < 0.012$) are shown bold. *Variable with highest r-value in step 1.*

Geographic Barriers

All three barriers were associated with discontinuities in peptide variation, but the pattern was weak when considering genetic distance and/or leg coloration. The barrier at Limón, isolating populations between NE and SE Costa Rica (Kohlmann et al., 2002; **Figure 1**) also delineates the break observed in leg coloration for red-eyed treefrogs (Robertson and Zamudio, 2009). The nature of the biogeographic break is poorly understood, but does mark the distributional limits for numerous invertebrate and vertebrate species, including amphibians (Kohlmann et al., 2002; Savage, 2002).

Surprisingly, we detected only a relatively weak association between skin peptide diversity and isolation due to the Talamanca mountain range when accounting for genetic distance. The Talamanca Mountain range forms the continental divide, extends 400 km along the length of Costa Rica and Western Panama (**Figure 1**; Kohlmann et al., 2002; Savage, 2002) and imposes strong distributional limits for certain terrestrial amphibians, reptiles, and insects (Zamudio and Greene, 1997; Wiens, 2000; Kohlmann et al., 2002; Crawford, 2003; Zeh et al., 2003). The Talamanca uplift is a strong barrier associated with genetic and phenotypic differentiation of *A. callidryas* populations (Robertson and Vega, 2011), however we did not find an effect of this mountain range on structuring peptide diversity. Similarly we found no evidence of a geographic barrier between northwestern and southwestern CR (Río Naranjo and Río Savegre) after accounting for genetic distance.

Peptide Diversity and Color Variation in a Nocturnal Frog

We detected the strongest association between leg color pattern and peptide diversity. The mechanistic relationship between leg color and peptide variation is beyond the scope of this work. However, bright coloration (conspicuous warning coloration) is often associated with the production or sequestering of toxic skin secretions in diurnal amphibians, including certain dendrobatids (Daly et al., 1987) and members of the genus *Mantella* (Mantellidae), *Melanophryniscus* (Bufonidae), *Pseudophryne* (Myobatrachidae), and *Eleutherodactylus* [26]. The red-eyed treefrogs exhibit bright coloration on their flanks, arms, and legs, whereas the green dorsal surface is known to serve as cryptic coloration against the leaves it sits on (Schwalm et al., 1977). Further, the role of bright color pattern in crepuscular/nocturnal taxa is less well understood.

It remains untested whether the bright coloration of red-eyed treefrogs serves as a social signal, antipredator signal, or both.

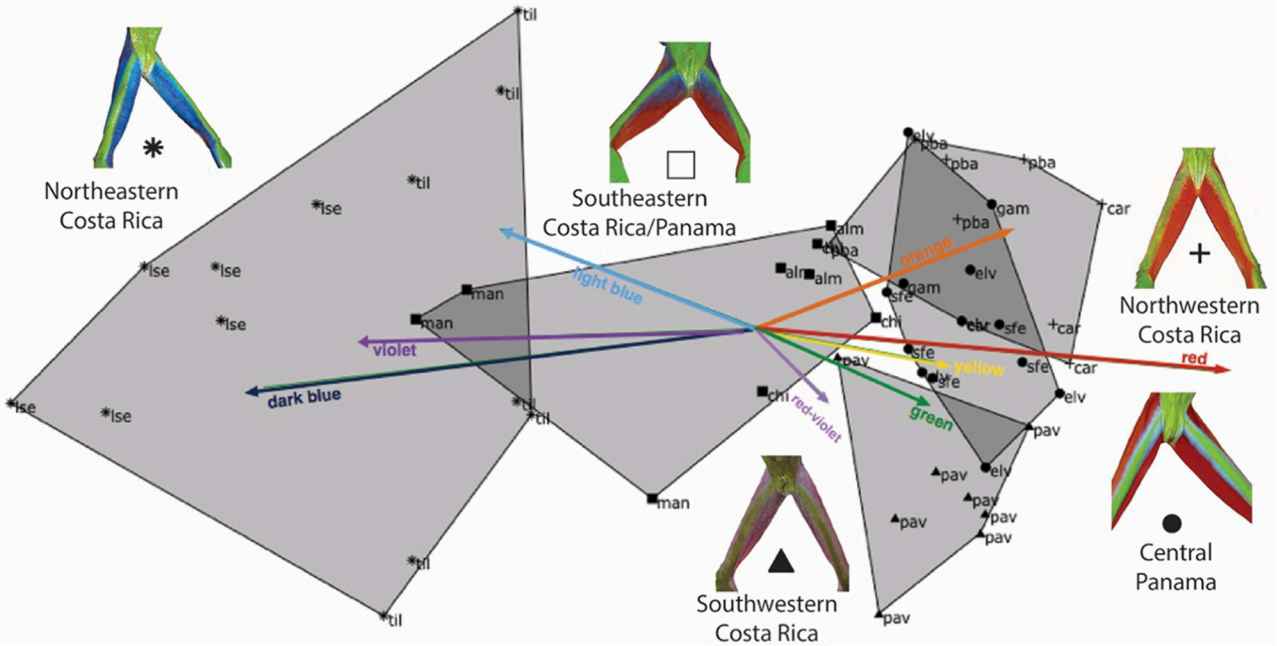

FIGURE 5 | Ordination biplot illustrating the peptide profiles of 54 individual frogs and eight explanatory co-variates divided into color-bins (orange, red, yellow, green, red-violet, violet, dark blue, and light blue). Each point represents an individual's peptide profile, identified to population (three-letter abbreviation) within one of five geographic regions (shaded hulls). Arrows indicate the direction and magnitude of the color hue component associated with peptide presence. The first two axes of the CCA together explain 55.71% of the peptide variation.

TABLE 3 | Results of simple and partial Mantel tests to investigate the relationship between geographic barriers and peptide variation in *Agalychnis callidryas*.

Barrier	Mantel Test	*r*	*P*-value
Talamanca Mountains	Talamanca × peptides	0.1818	**0.001**
	Talamanca × peptides (genetic dist.—patristic)	0.0446	0.128
	Talamanca × peptides (genetic dist.—pairwise F_{ST})	−0.0588	0.950
	Talamanca × peptides (leg color pattern)	0.1914	**0.002**
Riverine (Ríos Naranjo and Savegre)	Ríos × peptides	0.4526	**0.001**
	Ríos × peptides (genetic dist.—patristic)	−0.0432	0.676
	Ríos × peptides (genetic dist.—pairwise F_{ST})	−0.1754	0.999
	Ríos × peptides (leg color pattern)	0.4424	**0.002**
Limón	Limón × peptides	0.2737	**0.003**
	Limón × peptides (genetic dist.—patristic)	0.1976	**0.011**
	Limón × peptides (genetic dist.—pairwise F_{ST})	0.1855	**0.023**
	Limón × peptides (leg color pattern)	0.1744	**0.017**

For all barriers, a pairwise Mantel test was used to assess the correlation between location on either side of a biogeographic barrier and peptide variation. A partial Mantel test was used to assess the correlation between biogeographic region and peptide variation, while controlling for either genetic distance or leg color pattern, which was most significantly correlated with peptide variation in our matrix multiple regression model. Two measures of genetic distance were used: patristic distance, based on branch lengths among individuals in the Bayesian consensus tree, and population pairwise F_{ST} estimates based on six microsatellite loci. Significance values were determined based on 999 permutations. P-values that were significant ($p < 0.012$) after Bonferonni correction are shown bold.

Female mate choice trials in red-eyed treefrogs revealed a strong pattern of assortative mating for color morphs, indicating a possible role in social signaling (Jacobs et al., 2016). Further, as with other phyllomedusines, *A. callidryas* produces toxic secretions with reports of frogs being regurgitated by natural predators (e.g., snakes), supporting the possibility of aposematic coloration as a defense mechanism in this species (Sazima, 1974). However, the mechanistic properties of skin peptides for defense remain unknown (Woodhams et al., 2006). *A. callidryas* has adopted a defensive strategy to protect themselves from snakes,

which involves a "contracting defensive behavior" (Borteiro et al., 2014). This posture is akin to rolling up into a ball and is associated with toxic frogs, including the congeneric *A. saltator* (Gally et al., 2014). Red-eyed treefrogs exude copious skin peptides when their movement is restricted (e.g., captured by hand), suggesting that toxic skin peptides are used as the final defense to avoid predation (JR, LD, AV, and RS, *pers. obs.*). Future mechanistic studies that examine the role of color pattern as a social signal and aposematic signal would illuminate the connection between localized selection on color pattern and skin peptide diversification, as observed in *O. pumilio* (Maan and Cummings, 2008).

Future Directions

Defensive skin exudates in amphibians are extremely diverse and produced in copious quantities (Vanhoye et al., 2003). The diversification and maintenance of a broad spectrum of components could be an optimal evolutionary strategy for ensuring protection against multiple predators (e.g., snakes, spiders) and/or infectious microbes (Vanhoye et al., 2003). Peptides are unique and structurally diverse to the extent that the identical amino acid sequence of one peptide is rarely found in another host species, even closely related ones (Conlon, 2011a). Even peptides with very similar structures such as caerin 1.1 and caerin 1.11 will target specific microbes (Nicolas and Mor, 1995). In this system, given the regional variability we observed, one future research avenue is bioassay testing of specific AMPs to infectious pathogens and various macro predators present in each region.

Bioassays testing the emetic, antimicrobial, and antifungal properties of amphibian host defense peptides in red-eyed treefrogs would elucidate the role of single peptides in host defense, as found in other amphibians (Mignogna et al., 1997; Woodhams et al., 2006, 2007b), and holds conservation value. Pairwise peptide-pathogen growth assays, including peptide classes found in *A. callidryas,* contribute to host resistance to the fungal pathogen *Batrachochytrium dendrobatidis* (Wabnitz et al., 2000; Rollins-Smith et al., 2005; Woodhams et al., 2006), associated with global amphibian population declines. Further, variation among species peptide variation has been shown to account for differences in the susceptibility of species or resistance to *B. dendrobatidis* (Woodhams et al., 2007a).

We found a single conserved peptide (the only peptide detected in every individual) that could provide insight into an evolutionary-conserved host protection trait. This conserved peptide has a molecular mass that corresponds to the described tryptophyllin, AcT-3 (see Table S4), with antimicrobial and myotropic properties (Wang et al., 2015). Tryptophyllins are small peptides that range from 400 to 900 m/z (Apponyi et al., 2004), and are produced in copious amounts by phyllomedusines in Australia and the New World (Erspamer et al., 1985) but have not been detected in other frog families, thus far (Bowie et al., 2012; König et al., 2015) except for the primitive extant frog, *Ascaphus truei* (Conlon et al., 2005). The bioactive role of tryptophyllins is still unclear, as some tryptophyllins (Erspamer's FPPWM-NH2) induce sleep in birds, some have myotropic properties and still others have unidentified bioactive properties (Renda et al., 1985; Apponyi et al., 2004). Understanding the function of highly conserved peptides such as tryptophyllins would inform our understanding of the role of positive natural selection in amphibian host defense.

Conclusions

Skin peptide profiles in red-eyed treefrogs co-vary with several factors, including leg and flank color pattern, geography, and evolutionary history. The incongruence in phylogenetic history and peptide variation indicates the possible role of selection in shaping geographic patterns of peptide profiles. However, functional tests are essential for disentangling the interaction and role of natural selection in shaping patterns of HDP variation. Examining peptide variation of captive bred frogs in common garden experiments would be useful for assessing the plasticity in peptide expression and diversity and the heritability of this functional trait. Finally, determination of specific amino acid sequences, especially of those unique to specific regions and color morphs, could elucidate the presence or absence of specific peptide families within a region or color morph (similar to Daum et al., 2012). Our study emphasizes that skin peptide profiles in studies of population differentiation in a polymorphic species can contribute toward an understanding of the evolutionary processes that mediate population and lineage diversification.

AUTHOR CONTRIBUTIONS

JR, KZ, and LD conceived the original idea and outlined study. JR and KZ supervised research. JR, AV, and LD conducted fieldwork. LD and MH pre-processed peptides. HR and LD ran MALDI-TOF MS analysis. JR, KK, and LD performed statistical analyses with guidance from HR, KZ, KV, QS, and RS. LD wrote initial manuscript, prepared figures, and integrated ideas and revisions from ER, HR, KK, AV, JR, KZ into early drafts. All authors contributed to several versions of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2016.00097

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