Evolutionary drivers of sexual signal variation in Amazon Slender Anoles

Ivan Prates,1,2,3 Annelise B. D’Angiolella,4 Miguel T. Rodrigues,5 Paulo R. Melo-Sampaio,6 Kevin de Queiroz,1,* and Rayna C. Bell1,7,*

1 Department of Vertebrate Zoology, National Museum of Natural History, Smithsonian Institution, Washington, District of Columbia 20560
2 Department of Ecology and Evolutionary Biology and Museum of Zoology, University of Michigan, Ann Arbor, Michigan 48109
3 E-mail: ivanprates@gmail.com
4 Universidade Federal Rural da Amazônia campus Capitão Poço, Capitão Poço, Pará, Brazil
5 Departamento de Zoologia, Instituto de Biociências, Universidade de São Paulo, São Paulo, São Paulo, Brazil
6 Departamento de Vertebrados, Museu Nacional, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil
7 Herpetology Department, California Academy of Sciences, San Francisco, California 94118

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Phenotypic variation among populations, as seen in the signaling traits of many species, provides an opportunity to test whether similar factors generate repeated phenotypic patterns in different parts of a species’ range. We investigated whether genetic divergence, abiotic gradients, and sympatry with closely related species explain variation in the dewlap colors of Amazon Slender Anoles, Anolis fuscoauratus. To this aim, we characterized dewlap diversity in the field with respect to population genetic structure and evolutionary relationships, assessed whether dewlap phenotypes are associated with climate or landscape variables, and tested for nonrandom associations in the distributions of A. fuscoauratus phenotypes and sympatric Anolis species. We found that dewlap colors vary among but not within sites in A. fuscoauratus. Regional genetic clusters included multiple phenotypes, while populations with similar dewlaps were often distantly related. Phenotypes did not segregate in environmental space, providing no support for optimized signal transmission at a local scale. Instead, we found a negative association between certain phenotypes and sympatric Anolis species with similar dewlap color attributes, suggesting that interactions with closely related species promoted dewlap divergence among A. fuscoauratus populations. Amazon Slender Anoles emerge as a promising system to address questions about parallel trait evolution and the contribution of signaling traits to speciation.

KEY WORDS: Anolis, dewlap, parallel evolution, polytypism, reproductive isolation, species interactions.

Phenotypic variation within species is pervasive. This variation can occur in the form of polymorphism, when conspecific individuals in the same locality exhibit alternative traits (e.g., Sinervo and Lively 1996; Galeotti et al. 2013). Alternatively, phenotypes can vary between localities across a species’ range, a situation traditionally referred to as polytypism (Mayr 1963). Polytypic species show marked population differences in traits, such as coloration, vocalization, and chemical defenses, which often vary across short geographic distances (e.g., Schiotz 1971; Galeotti et al. 2003; Seehausen et al. 2008; Prates et al. 2019). Some remarkable cases of polytypism involve sexual signals, used by organisms to attract, identify, and choose suitable mates (Hill 1994; Kwiatkowski and Sullivan 2002; Jiggins et al. 2001).
Within-species variation in sexual signals might be unexpected because it could disrupt mate choice (Gleason and Ritchie 1998; Hoskin et al. 2005; Jiggins et al. 2001). Nevertheless, population differences in visual and acoustic signaling traits have been reported in many organisms (e.g., Ryan et al. 1996; Maan and Cummings 2008; Arnqvist and Kolm 2010; Scordato and Safran 2014). Given the potential contribution of sexual signals to premating reproductive isolation, uncovering the factors behind signaling trait divergence can provide insight into how new species arise. In particular, widespread species that show repeated variation in sexual signals in different parts of their range provide promising evolutionary and ecological replicates for testing hypotheses about the origins of trait diversity.

Several hypotheses have been proposed to explain how polytypic signaling traits originate. Empirical and theoretical studies have proposed that geographically structured phenotypes can evolve through nonadaptive processes such as genetic drift in isolated populations or isolation-by-distance across continuously distributed populations (Lande 1982; Campbell et al. 2010; Tazzyman and Iwasa 2010; Gehara et al. 2013). This hypothesis is consistent with observations that signal divergence scales directly with time since population divergence and inversely with gene flow in several species (e.g., Ryan et al. 1996; Bernal et al. 2005; Warwick et al. 2015). An alternative hypothesis postulates that signaling trait diversity can be adaptive, particularly when these traits vary along abiotic and biotic landscape gradients (Boughman 2002). For instance, colorful signals vary with the light environment in birds and fishes (Marchetti 1993; Boughman 2001; Seehausen et al. 2008), suggesting optimized signal transmission at a local scale in both terrestrial and aquatic habitats. Last, it has been proposed that sexual signals might diverge via reproductive character displacement, particularly when two closely related lineages overlap geographically (Grant 1972). For instance, studies in frogs have found that closely related lineages produce similar calls in allopatry and divergent calls in sympatry (Höbel and Gerhardt 2003; Hoskin et al. 2005), which suggests that biotic interactions may select for increased signal discrimination at a local scale. Although studies focusing on different organisms have provided support for each of these hypotheses, few empirical investigations have attempted to explore their relative contributions to signaling trait diversity within a single polytypic species.

Variable colorful signals are common in visually oriented diurnal organisms including several lizard clades (Stuart-Fox et al. 2007; Stuart-Fox and Moussallai 2008; Edwards et al. 2015). An iconic example of a diverse sexual signal is the dewlap, a colorful and extensible flap of skin positioned along the underside of the throat in some groups of lizards (reviewed in Tokarz 1995). The largest diversity of dewlap color and pattern is seen in Anolis, which perform dewlap displays in courtship and agonistic interactions (Nicholson et al. 2007; Losos 2009). Behavioral and physiological experiments, as well as visual modeling, indicate that anole lizards have excellent color discrimination (Hodgkinson and Still, 1980; Macedonia and Stamps, 1994; Fleishman and Persons 2001; Loew et al. 2002; Macedonía et al. 2013; Baruch et al. 2016; Fleishman et al. 2016), although visual acuity decreases with increasing distances (Fleishman et al. 2020). It has been hypothesized that dewlap coloration is associated with optimizing signal transmission in relation to the light environment (Ng et al. 2013a). In support of this hypothesis, Caribbean anole species that inhabit shaded forests more often have dewlaps with white or yellow skin color (Fleishman 1992), which reflect a high total number of photons and are, thus, brighter (Fleishman et al. 2009). By contrast, species from open habitats more frequently have dewlaps with red or blue skin color (Fleishman 1992), which are less reflective and, thus, darker (Fleishman et al. 2009). However, other studies have hypothesized that dewlap coloration diversity in Anolis has evolved through selection for reduced phenotypic overlap among sympatric species, leading to reproductive character displacement (Webster and Burns 1973; Nicholson et al. 2007; Lambert et al. 2013). This hypothesis is consistent with the observation that sympatric anoles rarely share the same dewlap pattern (Rand and Williams 1970). In this case, dewlap divergence might be particularly important for codistributed species with more similar dorsal coloration and body sizes (Fleishman et al. 2009).

Despite the presumed role of dewlap coloration in mate choice and reproductive isolation, some anole species show geographic dewlap variation (e.g., Vanhooydonck et al. 2009; Ng and Glor 2011; Prates et al. 2015; Driessens et al. 2017; Ng et al. 2017; White et al. 2019). Among them is Anolis fuscoauratus, the Amazon Slender Anoles. Taxonomic compendiums have reported that males of this species have dewlaps with grayish, yellowish, or reddish shades (Avila-Pires 1995). However, there has been no attempt to systematically characterize dewlap color variation over this species’ expansive range, and it is unclear whether different phenotypes are geographically restricted. If populations that share a given dewlap pattern are more closely related to each other than to populations with distinct phenotypes, dewlap variation in A. fuscoauratus may reflect genetic divergence and the formation of incipient species. Alternatively, because widespread South American anole species span pronounced environmental gradients (Prates et al. 2018), divergent dewlap phenotypes may have been selected to increase signal transmission at a local scale. Finally, dewlap color diversity might reflect character displacement, because A. fuscoauratus co-occurs with at least 11 other Anolis species across its distribution in lowland South American rainforests (Avila-Pires 1995; Ribeiro-Júnior et al. 2015; Prates et al. 2017). The apparent dewlap coloration polytypism of A. fuscoauratus provides a promising system to test hypotheses about
how similar factors acting in different parts of a species’ range might have generated repeated phenotypic patterns.

This study seeks to test whether evolutionary divergence, landscape gradients, and the composition of local Anolis assemblages explain sexual signal variation in Amazon Slender Anoles. We first comprehensively surveyed dewlap diversity and geographic variation on the basis of herpetological inventories that we performed over the last two decades in South America. After confirming that dewlap coloration shows large variation across the range of A. fuscoauratus, we proceeded to test the hypothesis that populations with similar dewlaps are more closely related. To this end, we generated genome-wide data through a reduced representation method to infer patterns of genetic structure and evolutionary relationships. To test the hypothesis that dewlap coloration in Amazon Slender Anoles varies as a function of landscape gradients due to locally adapted signals, we estimated multidimensional environmental space occupancy by different dewlap phenotypes based on geospatial descriptors of climate, topography, and vegetation. Last, we leveraged the results of our extensive herpetological inventories to test the hypothesis that dewlap colors in A. fuscoauratus vary as a function of local co-occurrences with other Anolis species, consistent with a scenario of reproductive character displacement.

Material and Methods

FIELD ASSESSMENT OF DEWLAP VARIATION AND ANOLE ASSEMBLAGE COMPOSITION

Anolis fuscoauratus is found in both primary and secondary rainforests in South America, where it usually is the most abundant Anolis species locally. To characterize geographic dewlap color variation in this species, we used data from our comprehensive herpetofaunal inventories in Amazonia and the Atlantic Forest over the last two decades. To this purpose, we sampled individuals by hand or pitfall traps. No quantitative color data (e.g., spectrometric measurements) were obtained due to constraints of field sampling and infrastructure. Therefore, in our environmental and species co-occurrence analyses we only included 32 sites for which dewlap color information was available (pictures or field notes) from the 63 sites that were included in genetic analyses (see below).

One of the goals of this study was to test whether dewlap variation in A. fuscoauratus is linked to the presence of other Anolis species across regions. To test this hypothesis, we obtained data on species presence at a given site based on our field inventory data. To reduce the chance of undetected species, we only included data from surveys that lasted a minimum of one week and involved at least three herpetologists searching for animals both night and day. We found all Anolis species expected to occur in the sampled regions based on species ranges (Avila-Pires 1995; Ribeiro-Junior 2015). Attesting to the thoroughness of our sampling, over the course of our expeditions we sampled two Anolis species thought to have been extinct and one new to science (Prates et al. 2017, 2020). The final dataset included occurrence data for 11 other anole species at the 32 sites for which A. fuscoauratus dewlap coloration data were available: Anolis auratus, A. chrysolepis, A. dissimilis, A. nasofrontalis, A. ortoni, A. planiceps, A. punctatus, A. scyphus, A. tandai, A. trachyderma, and A. transversalis (Supporting information Table S1).

GENETIC SAMPLING AND DATA COLLECTION

For genetic analyses, sampled individuals were euthanized by injection of 5% lidocaine solution, fixed in 10% formalin, and preserved in 70% ethanol. Prior to fixation, a sample of liver or muscle tissue was removed and preserved in 95% ethanol. Animal handling procedures were approved by the Institutional Animal Care and Use Committee of the City University of New York and Smithsonian National Museum of Natural History. Voucher specimens were deposited in the collections of the Herpetology Laboratory and Museum of Zoology of the University of São Paulo and the Federal University of Acre.

To improve inferences of genetic structure and phylogenetic relationships in Amazon Slender Anoles, we included in the genetic analyses samples from sites with both known and unknown male dewlap coloration, as well as females, which have rudimentary dewlaps in A. fuscoauratus. Our combined sampling for genetic analyses included 164 individuals of A. fuscoauratus sampled at 63 sites (Fig. 2A), encompassing most of the species’ distribution (Ribeiro-Júnior 2015). Most of these samples came from sites where dewlap coloration was known (N = 108). We used A. auratus (N = 2), A. brasilienis (1), A. chrysolepis (2), A. meridonialis (1), A. ortoni (1), A. planiceps (2), A. polyplepis (1), A. quagguulus (1), A. scyphus (1), and A. trachyderma (1) as outgroups for phylogenetic analyses (see below) based on relationships found by Poe et al. (2017). Specimen and locality information are given in Supporting information Table S2.

Genomic DNA was extracted from each tissue sample through a protein precipitation extraction protocol following proteinase K and RNAase treatment (Supporting information Text S1). After examining DNA fragment size using agarose gels, DNA concentration was measured using a Qubit fluorometer (Invitrogen, Waltham) and diluted to ensure a final concentration of 20-50 ng DNA per microliter in a total volume of 15 µL (in TE buffer). A double-digest restriction site associated DNA library (Peterson et al. 2012) was generated at the University of Wisconsin Biotechnology Center. Briefly, DNA extractions were digested with the restriction enzymes PstI and MspI, and the resulting fragments were tagged with individual barcodes, PCR-amplified, multiplexed, and sequenced.
in a single lane on an Illumina HiSeq 2500 platform. The number of paired-end reads ranged from ~1.15 to 8.85 million per individual, with a read length of 100 base pairs. Demultiplexed raw sequence data were deposited in the Sequence Read Archive (BioProject PRJNA492310; BioSample accessions SAMN18340748-18340924).

INFERRING POPULATION GENETIC STRUCTURE AND EVOLUTIONARY RELATIONSHIPS

We used Ipyrad version 0.7.30 (Eaton and Overcast 2020) to demultiplex and assign reads to individuals based on sequence barcodes (allowing no mismatches from individual barcodes), perform de novo read assembly (minimum clustering similarity threshold = 0.95), align reads into loci, and call single nucleotide polymorphisms (SNPs). A minimum Phred quality score (= 33), sequence coverage (= 6x), read length (= 35 bp), and maximum proportion of heterozygous sites per locus (= 0.5) were enforced, while ensuring that variable sites had no more than two alleles (i.e., a diploid genome). Moreover, for inclusion in the final datasets, we ensured that each locus was present in at least 70% of the sampled individuals. Following the demultiplexing step in Ipyrad, read quality and length were ensured for each sample using FastQC (available at http://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

To estimate population genetic structure and admixture in A. fuscoauratus, we generated in Ipyrad a final dataset composed of 118 434 SNPs at 16 368 loci (including no outgroups). A single SNP was then extracted from each locus to minimize sampling of linked SNPs. We used VCFtools version 0.1.16 (Danecek et al. 2011) to filter out SNPs whose minor allele frequency was lower than 0.05 (Ahrens et al. 2018). After the filtering steps, 2157 SNPs were retained across 162 individuals, with around 23% missing data across samples. To quantify missing data, we used the Matrix Condenser tool (Medeiros and Farrell 2018). Based on the SNP data, we estimated the best-fit number of genetic clusters (K) using sNMF (Sparse Nonnegative Matrix Factorization) (Frichot et al. 2014) as implemented in the R package LEA version 2 (Frichot and François 2015). We tested K = 1–12, with 100 replicates for each K. The run with the lowest entropy value, estimated by masking 5% of the samples, was considered to identify the best K (Frichot et al. 2014). To examine the robustness of sNMF to the regularization parameter (alpha), we ran preliminary analyses with alpha = 1, 25, 50, 100, 200, 400, 800, 1600, and 3200. Best-fit K were consistent across values of alpha, with remarkably similar model fit (entropy score range = 0.49–0.52).

We implemented a phylogenetic approach to assess whether individuals with similar dewlaps are more closely related to each other than to other color phenotypes across the range of A. fuscoauratus. We refrained from using species-tree methods because these approaches assume that shared molecular polymorphisms among lineages reflect incomplete lineage sorting and not gene flow. Our clustering analyses identified six broad geographic demes (see Results), and treating sampled sites as “species” would split localities inferred to belong to the same genetic pool. In addition, treating those six demes as “species” would not address our question of whether lizards with similar dewlaps are more closely related, because each deme included multiple phenotypes (see Results). Consequently, we implemented an individual-based phylogenetic approach that allows us to identify finer levels of phylogenetic structure and test whether phenotypically similar individuals cluster together in the phylogeny. To this end, we generated in Ipyrad a second dataset composed of 135 952 SNPs at 17 302 RAD loci (now including outgroup taxa and linked SNPs), ensuring that each locus was present in at least 70% of the sampled individuals. We performed phylogenetic inference under Maximum Likelihood on the concatenated dataset using RaxML-HPC version 8.2.12 (Stamatakis 2014) through the CIPRES Science Gateway (Miller et al. 2010). The GTRCAT model of nucleotide evolution was used and node support was estimated with 1000 bootstrap replicates.

ESTIMATING ENVIRONMENTAL SPACE OCCUPANCY ACROSS PHENOTYPES

Previous studies that performed spectral measurements in situ suggested that ambient light varies between forest strata (Fleishman et al. 1997; but see Fleishman et al. 2009 for a negative result). Thus, selection for dewlap detectability might vary with microhabitat use in anoles. In the case of Amazon Slender Anoles, previous studies found uniform microhabitat use among populations across the Amazon basin, with individuals preferably foraging on low vines, small twigs in the understory, and at the base of tree trunks (Avila-Pires 1995; Vitt et al. 2003; Duellmann 2005). Therefore, geographic dewlap variation in A. fuscoauratus does not appear to be explained by differential microhabitat use among populations. We, thus, focused on whether sexual signal variation in this species is linked to landscape gradients at large spatial scales, an approach that found local adaptation in the dewlap colors of the Caribbean Anolis distichus (Ng et al. 2013a). Specifically, we tested whether populations that show distinct dewlap colors segregate in a multidimensional environmental space defined by vegetation cover, climate, and topography. While vegetation cover variables are expected to more closely reflect local light environments, they may not capture all of the variation in vegetation composition and structure across the distribution of A. fuscoauratus. We, therefore, included climate and topography variables in our analyses because these factors are known to strongly affect spatial vegetation patterns across the Amazon basin (ter Steege et al. 2006; Butt et al. 2008; Laurance et al. 2010).
We used 17 variables in environmental analyses (Supporting information Table S3): cover of evergreen broadleaf trees, deciduous broadleaf trees, shrubs, herbaceous vegetation, and regularly flooded vegetation, annual cloud cover, elevation, slope, terrain roughness, and terrain ruggedness (Robinson et al. 2014; Tuanmu and Jetz 2015; Wilson and Jetz 2016; Amatulli et al. 2018), all obtained from the EarthEnv database (http://www.earthenv.org). As climatic variables, we used annual mean temperature, maximum temperature of the warmest month, mean temperature of the warmest quarter, annual precipitation, precipitation of the wettest month, and precipitation of the wettest quarter (Karger et al. 2017), obtained from the Chelsa database (http://chelsa-climate.org), as well as the climatic moisture index, a metric of relative wetness (Title and Bemmels 2018), obtained from the ENVIREM database (http://envirem.github.io).

Values were extracted for each environmental variable from the 32 sites for which A. fuscoauratus dewlap color information was available using the Point Sampling Tool plugin in QGIS version 3.4.5. Because certain variables were correlated (i.e., Pearson correlation coefficient >0.7), and to more easily visualize and compare environmental space occupancy across the range of A. fuscoauratus, we performed a principal component analysis (PCA) on the environmental variables and retained the three first principal components (PC) for downstream analyses. Environmental variables were standardized before applying PCA using a z-score transformation. Based on the three first PCs, we generated violin and scatter plots using R (R Core Team 2020) and compared mean values between dewlap phenotypes based on an analysis of variance (ANOVA) using the aov R function. We visually inspected quantile-quantile (Q-Q) plots to detect outliers and verify that model residuals were normally distributed. To account for evolutionary relationships in these analyses, we also performed a phylogenetic ANOVA (Garland et al. 1993) using the phytools R package (Revell 2012) based on our SNP-based phylogenetic tree (pruned to include one random terminal sample per site) and 1000 simulations to estimate significance. Last, we repeated these analyses by focusing only on the vegetation cover variables (i.e., not including climate or topography) given the potentially more direct effect of vegetation on the light environment.

**TESTING PATTERNS OF SPECIES CO-OCCURRENCE**

To perform a quantitative test of whether A. fuscoauratus dewlaps vary geographically as a function of co-occurrences with other Anolis species, we used the probabilistic model implemented in the cooccur package in R (Veech 2013; Griffith et al. 2016). To test for negative or positive associations between classes (e.g., species), this method calculates the observed and expected frequencies of co-occurrence between pairs of classes; the expected frequencies are calculated assuming that the distribution of a class is independent of, and random relative to, that of another class. The method returns the probabilities that lower or higher values of co-occurrence (relative to expected values) could have been obtained by chance (Griffith et al. 2016). Our field surveys of dewlap color diversity and variation in A. fuscoauratus found three phenotypes over this species’ range: gray, yellow, and pink (see Results). We treated each of these phenotypes as a distinct class in all co-occurrence analyses.

We initially ran an analysis to test for negative co-occurrences between each of the three A. fuscoauratus color phenotypes (gray, N = 11 sites; pink, N = 12 sites; yellow, N = 9 sites) and each of the five most common codistributed Anolis species. Each of these species was detected in at least eight of the 32 sites where A. fuscoauratus dewlap data were available and represent a broad range of dewlap coloration, as follows: A. ortonii (red dewlap background, N = 12 sites), A. punctatus (yellow, N = 19 sites), A. tandai (blue, N = 12 sites), A. trachyderma (yellow and orange, N = 8 sites), and A. transversalis (yellow, N = 13 sites). Behavioral and physiological experiments and visual modeling have shown that anole lizards can perceive and discriminate all of the colors present in the dewlaps of A. fuscoauratus and codistributed Anolis species (Hodgkinson and Still 1980; Macedonia and Stamps 1994; Fleishman and Persons 2001; Loew et al. 2002; Macedonia et al. 2013; Baruch et al. 2016; Fleishman et al. 2016).

We performed a second analysis grouping all 11 Anolis species detected in sympatry with A. fuscoauratus into two groups: one group (N = 24 sites) of species where males have dewlaps with brighter, more reflective background skin colors (as per Fleishman 1992; Fleishman et al. 2009), namely A. dissimilis (white), A. planiceps (orange), A. punctatus (yellow), A. trachyderma (yellow and orange), and A. transversalis (yellow); and a second group (N = 20 sites) of species whose dewlaps have relatively darker, less reflective background skin colors (Fleishman 1992; Fleishman et al. 2009), namely A. auratus (blue), A. chrysopela (blue), A. nasofrontalis (pinkish-brown), A. ortonii (red), A. scypheus (red and blue), and A. tandai (blue). By grouping species into color classes, we were able to incorporate data from species with narrow distributions that were represented by fewer than eight sites (namely A. auratus, A. planiceps, A. dissimilis, A. nasofrontalis, A. planiceps, and A. scypheus). Moreover, this approach accommodates the possibility that the dewlaps of A. fuscoauratus are influenced by multiple similar Anolis species jointly, rather than individual species only.

In a third analysis, we grouped the 11 sympatric Anolis species based on relative dewlap background color brightness and degree of overall morphological similarity to A. fuscoauratus. In South America, Anolis species belong to two major clades: Draconura (Poe et al. 2017), represented in our study area by generally small, slender, brown or gray anoles including
A. fuscoauratus and seven other species (A. auratus, A. chrysolepis, A. ortonii, A. planiceps, A. sycpheus, A. tandai, and A. trachyderma); and Dactyloa (Poe et al. 2017), represented in our study area by four anoles with generally green or greenish-gray bodies (A. dissimilis, A. nasofrontalis, A. punctatus, and A. transversalis, the latter two attaining larger body sizes than all other sampled Anolis). Based on overall morphological similarity (which correlates with clade membership), we expected that dewlap variation in A. fuscoauratus might be more strongly affected by sympatric Draconura than Dactyloa species. Therefore, in this third co-occurrence analysis, we grouped Anolis species into three classes: Draconura with bright background dewlap colors (N = 9 sites), Draconura with darker background dewlap colors (N = 19 sites), and Dactyloa (N = 23 sites).

Environmental data, species co-occurrence data, filtered genetic data, and detailed specimen information are available as Supporting Information online and through the Dryad Digital Repository (available at https://doi.org/10.5061/dryad.0zpc866X8) and GitHub (available at https://github.com/ivanprates/2021_fusco_dewlaps). R and Unix shell scripts used to prepare and filter the data and perform all analyses are available online through GitHub.

Results

DEWLAP VARIATION AMONG POPULATIONS OF ANOLIS FUSCOAURATUS

Our field inventories found remarkable geographic turnover in dewlap color over the range of A. fuscoauratus. Across South American lowland forests, we found three dewlap phenotypes, each present at multiple sites: gray, pink, and yellow (Fig. 1). Each of these three phenotypes was sampled in regions separated by hundreds to thousands of kilometers (Fig. 2A). Individuals from sites close to each other often had similar dewlap colors, but there were also instances of phenotypic turnover within tens of kilometers. Based on samples from two to 24 individuals per site (mean = 8.3; sample sizes given in Supporting information Table S1), intrasite variation was small (Fig. 1). In no circumstance did we observe more than one color phenotype (gray, pink, yellow) at the same site. Among the 32 sites with documented dewlap information, anoles from 11 sites had gray dewlaps, those from 12 had pink dewlaps, and those from nine had yellow dewlaps. Three sites were visited twice over six years, at the same time of the year (January); local dewlap patterns remained the same over time. Dewlap color at sites was consistent between juvenile and adult males, suggesting no ontogenetic changes.

Dewlap coloration was consistent across sites in all of the other anole species that co-occur with A. fuscoauratus, with the exception of two populations of A. punctatus (Amazon Green Anoles). Across most of the range of A. punctatus, individuals have yellow dewlaps; however, in one population from the Içá River (state of Amazonas, Brazil), the lizards had light green dewlaps, and in one from the Ariquara River (state of Mato Grosso, Brazil) they had creamy-white dewlaps (Rodrigues et al. 2002). Of the two unique A. punctatus populations, only the Içá River population overlapped with the known A. fuscoauratus dewlaps and was included in the co-occurrence analyses.

Figure 1. Dewlap phenotypes in Amazon Slender Anoles (Anolis fuscoauratus) and examples of limited intrasite dewlap coloration variation. (A) The three dewlap phenotypes recorded in our field inventories: yellow, pink, and gray. (B) Intrasite variation in Rio Branco, Acre, Brazil. (C) Intrasite variation in Senador Guiomard, Acre, Brazil. These two sites are separated by around 25 km of continuous Amazonian rainforest.
Figure 2. Spatial patterns of genetic and phenotypic structure in Amazon Slender Anoles. (A) Geographic dewlap color variation based on field inventories performed over the last two decades in South American rainforests. (B) Distribution of the six genetic clusters inferred from genetic cluster analysis (each cluster shown in a different color); pies indicate the average proportions of alleles (i.e., ancestry proportions) from each cluster at a given site (based on all individuals sampled at that site). Light green background on the maps depicts rainforest distribution. (C) Ancestry proportions from genetic cluster analyses; each bar represents an individual. When known, dewlap color is indicated with colored circles below each individual bar. (D) Phylogenetic relationships among samples inferred under a Maximum Likelihood framework based on the SNP data. For clarity, nodal support is shown only for the relationships between major groups inferred by genetic cluster analyses (a complete phylogeny, including outgroups, with support for all nodes is provided in Supporting information Text S2). Asterisks indicate bootstrap support > 95.

(both light-green and yellow were classified as relatively brighter dewlap colors).

**PATTERNS OF GENETIC STRUCTURE**
Cluster analyses using sNMF inferred six major genetic clusters across the geographic distribution of *A. fuscoauratus* (Fig. 2B and C), with some admixture or mixed assignments (as indicated by the ancestry coefficients of individuals) across clusters. Dewlap phenotypes (gray, pink, yellow) did not compose distinct genetic clusters; instead, all six clusters were made up of anoles with two or three different dewlap color phenotypes, and each phenotype was found in three to six genetic clusters. The three dewlap phenotypes occurred in both admixed and nonadmixed individuals.

Inferred genetic clusters segregated in geographic space (Fig. 2B and C). Samples from the coastal Atlantic Forest formed one cluster (represented by lighter green in Fig. 2B and C), whereas the five remaining clusters occur in different parts of Amazonia: (1) the Guiana Shield in northern South America (cream); (2) westernmost Brazilian Amazonia (blue); (3) southwestern Brazilian Amazonia, west of the Madeira river (lighter purple); (4) south-central Brazilian Amazonia, east of the Madeira river and west of the Tapajós river (darker purple); and
(5) central Amazonia south of the Amazon river, extending to Ecuador in the west and to the Xingu River in the east (darker green). Admixture was inferred primarily between clusters that have adjacent geographic distributions (Fig. 2C).

**PHYLOGENETIC PATTERNS**

Similar to the genetic cluster analyses, phylogenetic analyses inferred that none of the three *A. fuscoauratus* dewlap phenotypes (gray, pink, and yellow) forms a clade. Instead, each phenotype is located in multiple parts of the tree (Fig. 2D; a phylogeny including outgroup taxa and support for all nodes is provided in Supporting Information Text S2). Samples from the same site shared the same dewlap coloration and grouped together. At deeper phylogenetic levels, major clades included samples having two or three different dewlap phenotypes; within each major clade, samples with the same phenotype often were not closely related (Fig. 2D).

Mirroring the results from the genetic cluster analyses, major clades corresponded to different parts of the geographic distribution of *A. fuscoauratus*. Samples from the Atlantic Forest (indicated in lighter green in Fig. 2) and Guiana Shield in northern Amazonia (cream) group together. The clade formed by these samples is sister to a clade formed by the remaining Amazonian samples. Within the latter clade, samples from westernmost Brazilian Amazonia (in western Acre; blue) group together, as do samples from southwestern Brazilian Amazonia (in eastern Acre; lighter purple) and south-central Brazilian Amazonia (in Rondônia; darker purple). Samples from central Brazilian Amazonian (darker green) comprise two primary clades that together are paraphyletic relative to the other Amazonian clades. Relationships among these major clades generally received high bootstrap support (Fig. 2D).

**ENVIRONMENTAL SPACE OCCUPANCY**

Climate, topography, and vegetation cover vary over the distribution of *A. fuscoauratus*. For instance, annual mean temperature at sampled sites ranged from 20.2 to 26.4°C, annual precipitation from 1258 to 3511 mm, elevation from 22.5 to 913.0 m, and cover of evergreen broadleaf trees from 3 to 100% (raw data for all 17 environmental variables are presented in Supporting information Fig. S1). After implementing PCA on the environmental data, the first three PCs explained 37, 22, and 15% (total of 74%) of the environmental variation across sampled sites, respectively (PCA loadings presented in Supporting information Table S4). PC1 increased with higher elevation, higher topographic complexity, and lower temperature, describing a lowland to highland axis; PC2 increased with higher precipitation and cloud cover, describing a dry to wet axis; and PC3 increased with more open and deciduous vegetation, describing an axis of evergreen forest to savanna and deciduous forest.

Plots of these first three PC axes indicated that each of the three dewlap phenotypes of *A. fuscoauratus* occur at localities that together exhibit a similar range of environmental conditions, with large overlap in environmental space (Fig. 3). An ANOVA based on all sampled sites found no significant differences between phenotypes in PC1 ($F_{2.29} = 0.28; p = 0.76$), PC2 ($F_{2.29} = 1.68; p = 0.20$), or PC3 ($F_{2.29} = 2.38; p = 0.11$). After eliminating four outlier sites based on the inspection of Q-Q plots, there was a statistically significant difference in PC3 (savannah and deciduous forest to evergreen forest) across phenotypes ($F_{2.25} = 3.47; p = 0.047$); however, post-hoc analyses using Tukey’s test did not support significant differences between groups in pairwise comparisons ($p > 0.05$ in all tests).

The same pattern of environmental overlap between color phenotypes was found when accounting for evolutionary relationships within *A. fuscoauratus* (phylogenetic ANOVA, PC1: $F_{2.29} = 0.28; p = 0.81$, PC2: $F_{2.29} = 1.68; p = 0.28$, PC3: $F_{2.29} = 2.38; p = 0.16$). Likewise, there were no significant differences between phenotypes when focusing on vegetation cover alone (i.e., not including climate or topography) when accounting for evolutionary relationships (phylogenetic ANOVA, PC1: $F_{2.29} = 0.96; p = 0.45$, PC2: $F_{2.29} = 0.02; p = 0.99$, PC3: $F_{2.29} = 0.69; p = 0.6$) or not (ANOVA, PC1: $F_{2.29} = 0.96; p = 0.40$, PC2: $F_{2.29} = 0.02; p = 0.98$, PC3: $F_{2.29} = 0.69; p = 0.51$).

**SPECIES CO-OCCURRENCES**

Co-occurrence analyses (Fig. 4) invariably found each of the three *A. fuscoauratus* phenotypes to be negatively associated with one another ($p < 0.010$), reflecting our field observation of a single phenotype at each sampled site.

Analyses including the three *A. fuscoauratus* dewlap phenotypes and the other five most common sympatric *Anolis* species (Fig. 4A) found a negative association between *A. fuscoauratus* with yellow dewlaps and *A. trachyderma* ($p = 0.047$); these two classes never co-occurred. *Anolis trachyderma* and *A. fuscoauratus* both exhibit brown dorsal coloration, slender bodies, and yellowish dewlaps. The distributions of the other two *A. fuscoauratus* phenotypes (gray and pink) were not associated negatively or positively with the five most common sympatric *Anolis* species ($p$-values ranging from 0.104 to 1.0). This analysis also found a positive association between the occurrences of *A. tandai* (blue dewlaps) and *A. transversalis* (yellow dewlaps) ($p = 0.003$), as well as between *A. ortonii* (red dewlaps) and *A. punctatus* (yellow dewlaps) ($p = 0.005$), consistent with the observation that these species pairs frequently co-occurred at sampled sites.

When grouping the other 11 *Anolis* species based solely on dewlap coloration (Fig. 4B), we found a negative association between *A. fuscoauratus* with gray dewlaps and anole species that have dewlaps with darker colors ($p = 0.005$). Other relationships were not significant ($p$-values ranging from 0.059 to 0.994).
Figure 3. Environmental space occupancy and overlap between dewlap phenotypes in Amazon Slender Anoles. (A) Violin plots showing, for each phenotype, the probability densities of the first three axes from an environmental PCA. PC1 describes a lowland to highland axis; PC2 describes a dry to wet axis; and PC3 describes an evergreen forest to deciduous forest and savanna axis. (B) Overlap in environmental space occupancy among dewlap phenotypes based on biplots of PC1, PC2, and PC3.

When grouping the other 11 Anolis species considering both relative color brightness and Anolis clade (Draconura: small, slender, brown anoles more similar to A. fuscoauratus; Dactyloa: greenish anoles that often attain larger body sizes than A. fuscoauratus) (Fig. 4C), we found a negative association between A. fuscoauratus with gray dewlaps and Draconura species that have darker dewlap colors (p = 0.011). We also found a negative association between A. fuscoauratus with yellow dewlaps and Draconura species that have brighter dewlap colors (p = 0.029). Other relationships were not significant (p-values ranging from 0.071 to 1).

Discussion
On the basis of biodiversity inventories at dozens of rainforest sites in northern South America, we found extensive dewlap color variation in A. fuscoauratus among sites, but limited variation within sites (Fig. 1). Similar dewlaps occur at sites hundreds to thousands of kilometers apart. In some cases, these sites are separated by unsuitable habitat; for instance, yellow and gray dewlaps occur in both Amazonia and the Atlantic Forest, two rainforest regions separated by open and dry grasslands and scrublands in which Amazon Slender Anoles do not occur (Fig. 2). A reduced representation genomic dataset indicated that phenotypically similar populations are often not closely related (Fig. 2), consistent with a history of repeated origin (or loss) of each of the three dewlap phenotypes. Moreover, a genetic cluster analysis indicated mismatches between dewlap phenotype and genetic structure: genetic clusters were composed of individuals with different dewlap colors, and each dewlap phenotype was distributed across multiple genetic clusters (Fig. 2). Estimates of environmental space occupancy found no separation by phenotype (Fig. 3), providing no clear support for the hypothesis of local adaptation to abiotic landscape gradients. By contrast, dewlap variation was associated with the presence of other Anolis species across the geographic distribution of A. fuscoauratus. Specifically, co-occurrence analyses found that A. fuscoauratus with yellow (bright) and gray (darker) dewlaps occur less frequently than expected at sites where sympatric species have relatively brighter or darker dewlap colors, respectively (Fig. 4).

POPULATION ISOLATION AND SEXUAL SIGNAL DIVERGENCE
A pattern of geographically clustered phenotypic variation, as we report in Amazon Slender Anoles, could be generated by genetic isolation between populations due to stochastic or nonadaptive evolutionary processes. For instance, genetic drift can lead to the fixation of alternative phenotypes in isolated populations, a
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Figure 4. Results of co-occurrence tests between Anolis fuscoauratus dewlap phenotypes and sympatric Anolis species. Each square represents a pairwise comparison between each of the three A. fuscoauratus phenotypes (gray, pink, yellow) and (A) each of five codistributed and common Anolis species (detected in at least eight out of 32 sites); (B) 11 Anolis species that occur sympatrically with A. fuscoauratus grouped into dewlaps with relatively brighter (yellow, orange, white) or darker (blue, red, pinkish-brown) colors; and (C) the same 11 species grouped based on both relative dewlap color reflectivity and major Anolis clade (Draconura: brown or gray small, slender anoles, more similar and more closely related to A. fuscoauratus; Dactyloa: greenish larger, stockier anoles, less similar and more distantly related to A. fuscoauratus). The dewlaps of the five anole species most frequently found in sympatry with A. fuscoauratus are also illustrated: (D) Anolis ortonii, (E) Anolis tandai, and (F) Anolis trachyderma (all three in the Draconura clade); and (G) Anolis punctatus; and (H) Anolis transversalis (both in the Dactyloa clade).

process that has been invoked to explain sexual signal divergence in island species (Gehara et al. 2013). This scenario predicts genetic discontinuity (i.e., allele frequency differences) between phenotypically distinct populations. However, our analyses often inferred different phenotypes of A. fuscoauratus as part of the same genetic cluster, which contradicts the hypothesis of phenotypic divergence between genetically isolated populations. Alternatively, trait diversity can arise as a result of isolation-by-distance. In this case, phenotypic divergence is predicted to correlate with geographic separation (Campbell et al. 2010). However, our field surveys found dewlap turnover among sites that were assigned to the same genetic cluster and are separated by only tens of kilometers of rainforest habitat, with no apparent geographic features that would constitute barriers to gene flow. Moreover, distinct genetic clusters occurring in distant geographic regions often showed similar phenotypes. Taken together, these findings suggest that genetic or geographic isolation is insufficient to explain the sexual signal diversity seen in Amazon Slender Anoles. Discordance between population genetic divergence and geographic trait variation has been documented in many studies investigating phenotypes ranging from bird bill morphology and plumage (Mason and Taylor 2015) to fish body shape (Faulks et al. 2015). Such mismatches between genetic and phenotypic structure have been attributed to phenotypic plasticity or convergent local adaptation (reviewed by Zamudio et al. 2016), two processes that might also contribute to dewlap polytypism in A. fuscoauratus (see below).

ENVIRONMENTAL FACTORS AND SEXUAL SIGNAL VARIATION

A pattern of phenotypic divergence not accompanied by genetic divergence, as seen in A. fuscoauratus, can result from environmental factors that vary geographically. For instance, dewlap color variation might stem from local differences in diet. In birds and fishes, pigments that bestow yellow, orange, and red coloration depend on dietary sources of carotenoids, in certain
cases leading to geographic population variation (Endler 1980; Hill et al. 2002; Hill 1993). In the case of anoles, these colors can also be produced via endogenously synthesized pteridins (Macedonia et al. 2000; Steffen and McGraw 2007; Alfonso et al. 2013). Experiments with Anolis distichus and Anolis sagrei found no change in dewlap color or pattern under alternative dietary regimes of carotenoid supplementation (Steffens et al. 2010; Ng et al. 2013b). Moreover, breeding experiments showed that dewlap coloration is heritable in Anolis distichus and A. sagrei (Ng et al. 2013b; Cox et al. 2017). While these studies suggest that dewlap colors are genetically determined and not plastic in Anolis, no such data are currently available for A. fuscoauratus. Future experimental studies could elucidate whether dietary pigments contribute to dewlap diversity in Amazon Slender Anoles, which show higher levels of geographic color variation than the previously studied species.

Alternatively, mismatches between genetic and phenotypic structure, as seen in Amazon Slender Anoles, may stem from adaptive divergence with gene flow (reviewed in Zamudio et al. 2016). In Caribbean anoles, highly reflective (brighter) dewlap colors (e.g., white and yellow) are more frequent in species that inhabit dense forests, while less reflective (darker) dewlap colors (e.g., red and blue) appear more common in species from dry scrublands (Fleishman 1992). A similar pattern may hold for populations within species. In Anolis cristatellus and A. distichus, for instance, dewlap spectral properties co-occur with habitat type at the intraspecific level, suggesting that signaling traits are locally adapted for increased detectability (Leal and Fleishman 2004; Ng et al. 2013a). Importantly, local adaptation in sexual signals can disrupt mate choice and promote reproductive isolation among populations, leading to speciation through sensory drive (reviewed in Boughman 2002). However, we found no association between dewlap color and spatial gradients of climate, topography, and vegetation cover in A. fuscoauratus. This result is inconsistent with the hypothesis that local adaptation to landscape gradients was a driver of sexual signal diversity in this species. It is worth noting that Amazon Slender Anoles are restricted to moist forests; the driest habitats where we sampled this species were forest patches in forest-savanna transitional areas (e.g., in Brazil’s state of Roraima). By contrast, previously studied anole species with locally adapted dewlaps have ranges that span mesic forests to open xeric habitats (Leal and Fleishman 2004; Ng et al. 2013a). Therefore, environmental factors may be a more important driver of dewlap variation in Anolis species that are more ecologically diverse than is A. fuscoauratus (e.g., Fleishman et al. 2009). Likewise, iconic cases of locally adapted phenotypes occur along pronounced environmental transitions including dorsal coloration matching dark versus light soils in lizards and rodents (Hoekstra et al. 2006; Rosemblum 2006) and armor plate patterning in conspecific freshwater and marine fish populations (Colosimo et al. 2005). Consequently, if phenotypic and environmental variation are coupled across the distribution of A. fuscoauratus, it may be challenging to detect this relationship if the relevant spatial abiotic gradients are subtle.

**SPECIES CO-OCCURRENCE AND SEXUAL SIGNAL DIVERGENCE**

Our results suggest that sexual signal variation in Amazon Slender Anoles may be tied to spatial turnover in the composition of ecological assemblages. Specifically, we found negative associations between the distributions of the gray and yellow A. fuscoauratus phenotypes and Anolis species with similarly bright or dark dewlap colors. These associations may be influenced by the degree of overall morphological similarity among species. For instance, we found negative associations between A. fuscoauratus dewlap phenotypes with other Amazonian Draconura species, which also have brown or gray dorsa and slender bodies, but not with the more distantly related Amazonian Dactyloa species, which are green and stockier. These findings suggest that dewlap colors in A. fuscoauratus may adapt to reduce sexual signal similarity with codistributed species at a local scale, potentially decreasing the frequency of cross-species interactions (e.g., Rand and Williams 1970; Webster and Burns 1973; Lambert et al. 2013). Other studies have invoked reproductive character displacement to explain divergent signaling traits among closely related lineages in sympathy. This is the case, for instance, with colorful signals in Australian agamid lizards (Edwards et al. 2015) and vocalizations in birds and frogs (Wallin 1986; Höbel and Gerhardt 2003; Hoskin et al. 2005; Kirshel et al. 2009). Moreover, our results are consistent with studies showing that local selective regimes can lead to phenotypic mosaics when species interactions vary geographically (Brodie Jr et al. 2002; Thompson 2005).

Our co-occurrence results pose the question of why A. fuscoauratus seems to be the only Amazonian Anolis whose signaling traits vary as a function of the distributions of closely related species whereas the vast majority of other species have uniform dewlap coloration. One possibility is that dewlap diversity in Amazon Slender Anoles is related to relationships of behavioral dominance among species. Anolis fuscoauratus is the smallest and most slender of lowland Amazonian anole species (Avila-Pires 1995; Prates et al. 2017, 2020). By evolving divergent dewlaps, A. fuscoauratus might reduce agonistic interspecific interactions and, thus, avoid aggression from its larger relatives. Integrative behavioral and phenotypic experimental approaches could be used to test the hypothesis that body size predicts dominance (or subordination) and dewlap coloration divergence among sympatric Anolis species, in Amazonia and elsewhere.
In contrast to the gray and yellow dewlaps, we found no evidence of geographic associations between A. fuscoauratus with pink dewlaps and codistributed Anolis species. This pattern may indicate that pink dewlaps have intrinsic spectral properties that result in lower interference with sympatric anoles (e.g., Fleishman 1992; Fleishman et al. 2009). For instance, beyond relative brightness, Anolis dewlap colors vary along additional axes that contribute to signaling such as chroma (“colorfulness”) (Fleishman et al. 2009). Furthermore, pink dewlaps might be associated with factors not considered in this investigation including signal detection by nontarget viewers. For instance, geographic differences in predation intensity have led to polytypic signaling traits in fishes and frogs (Trillo et al. 2013; Heinen-Kay et al. 2015; Johnson and Candolin 2017), albeit not in brown anoles, A. sagrei (Baeckens et al. 2018). Future studies of A. fuscoauratus will benefit from characterizing dewlap color spectra, identifying key predators, and quantifying differences in predation intensity among dewlap color phenotypes and localities, for instance, using clay models (Steffen 2009; Paemelaere et al. 2013).

SEXY SIGNAL DIVERGENCE AND REPRODUCTIVE ISOLATION

Our genetic analyses suggest that patterns of genetic structure do not match phenotypic structure among populations of Amazon Slender Anoles, contradicting the expectation that populations with distinct sexual signals are reproductively isolated. Yet, it is widely accepted that the dewlap plays a key role in premating reproductive isolation in Anolis lizards (reviewed by Tokarz 1995; Losos 2009). Supporting this view, behavioral experiments with A. cybotes, A. marcanoi, and A. grahami found stronger responses of individuals to dewlap displays of their own species than to those of other species (Losos 1985; Macedonia and Stamps 1994). Nevertheless, it remains unclear whether dewlap divergence can ultimately disrupt gene flow between lineages. In the case of Amazon Slender Anoles, multiple dewlap phenotypes are present within each of the six genetic clusters across the species range, suggesting that genetic divergence within A. fuscoauratus is not associated with differences in dewlap coloration at broad or narrow spatial scales. It is worth noting that, despite dewlap color variation, populations across the range of A. fuscoauratus have homogeneous hemipenes, a trait linked to reproductive isolation in lizards (D’Angiolella et al. 2016). Signal variation among interbreeding populations, as seen in A. fuscoauratus, has been documented in other Anolis species (Thorpe and Stenson 2003; Ng and Glor 2011; Stapley et al. 2011; Ng et al. 2017) as well as other organisms that rely on visual signals such as birds and fishes (Hermansen et al. 2011; Morgans et al. 2014). These studies support the idea that divergent signaling traits do not necessarily impose strong barriers to interbreeding and gene flow, even when sexual signals are locally adapted (Muñoz et al. 2013; Ng et al. 2016).

CONCLUDING REMARKS

On the basis of phenotypic, genetic, and ecological data, we found evidence that certain dewlap colors in a widespread anole lizard species are negatively associated with the local occurrence of phenotypically similar closely related species. Our finding of extensive mismatches between genetic and phenotypic structure in Amazon Slender Anoles at both broad and narrow spatial scales raises questions about the presumed role of the dewlap in reproductive isolation in anole lizards (Tokarz 1995; Losos 2009). Correspondingly, our results also call into question the extent to which dewlap coloration is informative for species delimitation and taxonomy in Anolis, as previously suggested based on other anole species complexes (Prates et al. 2015).

This investigation highlights several knowledge gaps to be addressed by future studies. First, we still know little about how divergent visual signals affect agonistic interactions and mate choice in Anolis, which will require additional behavioral experimentation (e.g., Losos 1985). Moreover, the genetic basis of dewlap color variation remains unclear. Genomic analyses of phenotypically diverse species can elucidate the genetic mechanisms behind parallel trait evolution including the contribution of standing genetic variation (reviewed in Zamudio et al. 2016) and differential gene flow across genomic regions in the face of selection (reviewed in Harrison 2012; Harrison and Larson 2014). The geographically variable dewlaps of Amazon Slender Anoles emerge as a promising system to address these questions. Future investigations of this compelling system will benefit from quantitative assessments of sexual signal variation, behavioral experiments, and comparative genomic analyses.

AUTHOR CONTRIBUTIONS

IP, ABD, and MTR conceptualized the study. IP, MTR, KdQ, and RCB acquired funding. IP, MTR, and PRMS designed data collection and obtained the data. IP, ABD, KdQ, and RCB designed the analyses. IP wrote computer scripts, performed the analyses, prepared figures, and led the writing of the initial draft. IP, ABD, MTR, PRMS, KdQ, and RCB interpreted the results and wrote the final draft.

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DATA ARCHIVING
Environmental data, species co-occurrence data, filtered genetic data, and detailed specimen information are available as Supplementary Information online and through the Dryad Digital Repository database (available at https://doi.org/10.5061/dryad.0zpc866x8) and GitHub (available at https://github.com/ivanprates/2021_fusco_dewlaps). Demultiplexed raw sequence data were deposited in the Sequence Read Archive (BioProject PRJNA492310; BioSample accessions SAMN18340748-18340924). R and Unix shell scripts used to prepare and filter the data and perform all analyses are available online through GitHub.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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Supporting Information
Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Violin plots depicting the ranges of all 17 environmental variables.
Table S1. Locality information for Anolis fuscoauratus and sympatric Anolis species used in the co-occurrence analyses.
Table S2. Specimen and locality information of individuals used in the genetic analyses.
Table S3. Locality information and used in the environmental analyses.
Table S4. Loadings of variables used in environmental principal component analyses.
Text S1. Protein precipitation protocol used for genomic DNA extraction.
Text S2. Phylogenetic tree including node support values and outgroup taxa.