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No Evidence of Genetic Differentiation Between Anoles With Different Dewlap Color Patterns

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Color variation across and within populations can play an important role in speciation and our understanding of the maintenance of genetic variation. Trait polymorphisms may be important in reproductive isolation and speciation. Conversely, if 2 morphs exist within a species, then the classical question of how the polymorphism is maintained in the face of drift and selection becomes relevant. In *Anolis* lizards, variations in dewlap size and color are often used as diagnostic markers of species and considered important traits in population divergence and speciation. The aim of this study was to describe dewlap color pattern variation in *Anolis apletophallus* and estimate gene flow between populations that have different dewlap color patterns. We confirmed that 2 dewlap morphs exist, a "solid" morph that has an orange dewlap and a "basal" morph that has a white dewlap with an orange basal spot. Throughout most of *A. apletophallus*' range, the morphs have non-overlapping distributions, except for one area where both morphs occur in equal frequencies. Analysis of reflectance spectra demonstrated that the color of the dewlap margin differed between morphs but that dewlap color and pattern did not differ across populations within morphs. Using 8 microsatellite markers, we found little genetic differentiation between populations or individuals with different dewlap morphs. In contrast, the small amount of genetic structure that does exist is due to current day geographic barriers. Therefore, dewlap color variation in *A. apletophallus* appears to be a polymorphism rather than an indicator of 2 fully or partially reproductively isolated populations.

Key words: Anolis apletophallus, Anolis limifrons, *color polymorphism, neutral loci, Panama, population structure, speciation*

Studying polymorphisms can provide valuable insight into fundamental processes in evolutionary biology including speciation and the maintenance of genetic variation. An important step to understanding their role in reproductive isolation is to quantify the degree of genetic differentiation between different morphs. Here, we investigate gene flow between populations of *Anolis* lizards that vary in dewlap color pattern.

In *Anolis* lizards, dewlap color polymorphisms are thought to play an important role in speciation (Losos and Schneider 2009). In most species of anoles, males (and sometimes females) possess a colorful dewlap—an extendable flap of skin on the neck—used in sexual signaling, territory defense, pursuit deterrence, and possibly interspecies recognition (Williams and Rand 1977; Leal and Rodríguez-Robles 1997; Losos and Schneider 2009). Dewlap size, pattern, and coloration are often diagnostic between anole species, and for this reason, many species classifications are based, at least partly, on dewlap color (Savage 2002; Köhler 2003). However, the role of dewlap color during species recognition and speciation remains unclear (Nicholson et al. 2007; Vanhooydonck et al. 2009).

The aim of this study was to quantify dewlap color pattern variation from multiple populations of *Anolis apletophallus* using spectrophotometry and to use microsatellite markers to measure gene flow between different dewlap morphs and different populations. If there was reduced gene flow between individuals with different dewlap colors, this would be an indication of (at least partial) reproductive isolation between color pattern morphs. Alternatively, if there was little evidence of genetic structure between morphs, this would indicate that the dewlap color pattern variation was a polymorphism maintained within a single panmictic population.

Materials and Methods

Study System

The slender anole, *A. apletophallus* (formerly *Anolis limifrons*) is a small lizard inhabiting lowland tropical rainforest in Central and eastern Panama. Its ecology and life history have been well studied for over 30 years (e.g., Andrews 1976, 1991); most lizards do not live longer than a year

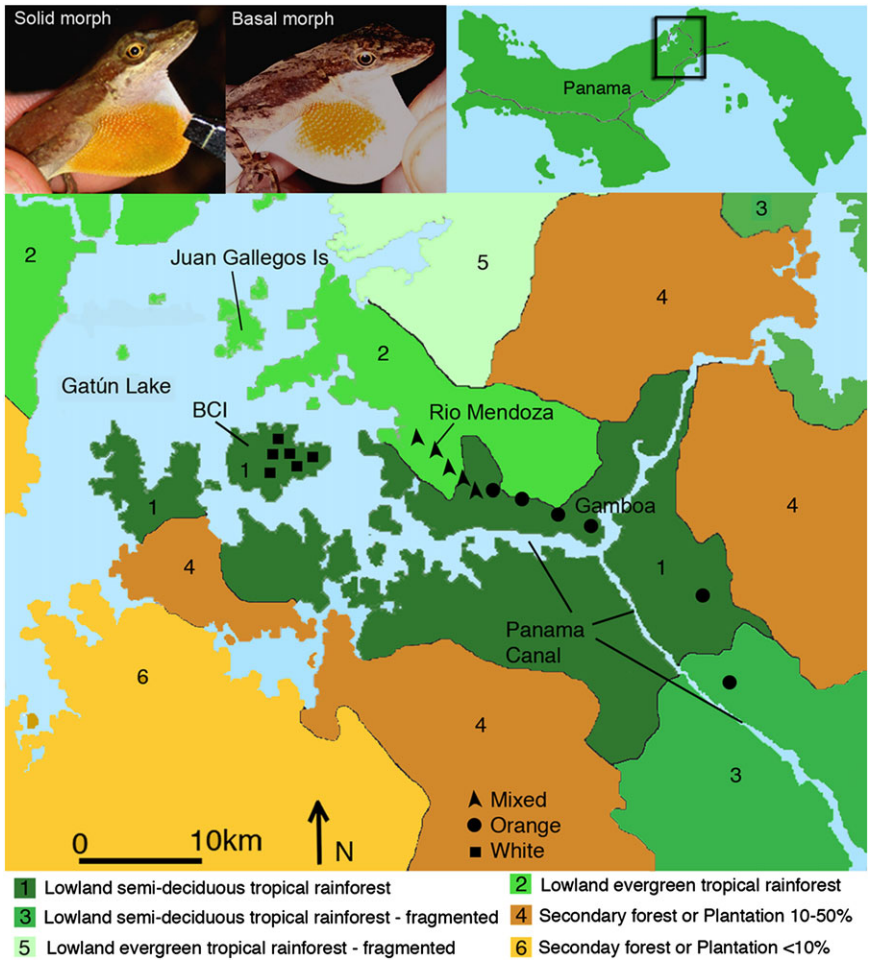

Figure 1. Sampling locations of *Anolis apletophallus* (closed symbols) overlaid on a vegetation map of central Panama. Distribution of vegetation types was obtained from the Panama Vegetation Map provided by the Herbarium of the Smithsonian Tropical Research Institute http://biogeodb.stri.si.edu/herbarium/article/vegetation+map+of+panama. Top left: photos of the 2 dewlap color morphs of *A. apletophallus*. Solid morph: dewlap is entirely orange. Basal morph: dewlap is white with an orange basal spot. Top right: Map of Panama.

(Andrews and Nichols 1990), they have limited dispersal (<20 m) (Andrews and Rand 1983) and females lack a dewlap.

Within *A. apletophallus* 2 morphs exist; herein referred to as the "solid" and "basal" morphs based on terminology in Nicholson et al. (2007). The dewlap of the solid morph is almost entirely orange. The dewlap of the basal morph has a white margin with a basal orange spot (see inset on Figure 1 for color photos). Sampling of more than 200 lizards from 16 sites in and around the Panama Canal by Brian Bock during 1985–1986 found most populations contained a single morph, with the exception of 2 sites which had almost equal proportions of both morphs; one was on the mainland near Rio Mendoza and one was an island in the canal (Juan Gallegos Island) (Figure 1). The distribution was confirmed and extended in 2005–2006 (J Stapley, unpublished data). In summary, the solid morph occurs to the east of the Panama Canal (designated "orange populations"), the basal morph occurs to the west of the Panama Canal and on at least one island in the Canal (designated "white populations"), and a region of overlap exists where both morphs occur at roughly equal frequencies to the east of the Panama Canal (designated "mixed populations") (Figure 1). From repeated sampling and breeding in captivity, it is apparent that these morphs represent a discrete trait; males have either one or the other type (no intermediate forms) and the color and/or pattern does not change during the lifetime of the adult male (once the dewlap is fully developed) or when animals are moved into captivity and experiencing different diets (Stapley J, unpublished data). Offspring born in captivity to wild-caught females (i.e., mated to males in the wild) from the single morph populations have offspring with dewlaps matching their population of origin. These observations suggest that the morph type is inherited but the exact mode of inheritance is unknown.

Sampling

Lizards were sampled from Soberania National Park (along Pipeline Road, around Gamboa and El Charco) and from Barro Colorado Island (BCI) within the Panama Canal (Figure 1) during 2005–2006. Sampling involved walking slowly through the forest and hand catching all lizards sighted. The sex and snout vent length of each lizard were recorded and a small amount of the tail was cut using sharp sterilized scissors and stored in 70% ethanol for later DNA extraction. Adult males were transferred into individual bags and brought back to the laboratory for measurements of dewlap color. Sites were visited on multiple occasions to record the morph frequencies and to sample more individuals. To ensure an individual was not recorded twice on subsequent visits to a site, only lizards with intact nonregenerating tails were sampled. DNA was extracted using the ammonium acetate method (modified from Bruford et al. 1998). Samples were collected under Collecting Permit number SE/A-50-05 and exported under the Export Permit number SEX/A-117-08 issued by Autoridad Nacional del Ambiente, Panama.

The sampling undertaken in this study covers a good proportion of the ideal habitat for *A. apletophallus* (Figure 1, Vegetation types 1–2) in this area. Across the region where samples were collected, the habitat is continuous lowland tropical rainforest, and the only barrier to the lizard's movement is the Panama Canal (completed in 1914) surrounding BCI. Outside of the protected area immediately adjacent to the Panama Canal (former Canal Zone), the rainforest is fragmented or has been cleared and replaced by secondary forest (Figure 1, Vegetation types 3–6) and is less suitable for lizards.

Color measurements

Dewlap color was measured with an SD2000 spectroradiometer with a pulsed Xeon (PX2) Lamp (Ocean Optics). Illumination and reflectance were measured at the same angle, 45° relative to the surface at a distance of 5 mm. Measurements were relative to an Ocean optics WS-1 diffuse white reflectance standard. Two different places on the dewlap were measured, one in the center of the dewlap, close to the lizard's body, and one at the outer margin of the dewlap (see lizard cartoon in Figure 2 for the location of measurements). For each center–margin measurement, 3 measurements were taken, each time removing and replacing the probe. To calculate reflectance spectra for the 2 dewlap regions, for each male, the mean of the 3 repeated measurements was calculated and the spectra were averaged every 5 nm by fitting a smoothing function to the reflectance data. The "standardized reflectance spectra" was reflectance spectra standardized for brightness by dividing by the area under the curve (Leal and Fleishman 2004).

Analysis of color measurements

Principle components analysis (PCA) was used to compare between standardized reflectance spectra of basal and solid morphs from each population. The center and the margin were each analyzed separately in a PCA. Differences in first principle component (PC1) between morphs and populations were tested using a linear model, with morph/population category (solid/orange, solid/mixed, basal/white, basal/mixed) as the explanatory variable.

Markers and Genotyping

Ten polymorphic microsatellites (Acar2, Acar8, Acar9, Acar14, Acar17, Acar27, Acar28, Acar30, Acar36, and Acar47) were arranged into multiplexes and genotyped in 99 individuals (Supplementary Table S1). The development of markers is described elsewhere (Wordley et al. 2010). We used a total polymerase chain reaction (PCR) volume of 2 μl, which contained ~10 ng DNA, 1 μl of primer mix (containing 0.02 μM of the forward and reverse primer), 1× Qiagen Multiplex Master mix and 0.5× Q-Solution (as supplied in the Qiagen Multiplex kit) (Kenta et al. 2008; Frantz et al. 2009). This was run on a touchdown thermal cycler (Hybaid) with the following touchdown PCR program; 95 °C for 15 min; followed by 10 cycles of: 94 °C for 30 s, 62 °C for 30 s (decreasing by 1 °C each cycle to 52 °C), and 72 °C for 45 s; and this was followed by a single extension step of 60 °C for 30 min.

Analysis of Genetic Data

All markers were checked for null allele frequencies using FreeNA (Chapius and Estoup 2007). Two (Acar14 and Acar47) of the 10 markers had estimated null allele frequencies of >0.1 and were excluded from further analysis (Table 1). ARLEQUIN (v2.0 http://anthro.unige.ch/arlequin) was used to check that they were in Hardy–Weinberg and linkage equilibrium. An excess of heterozygotes in the mixed population may be evidence of admixture between divergent populations, whereas a deficit of heterozygotes would be evidence of genetic structure, assortative mating, or reproductive isolation, so to test for heterozygote excess, the score test (*U* test) implemented in GENEPOP 4.0 (Raymond and Rousset 1995) was used. GENEPOP 4.0 was also used to calculate F_{ST} and test for population differentiation (Raymond and Rousset 1995).

We used STRUCTURE (v2.3.1) (http://pritch.bsd.uchicago.edu) to infer the number of populations based on 8 microsatellite loci. STRUCTURE was first run with no prior information regarding spatial location and then the program was implemented using new models developed by Hubisz (2009), which use location information to assist the clustering algorithm. This can be useful when there is some population structure (e.g., significant F_{ST} between locations), but the standard STRUCTURE models do not detect it, for example, due to limited data (small sample size, few markers) or because of very weak structure (Hubisz et al. 2009). STRUCTURE was run with default settings, with 100 000 MCMC reps, a burn in length of 20 000. The admixture model was used with the correlated allele frequencies option. Likelihood estimates were calculated for 1–6 populations (*K*) to ensure that likelihoods were

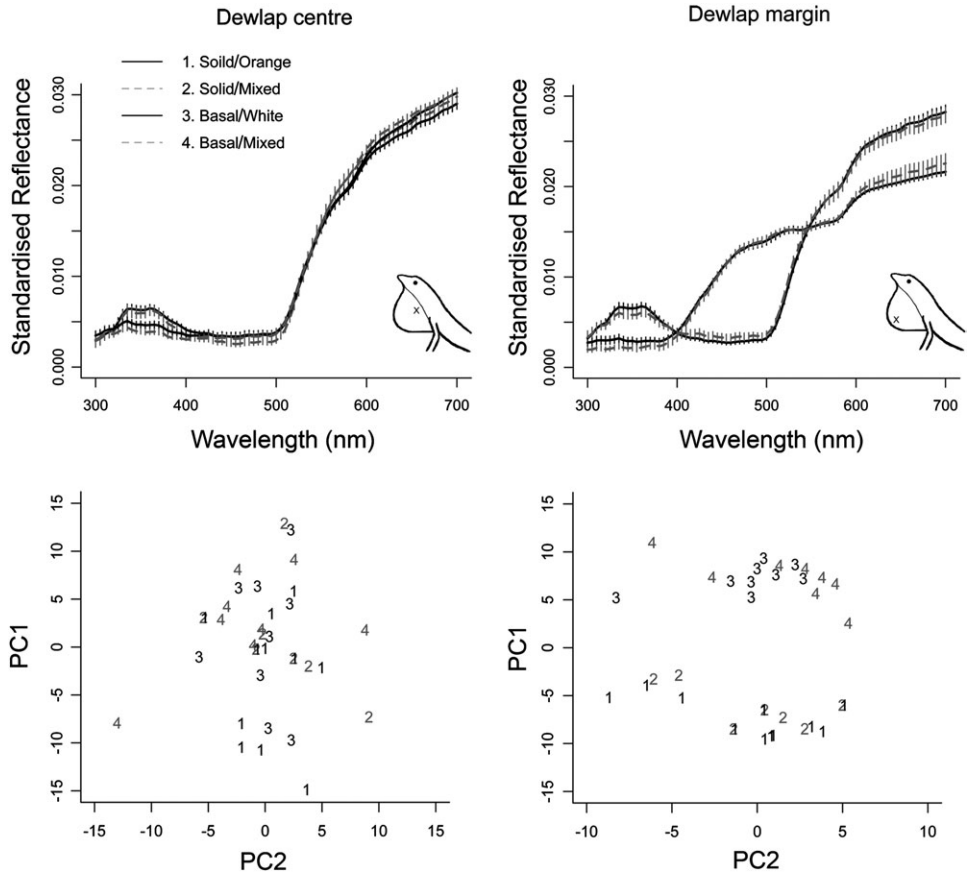

Figure 2. Reflectance spectra (upper panels) and plots of the first and second principle components (PC1, PC2) (lower panels) from the center and the margin of male dewlaps (cartoon of lizard shows where measurements were taken). Males of the solid morph have a completely orange dewlap, and males of the basal morph have a white dewlap with an orange basal spot. The reflectance spectra from the center of the dewlap does not differ between morphs or between males from white, orange, or mixed populations. The reflectance spectra from the margin of the dewlap does differ between basal and solid morphs but not between populations within morphs.

estimated for a range of *K* values spanning the predicted value of *K*. Each run was replicated 10 times to verify that the estimates were consistent across runs (see Documentation for Structure software: version 2.3). Previous studies have suggested that an indicator of the true number of populations is given by the change in the likelihood between models with different values of *K* (as an alternative to using the likelihood of each value of K) (Evanno et al. 2005).

Table 1 Summary statistics from microsatellite genotyping of 99 *Anolis apletophallus* individuals

Microsatellite	Number of alleles	Number of individuals	Observed heterozygosity	Expected heterozygosity	Null allele frequency
Acar2	8	98	0.67	0.69	0.00
Acar8	17	88	0.67	0.75	0.01
Acar9	17	94	0.95	0.90	0.00
Acar14[a]	3	99	0.28	0.40	0.26
Acar17	3	90	0.22	0.34	0.00
Acar27	12	95	0.53	0.77	0.08
Acar28	18	84	0.79	0.79	0.00
Acar30	22	94	0.83	0.85	0.00
Acar36	4	87	0.56	0.67	0.07
Acar47[a]	29	59	0.25	0.87	0.10

[a] Excluded from analysis.

However, this method is not appropriate in cases where the true K is 1 because change in likelihood between K and $K - 1$ cannot be estimated. Therefore, a graphical representation of the likelihood and its standard error were used to examine the results. STRUCTURE HARVESTER (http://users.soe.ucsc.edu/~dearl/software/struct_harvest/) was used to extract the relevant information from the STRUCTURE results files.

Results

Sampling and Distribution of Morphs

A total of 119 adult lizards were caught (Figure 1; Supplementary Table S1), and DNA samples were extracted from 99 of them (47 from the orange, 40 from the mixed, and 12 from the white populations). Among the DNA-sampled individuals, there were 27 basal and 39 solid dewlapped males. Only basal morphs ($n = 8$) were observed in the white populations and only solid morphs ($n = 26$) were observed in the orange populations. Of the 31 males sampled from the mixed populations, 14 had solid dewlap and 17 had a basal dewlap. Although relatively few males were sampled from the white population, we are confident that the solid morph is absent from BCI because this morph has never been observed there in over 30 years of extensive sampling (Andrews R, Jaramillo C, Rand S, personal communications).

Dewlap Color Pattern

The standardized reflectance spectra of the center of the dewlap was characteristic orange, the reflectance curves did not differ between solid and basal dewlapped males and there was no difference between the 3 populations (Figure 2). The margin of the dewlap, however, did differ between basal and solid morphs. The standardized reflectance spectra of the dewlap margin were clearly differentiated between the 2 morphs, and no differences are detected between populations (Figure 2). When the first and second principle components (PC1, PC2) of the dewlap margin are plotted against each other, the values form distinct clusters corresponding to the dewlap morph (Figure 2). PC1 from the center of the dewlap between the 4 morph/population categories (solid/orange, solid/mixed, basal/white, and basal/mixed) did not differ ($F_{1,31} = 1.42$, $P = 0.25$). In contrast, PC1 from the margin of the dewlap did differ between morphs ($F_{1,31} = 427.55$, $P < 0.001$) but not between populations within morphs (solid morph $F_{1,16} = 1.33$, $P = 0.26$; basal morph $F_{1,16} = 0.008$, $P = 0.92$; means: solid/orange = −7.28, solid/mixed = −6.11, basal/white = 7.26, basal/mixed = 7.18). In summary, the dewlap margin color differed between the morphs, but within a morph, there was no difference in color (margin or center) between populations.

Population Genetics

There was little genetic differentiation between the 3 populations of *A. apletophallus*. F_{ST} values between populations were low although significant pairwise genetic differentiation was detected between the white versus mixed populations and the white versus orange populations (Table 2a). No differences were detected between the orange and the mixed populations, which can be explained by the fact that there are no geographic barriers separating these populations.

Table 2 Summary of pairwise population genetic differentiation across 8 loci between a) the 3 populations of *Anolis apletophallus* and b) comparisons between morphs (basal/solid) and populations (white, orange, and mixed) using males only

	F_{ST}	Chi square	df	P value
a)				
Mixed versus orange	0.005	19.37	16	0.24
Mixed versus white	0.031	46.51	16	<0.001
Orange versus white	0.041	45.09	16	<0.001
b)				
Basal versus solid (all males)	−0.007	19.53	16	0.24
Basal/white versus basal/mixed	0.036	23.23	16	0.05
Basal/mixed versus solid/mixed	−0.010	15.17	16	0.51
Solid/orange versus solid/mixed	−0.005	18.58	16	0.29

df, degrees of freedom.

To test if genetic differentiation exists between different morphs, we restricted analysis to males only, ignored population information, and compared between all basal and solid males. Using this data set, we found no evidence of genetic differentiation between basal and solid morphs (Table 2b). We also compared the basal and solid males from the mixed population only and again found no evidence of genetic differentiation (Table 2b). Comparison between basal males from the white and mixed populations identified a marginal degree of differentiation, and no differentiation was detected between solid males from the mixed and orange populations. Taken together, these comparisons suggest that there is no evidence of reduced gene flow between the 2 dewlap color pattern morphs, and the small amount of genetic differentiation present between populations is related to the current day geographic barrier (Panama Canal).

Heterozygote excess can be indicative of recent admixture while a deficit is consistent with genetic structure. We found no evidence of departures from Hardy–Weinberg equilibrium across all populations combined ($P = 1.0$), or in the 3 populations ("mixed" $P = 0.99$, "orange" $P = 1.0$, "white" $P = 1.0$), or when males were used and split into 2 populations based on dewlap color pattern (basal $P = 1.0$, solid $P = 0.98$). The inferred number of populations from STRUCTURE was one; both runs of STRUCTURE with and without prior location information gave the same results (Figure 3). In summary, there is no evidence that populations of individuals with different dewlap morphs are genetically differentiated or that recent admixture between 2 genetically divergent morphs has occurred.

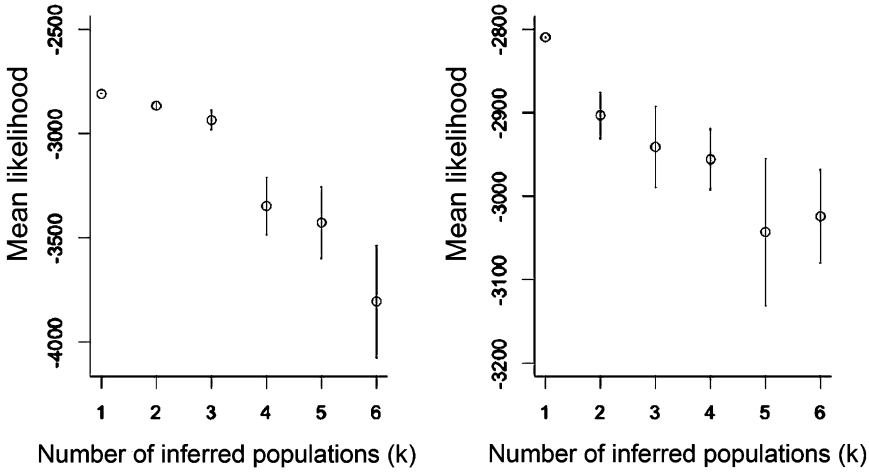

Figure 3. The mean likelihood ($L(K)$) ± standard deviation of STRUCTURE runs that simulate different numbers of populations (K) ranging from 1 to 6. Results are presented for STRUCTURE runs without (left panel) and with (right panel) prior information about population of origin. In both cases, the most likely number of populations is one.

Discussion

The aim of this study was to quantify the dewlap color pattern variation and then test whether it was likely to be an indicator of reproductive isolation between different morphs or whether it appeared to be maintained as a polymorphism within a single panmictic population. We identified a color difference in the outer margin of the dewlap between morphs, but males of the same morph did not vary in dewlap color pattern between populations. We found little genetic differentiation between morphs. The genetic differentiation that was present corresponded to a present day geographic barrier, the Panama Canal. This study represents one of only a few studies in *Anolis* (Webster and Burns 1973; Thorpe and Stenson 2003) to test whether dewlap variation is related to population divergence. Our results suggest that dewlap morph is not an indicator of reproductive isolation in this species, which contrasts with the widely held belief that dewlap color or pattern is an important factor in reproductive isolation and speciation in anoles (Losos 2009).

Although dewlap color and pattern have often been implicated in diversification and speciation in anoles (Losos and Schneider 2009), their role in reproductive isolation has rarely been tested. One of the earliest tests, in *A. brevirostris*, found that discontinuity in dewlap color did correspond closely with genetic differentiation as measured with 6 allozymes (Webster and Burns 1973). Another more recent study on *Anolis roquet* found genetic differentiation corresponding to geographic structure, but variation in dewlap color was associated with a certain habitat type and did not necessarily correspond to the genetic structure found (Thorpe and Stenson 2003).

Local adaptation appears to be the key driving force in anole speciation, and there is good support for the hypothesis that dewlap color/pattern is locally adapted to different light environments (Leal and Fleishman 2002, 2004). In the case of *A. cristatellus* and *A. cooki*, both dewlap color and spectral sensitivity are locally adapted to different light environments to maximize detectability for signaling (Leal and Fleishman 2002). Although there are little obvious differences in habitat across the species range of *A. apletophallus*, subtle differences in habitat light may exist and this could be a good avenue for future research.

The results presented here provide evidence of the presence of a dewlap color pattern polymorphism in *A. apletophallus* with no evidence of reduced gene flow that would indicate reproductive isolation between morphs. This study system presents a good opportunity to study the evolution and maintenance of dewlap polymorphisms in *Anolis*, which could provide insight into how selection acts on dewlap color pattern and the role of dewlap color pattern in adaptive radiations.

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

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