Are subspecies of *Anolis* lizards that differ in dewlap color and pattern also genetically distinct? A mitochondrial analysis

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ABSTRACT

Subspecies of *Anolis* lizards are often defined on the basis of geographic variation in the color and pattern of the dewlap, an extensible throat fan considered central to species recognition and sexual selection. Among the most impressive examples of this phenomenon are two species of trunk anoles found across Hispaniola and the Bahamas: *Anolis distichus* is divided into 16 subspecies with dewlap colors ranging from deep wine red to pale yellow while *Anolis brevirostris* is divided into three subspecies with dewlaps ranging from pale yellow to orange. Limited sampling of allozyme data indicates some genetic divergence among subspecies and suggests that they may deserve recognition at the species-level. Our goal here is to use more comprehensive geographic sampling of mtDNA haplotypes to test whether the five subspecies of *A. distichus* and three subspecies of *A. brevirostris* that occur in the Dominican Republic correspond with genetically distinct populations that may warrant recognition under the general lineage concept. We obtain an aligned dataset of 1462 bp comprised of the genes encoding ND2 and adjacent tRNAs from 76 individuals of *A. distichus* from 28 localities and 12 individuals of *A. brevirostris* from five localities. We find that haplotypes sampled from each Dominican subspecies of *A. distichus* form well-supported and deeply divergent clades (>10% uncorrected sequence divergence). Strong concordance between mtDNA haplotype structure and previously diagnosed phenotypic variation in traits central to interspecific communication (i.e., the dewlap) leads us to hypothesize that each of the presently recognized Dominican subspecies of *A. distichus* and *A. brevirostris* deserves elevation to full species status under the general lineage concept.

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1. Introduction

Evolutionary biologists traditionally used the subspecies category to diagnose geographically distinct populations that were thought to be in the early stages of speciation (Mayr, 1963). Whether subspecies carry any such evolutionary significance, however, has been debated for decades (Cronin, 2006; Wilson and Brown, 1953). One particularly serious challenge stems from the finding that phenotypically-defined subspecies sometimes do not correspond with boundaries between genetically distinct lineages identified by molecular markers (Burbrink et al., 2000; Zink, 2004). This discordance may result from subspecific diagnoses based on phenotypic traits that more accurately reflect adaptation to environmental variation than they do overall patterns of genetic differentiation or reproductive isolation (Burbrink et al., 2000; Pavlova et al., 2005; Pyron and Burbrink, 2009). While populations diagnosed in this manner may provide insight into local adaptation and other evolutionary processes, their continued recognition as distinct taxonomic entities cannot be justified. In other cases, however, phenotypically-defined subspecies do correspond with

the type of genetic differentiation expected among reproductively isolated populations and may warrant elevation to full species status (Burbrink, 2002; Pyron and Burbrink, 2009; Torres-Perez et al., 2009; Zink et al., 2009). This type of concordance seems particularly likely when the traits used to diagnose subspecies are directly involved in reproductive isolation.

In *Anolis* lizards, subspecies are often diagnosed by the color and pattern of the dewlap, an extensible throat fan that is considered essential to sexual selection and species recognition (Schwartz, 1968; Underwood and Williams, 1959; Williams, 1965). Two closely related species of Dominican trunk anoles – *Anolis brevirostris* and *Anolis distichus* – are among the most remarkable examples of this phenomenon (Fig. 1) (Arnold, 1980; Schwartz, 1968). *A. distichus* is presently divided into 16 subspecies characterized by dewlap colors that range from very pale yellow to deep wine red, whereas *A. brevirostris* is divided into three subspecies with dewlaps that vary from pale yellow to orange. Previous molecular genetic studies, however, suggest that both *A. distichus* and *A. brevirostris* may contain multiple genetically distinct populations that deserve recognition at the species-level. More than three decades ago, for example, Preston Webster and colleagues (Webster, 1977a; Webster and Burns, 1973; Webster et al., 1973) used allozymes to show that parapatrically distributed Haitian populations of *A. brevirostris* with different

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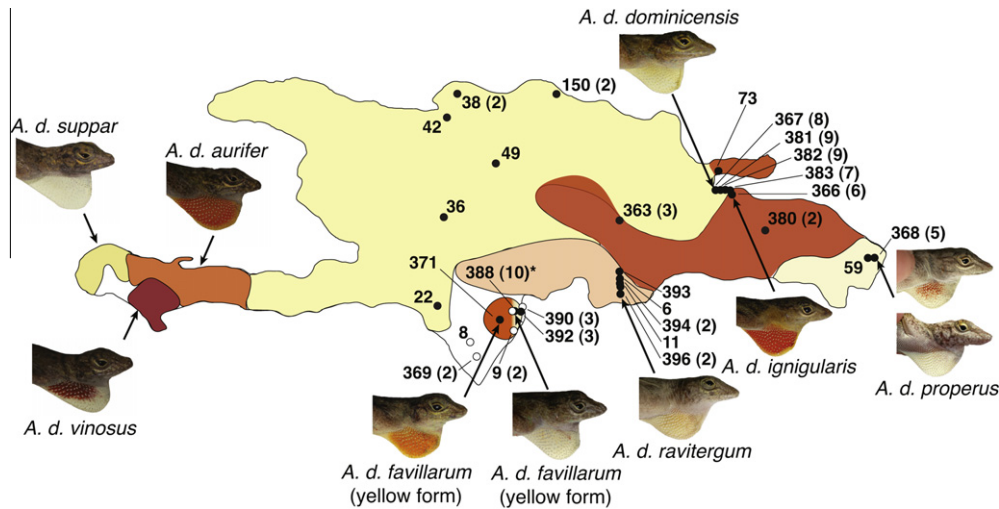 *E-mail address:* rglor@ur.rochester.edu (R.E. Glor).

Fig. 1. Sampling for this study. Circles indicate localities sampled, with associated labels indicating the locality number and number of individuals sampled (in parentheses for sample sizes exceeding one individual). Shaded regions indicate the ranges of each subspecies of *A. distichus*, with colors corresponding with the typical dewlap color exhibited by each form. Shaded regions were defined on the basis of the map and locality information in Schwartz (1968) as well as more recent field observations by REG. Photos are exemplars of dewlap color and pattern for each subspecies, with arrows indicating the locality from which the individual photographed was sampled.

dewlap colors and patterns were strongly genetically differentiated while exhibiting little or no hybridization where their ranges came into contact. This discovery ultimately led to the recognition of Haitian populations of *A. brevirostris* (*sensu lato*) as a complex of four species (*A. brevirostris, Anolis caudalis, Anolis marron*, and *Anolis websteri*) (Arnold, 1980; Powell et al., 1996; Webster, 1977a; Webster and Burns, 1973). The decision to distinguish these species was later reinforced by the finding that they exhibit strongly divergent display repertoires and mating behaviors (Jenssen, 1996; Jenssen and Gladson, 1984).

Comprehensive molecular and behavioral studies have yet to be conducted on populations of *A. distichus*, a species that exhibits even more impressive variation than *A. brevirostris* ever did. Nevertheless, the available allozymic data suggest that some of the existing subspecies of *A. distichus* are genetically distinct. First, these allozyme studies recover genetic divergences among Hispaniolan subspecies of *A. distichus* that rival the level of divergence seen between *A. distichus* and *A. brevirostris*, and among the Haitian forms related to *A. brevirostris* (Nei's *D* values of approximately 0.8) (Case, 1990; Webster, 1977b). Second, allozyme analyses of *Anolis distichus ignigularis* (orange dewlap) and *Anolis distichus dominicensis* (yellow dewlap) in central Hispaniola recover two loci that reliably distinguish these subspecies across much of their ranges in spite of gene flow along a relatively narrow contact zone (Case and Williams, 1984).

Our goal here is to use a mitochondrial phylogeographic analysis of the five Dominican subspecies of *A. distichus* to test the hypothesis that these populations are genetically distinct and deserving of recognition as species under the general lineage concept (de Queiroz, 1998, 1999) and associated operational species-delimitation criteria (e.g., Wiens and Penkrot, 2002). This hypothesis specifically predicts that each subspecies will correspond with distinct, well-supported, and deeply divergent mtDNA haplotype clades. We also use more limited sampling to address the status of two Dominican subspecies of *A. brevirostris*.

2. Materials and methods

2.1. Sampling

The *distichus* species group is composed of six species: *Anolis altavalensis, A. brevirostris, A. caudalis, A. distichus, A. marron*, and

A. websteri. With the exception of *A. altavalensis*, which is endemic to the Hispaniolan satellite island of Alta Velo, and several subspecies of *A. distichus* that are endemic to other Hispaniolan satellite islands and the Bahamas, this diversity is restricted to mainland Hispaniola (Schwartz and Henderson, 1991). Previous studies suggest that the *distichus* species group is closely allied with two other clades: the *cristatellus* series, a clade of 13 species found on Puerto Rico, its satellites on the Puerto Rico bank, the southern Bahamas, and St. Croix in the northern Lesser Antilles (Brandley and DeQueiroz, 2004) and the *bimaculatus* series, a clade of 13 species from the northern Lesser Antilles (Stenson et al., 2004). We included a single representative of the *cristatellus* series (*Anolis cristatellus*) and *bimaculatus* series (*Anolis marmoratus*), as well as two more distantly related anole species (*Anolis occultus* and *Anolis punctatus*) as outgroups in our analyses. Sampling of the *distichus* species group comprised 91 individuals, including one individual from each of the three species endemic to Haiti (*A. marron, A. caudalis*, and *A. websteri*) and broad geographic sampling from Dominican populations of *A. brevirostris* and *A. distichus* (Fig. 1, Table 1, and Appendix A). Populations were assigned subspecific status based on location and phenotype. Populations located where two subspecies come into contact with males characterized by intermediate dewlap color and pattern were classified as putative hybrids.

2.2. Molecular methods

We amplified and sequenced a ~1200 bp fragment of mtDNA that included complete sequence for the genes encoding ND2, tRNA^{Trp} and tRNA^{Ala}. This entire region was amplified using primers located in tRNA^{Met} (L4437 from Macey et al. (1997)) and tRNA^{Asn} (H5934 from Glor et al. (2004)). Our PCR protocol involved initial denaturation at 95 °C for 180 s followed by 30 cycles of 95 °C for 35 s, 53 °C for 35 s, and 72 °C for 150 s. Each of our 25 µl PCR reactions included 1–2 µl of genomic DNA, 11.4 µl H$_2$O, 0.125 µl of Taq DNA polymerase (New England Biolabs), 2.5 µl M190G thermophilic DNA polymerase 10× buffer, 2.5 µl of 25 mM MgCl$_2$, 2.5 µl 0.5 mM dNTP solution, and 2.5 µl of each 2 µM primer mix. Successful PCR product was purified using the ExoSAP (USB Corp.) method. Sequencing reactions were conducted with purified PCR product using a standard Big Dye Terminator sequencing protocol (PerkinElmer). Product from Big Dye reactions was cleaned using the ExcelaPure™ UF PCR Purification System (Edge

Table 1
Sampling for this study and dewlap color and pattern typical of each taxon investigated.

Taxon	Localities sampled	Individuals sampled	Dewlap color and pattern
A. brevirostris	5	12	
A. b. deserticola	2	7	Pale yellow with orange blush of variable size
A. b. wetmorei	1	2	Orange fading to yellow margin
A. b. deserticola × *A. b. wetmorei*	2	3	Pale yellow with orange blush of variable size
A. distichus	28	76	
A. d. dominicensis	8	16	Geographically polymorphic; typically yellow, but often with varying degrees of orange at some localities in the Cordilla Central and northern coast of Hispaniola
A. d. favillarum	3	10	Geographically polymorphic; pale yellow along eastern slopes of Sierra de Bahoruco, orange in central Sierra de Bahoruco
A. d. ignigularis	6	23	Orange with narrow yellow margin
A. d. properus	2	6	Polymorphic at most localities, typically pale yellow with orange blush
A. d. ravitergum	3	5	Pale yellow or peach
A. d. dominicensis × *A. d. ignigularis*	4	14	Pale yellow with orange spot or blush
A d. ignigularis × *A. d. ravitergum*	2	2	Pale yellow with orange spot or blush

Biosystems, Gaithersburg, Maryland). Sequences were visualized on an ABI 3700 (Applied Biosystems, Foster City, California) at the University of Rochester Medical Center's Functional Genomics Center (http://fgc.urmc.rochester.edu/).

2.3. Phylogenetic analyses

We inferred relationships among mtDNA haplotype sequences using two methods: parsimony and Bayesian inference. Parsimony trees were reconstructed used PAUP* 4.0b10 (Swofford, 2002), with TBR branch swapping and 100 random taxon addition replicates. Because the presence of similar sequences causes endless swapping after the tree's core topology is established, we limited the time spent on heuristic searches to 1×10^8 rearrangements. Bootstrap resampling was implemented with 1000 replicates to provide support values for individual nodes.

To infer relationships under Bayesian inference we used MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001). We optimized several parameters prior to conducting our final analyses using the Metropolis-coupled Markov Chain Monte Carlo (MCMCMC) method implemented by MrBayes, including partitioning strategy, models of molecular evolution, length of MCMCMC analyses, and temperature of incrementally heated chains. We considered two alternative partitioning strategies: (1) a single partition and (2) a four-partition strategy that applied a single partition to each of the three codon positions of ND2 and a fourth partition for tRNA sequence.

We selected the most appropriate model of sequence evolution for each individual partition using the AIC scores generated by MrModeltest 1.1b (http://www.abc.se/~nylander/, see Posada and Crandall, 1998). To compare the two alternative partitioning strategies, we conducted four independent analyses of each strategy in MrBayes, each consisting of three heated chains and one cold chain run for 10 million generations with sampling every 10,000 generations. We optimized heating for each analysis to achieve the recommended swapping frequency of 10–70% (MrBayes manual, www.mrbayes.net). To ensure sampling of the posterior distribution, a burn-in period for all analyses was diagnosed in two ways: (1) by the average standard deviation of split frequencies between two MCMC analyses run independently, with levels below 0.01 being considered indicative of convergence (http://mrbayes.csit.fsu.edu/wiki/index.php/Tutorial), and (2) via direct visualization of split frequencies throughout the course of each analysis using the cumulative plotting feature of the on-line application AWTY (http://king2.scs.fsu.edu/CEBProjects/awty/awty_start.php), with burn-in being diagnosed as the point after which split

frequencies for the 20 most variable nodes have achieved stable values. To compare performance of the two partitioning strategies we calculated Bayes Factors from the harmonic mean likelihood scores calculated using the sump command in MrBayes (Brandley et al., 2005; Brown and Lemmon, 2007).

After selecting the optimal partitioning strategy, we conducted four analyses using the optimized parameters. We then used the sumt command in MrBayes to generate a consensus topology and associated posterior probabilities from a pooled post-burnin sampled from all four analyses (eight independent MCMC analyses in total).

3. Results

We obtain an aligned dataset of 1462 bp comprised of 95 unique haplotypes. Parisimony analyses of 592 parsimony informative characters produce >1000 trees of 2377 steps. AIC analyses conducted with the aid of MrModeltest 1.1b identified the GTR + I + Γ model as the most appropriate model for all of our data partitions. However, we employed the GTR + Γ model in our analyses due to the potential for problematic interaction between the Γ and I parameters (Yang, 2006). Bayesian analyses were conducted for 10 million generations, with a conservative burnin of five million generations that provided dense sampling of the posterior distribution. Comparison of alternative partitioning strategies strongly favored the four-partition model over the one partition model (Bayes factor >400). Because both parsimony and Bayesian methods yielded similar trees, we present only the Bayesian tree here with support from parsimony indicated where appropriate (Fig. 2). Topological conflict between Bayesian and parsimony trees is restricted to relatively recent nodes that are poorly supported in both analyses. Haplotypes sampled from the *brevirostris* species group (*A. brevirostris, A. caudalis, A. marron*, and *A. websteri*, sensu Arnold, 1980) as well as from within both *A. brevirostris* and *A. distichus* form well-supported clades (Fig. 2). We observe high levels of sequence divergence between the five previously recognized species in the *distichus* species group (often >10% uncorrected divergence, Table 2). Within *A. brevirostris*, haplotypes from *Anolis brevirostris wetmorei* and *Anolis brevirostris deserticola* form deeply divergent, well-supported sister clades (Fig. 2). Haplotypes from the putatively intermediate population form a third clade that is the sister taxon to the a clade composed of haplotypes from *A. b. wetmorei* and *A. b. deserticola* (Fig. 2).

Within *A. distichus*, haplotypes sampled from all five Dominican subspecies form deeply divergent and strongly-supported clades, with three exceptions (Fig. 2, and Table 2). The first exception
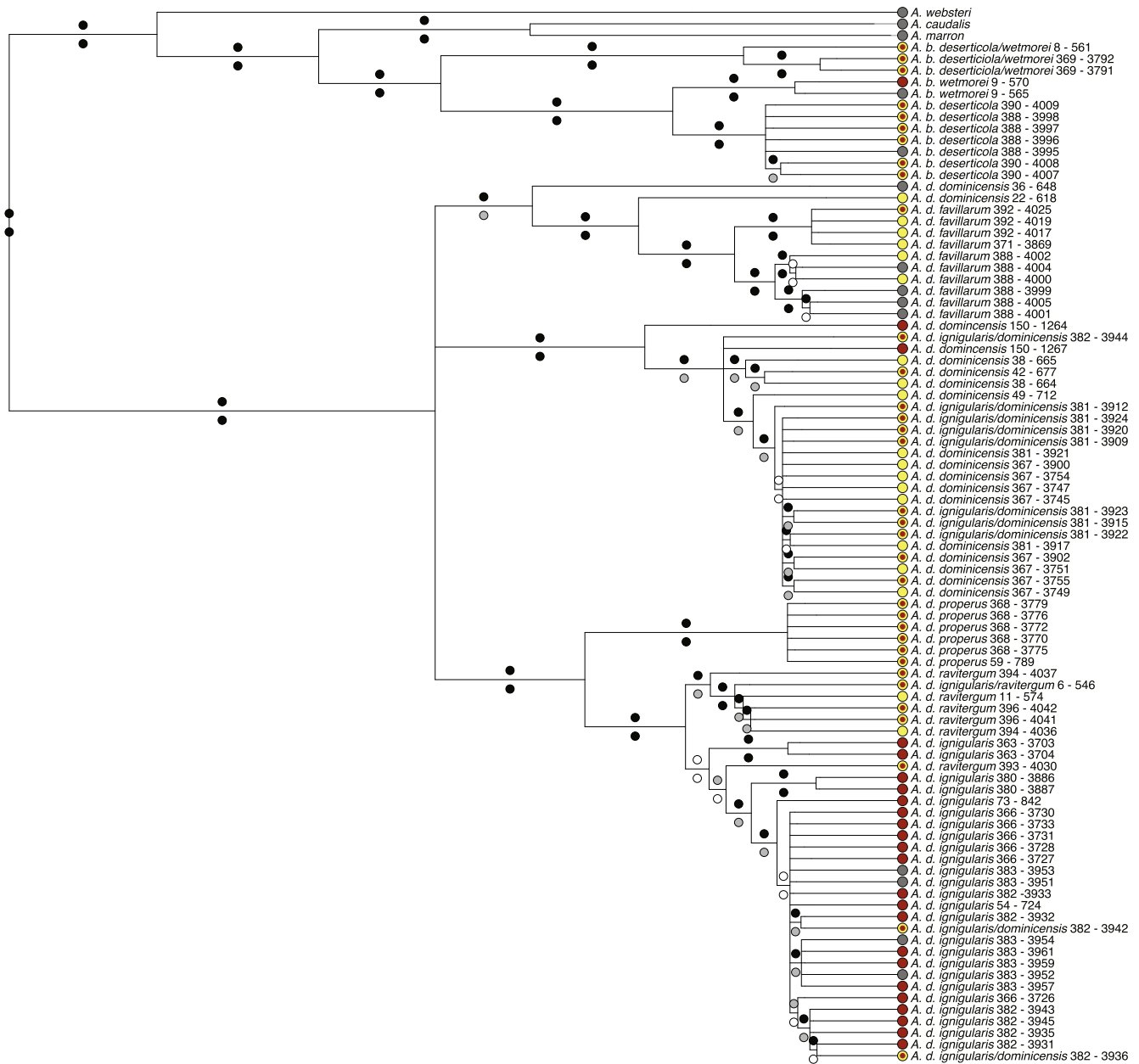
Fig. 2. Consensus phylogram obtained from the posterior distribution of four analyses run independently in MrBayes. Taxon labels include: subspecies classification, sampling locality, and specimen number. Circles at each terminal node represent dewlap color (gray = female [no dewlap], yellow = dewlap nearly completely yellow dewlap, yellow with orange spot in center = yellow dewlap with orange spot or blush, orange = dewlap nearly completely orange. Taxa in bold are from contact zones between two subspecies and are characterized by intermediate dewlap color and pattern (pale yellow dewlap with orange spot or blush [OY]). Circles above nodes indicate posterior probability (pp) values: black circles (pp ⩾ 0.95), gray circles (0.70 ⩽ pp < 0.95), white circles (pp < 70%). Circles below indicate support from parsimony bootstrap (bs) analyses: black circles (bs ⩾ 0.95), gray circles (0.70 ⩽ bs < 0.95), white circles (bs < 70%).

involves haplotypes from *A. d. dominicensis*, which are rendered paraphyletic by *Anolis distichus favillarum*. Two other possible exceptions involve possible instances of hybridization between subspecies. The first such example involves two populations from a contact zone between *A. d. ignigularis* and *A. d. dominicensis*, where individuals with intermediate dewlap phenotypes are characterized by haplotypes that group with others sampled from both *A. d. dominicensis* and *A. d. ignigularis*. A second possible example of hybridization among subspecies involves the single individual sampled from a contact zone between *A. d. ignigularis* and *Anolis distichus ravitergum*, which appears to have an intermediate dewlap while being characterized by a mtDNA haplotype that clusters with others sampled from *A. d. ignigularis*.

4. Discussions

Mitochondrial haplotypes sampled from *A. distichus* form well-supported clades that closely correspond with subspecific boundaries delimited primarily on the basis of dewlap color and pattern (Fig. 2). The haplotype clades associated with each subspecies are deeply genetically divergent, being differentiated by mean uncorrected levels of sequence divergence that often exceed 10% (Table 1). This degree of divergence suggests that evolutionary separation of most subspecies occurred millions of years ago, likely sometime during the Miocene based on a molecular clock calibration of 1.3% pairwise divergence per million years for a homologous mtDNA fragment in other lizard species (Macey et al., 1998)

Table 2
Genetic distances within and between species and subspecies included in this study. Mean uncorrected (*p*) distances are below diagonal; distances calculated using MEGA's Maximum Composite Likelihood model with rate variation among sites defined by the gamma shape parameter are above the diagonal. Mean maximum likelihood divergences within each species or subspecies are in the diagonal with bold text.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	Outgroups	**0.287**	0.85	0.907	0.832	0.826	0.812	0.859	0.862	0.849	0.91	0.904	0.9	0.868	0.913
2	*A. caudalis*	0.27	**NA**	0.169	0.289	0.198	0.206	0.239	0.361	0.354	0.349	0.351	0.337	0.360	0.348
3	*A. marron*	0.283	0.124	**NA**	0.290	0.221	0.244	0.245	0.377	0.355	0.388	0.405	0.374	0.381	0.387
4	*A. websteri*	0.269	0.168	0.170	**NA**	0.265	0.273	0.272	0.326	0.306	0.318	0.329	0.307	0.326	0.318
5	*A. b. deserticola*	0.268	0.135	0.145	0.162	**0.004**	0.053	0.162	0.371	0.34	0.375	0.376	0.358	0.375	0.366
6	*A. b. wetmorei*	0.266	0.14	0.157	0.166	0.048	**0.006**	0.163	0.374	0.344	0.379	0.368	0.358	0.377	0.372
7	*A. b. deserticola × A. b. wetmorei*	0.275	0.155	0.159	0.169	0.118	0.12	**0.029**	0.364	0.358	0.362	0.362	0.340	0.364	0.355
8	*A. d. dominicensis*	0.275	0.195	0.201	0.182	0.197	0.199	0.198	**0.041**	0.135	0.140	0.149	0.141	0.051	0.146
9	*A. d. favillarum*	0.274	0.193	0.194	0.175	0.188	0.189	0.197	0.102	**0.019**	0.141	0.153	0.147	0.141	0.151
10	*A. d. ignigularis*	0.284	0.194	0.206	0.180	0.201	0.203	0.201	0.106	0.107	**0.013**	0.084	0.032	0.119	0.035
11	*A. d. properus*	0.28	0.195	0.211	0.184	0.200	0.199	0.200	0.111	0.115	0.072	**0.004**	0.077	0.139	0.084
12	*A. d. ravitergum*	0.279	0.188	0.201	0.176	0.194	0.195	0.193	0.106	0.111	0.031	0.067	**0.006**	0.125	0.019
13	*A. d. ignigularis × A. d. dominicensis*	0.276	0.195	0.203	0.182	0.199	0.200	0.198	0.041	0.106	0.090	0.105	0.094	**0.04**	0.128
14	*A. d. ignigularis × A. d. ravitergum*	0.282	0.191	0.205	0.179	0.197	0.200	0.197	0.109	0.113	0.032	0.072	0.018	0.096	**0.031**

(internal calibrations are presently unavailable for anoles due to the absence of a fossil record for this group).

Concordance between strongly differentiated mtDNA haplotype clades and phenotypic variation supports the hypothesis that each of the five distinct Dominican subspecies of *A. distichus* deserves recognition at the species-level under the general lineage concept of species (de Queiroz, 1998, 1999) and associated operational species-delimitation criteria (Wiens and Penkrot, 2002). More limited sampling of Dominican populations of *A. brevirostris* also suggests that subspecies form well-supported and deeply divergent haplotype clades, but the position of haplotypes from putative hybrids as the sister clade to haplotypes sampled from existing subspecies suggests that more detailed sampling is required to interpret the taxonomic status of *A. brevirostris* (Fig. 2).

Although prevailing concordance with dewlap color variation suggests that mtDNA haplotype structure is not merely a consequence of the unique properties of this marker (Irwin, 2002), several exceptions to monophyly of mtDNA haplotypes deserve further consideration. One exception involves haplotypes sampled from populations of *A. dominicensis* along the western slopes of the Sierra de Bahoruco (locality 22) and the Valle de San Juan (locality 36), which are more closely related to haplotypes from *A. favillarum* than to haplotypes from other populations of *A. dominicensis*. Additional sampling from Haiti and the western Dominican Republic is required to determine whether *A. dominicensis* is composed of multiple distinct haplotype clades or if some populations presently recognized as *A. dominicensis* are better recognized as *A. favillarum* (the two species are difficult to distinguish morphologically and both are frequently characterized by pale yellow dewlaps).

Two other apparent exceptions to monophyly of subspecies may result from hybridization and resulting mtDNA introgression where representatives of two distinct lineages come into contact. The first such example involves two populations (localities 381 and 382) sampled along a coastal contact zone in Parque Nacional Los Haitises between *A. dominicensis* populations with dark orange dewlaps and *A. ignigularis* populations with pale yellow dewlaps. Males from these two populations are characterized by phenotypically intermediate dewlap color and pattern and were classified here as hybrids between the two forms (i.e., yellow with a basal orange blush or spot). These populations also include a mix of haplotypes sampled from both the *A. dominicensis*- and *A. ignigularis*-associated clades. These observations suggest that some degree of hybridization is occurring in the narrow (<2 km) contact zone between otherwise distinct populations of *A. dominicensis* and *A. ignigularis*. Previous allozyme based studies of another broader contact zone between *A. dominicensis* and *A. ignigularis* suggest a similar scenario, with evidence for genetic distinctness of the two populations accompanied by some degree of intergradation (Case and Williams, 1984).

The second example of potential hybridization involves single individuals sampled from localities 6 and 393, which occur at a contact zone between *A. ravitergum* with pale yellow dewlaps and *A. ignigularis* with dark orange dewlaps. The dewlaps of most males sampled from these populations are visibly intermediate (i.e., pale yellow with an orange spot of varying size and intensity) and were classified as putative hybrids. The one haplotype sampled from locality 6 groups with mtDNA haplotypes from other populations of *A. ravitergum*, whereas the haplotype sampled from locality 393 groups with other haplotypes sampled from *A. ignigularis*. To clarify the dynamics of these putative hybrid zones, studies of nuclear gene flow across contact zones between phenotypically distinct populations of *A. distichus* are required.

5. Conclusions

We recommend species-level recognition for each of the five phenotypically and genetically distinct populations previously recognized as Dominican subspecies of *A. distichus*: *A. dominicensis, A. favillarum, A. ignigularis, Anolis properus*, and *A. ravitergum*. We do so recognizing that more comprehensive geographic sampling and investigation of additional molecular markers are required to clarify hypothesized species boundaries and that reproductive isolation may be incomplete. Detailed ecological and behavioral studies of regions where these species come into contact will also be crucial to contextualizing observed genetic divergence.

Although some important questions remain unanswered, evolutionary, ecological, and behavioral studies that continue to treat *A. distichus* (*sensu lato*) as a single reproductively continuous unit will be problematic. Formal recognition of phenotypically and genetically distinct populations within *A. distichus* (*sensu lato*) is also important for effective conservation management. Although all of the species identified in the present study are locally abundant and in no imminent danger of extinction, they are also endemic to a single island, and some are restricted to extraordinarily small geographic areas (e.g., *A. favillarum*).

Although the species examined in this study appear to be one of few examples of anole hybridization along a zone of contact between parapatrically distributed populations (Glor et al., 2004), we suspect that many more such examples remain unrecognized due to strict application of the biological species concept by anole taxonomists. For decades, geographically distinct anole populations that undergo any hybridization were recognized at the subspecific level, or not at all (Heatwole, 1976; Schwartz, 1968; Underwood and Williams, 1959). These populations warrant renewed attention and consideration.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2010.11.004.

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