Using genomic data to revisit an early example of reproductive character displacement in Haitian Anolis lizards

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Abstract

The pattern of reproductive character displacement (RCD)—in which traits associated with reproductive isolation are more different where two species occur together than where they occur in isolation—is frequently attributed to reinforcement, a process during which natural selection acting against maladaptive mating events leads to enhanced prezygotic isolation between species or incipient species. One of the first studies of RCD to include molecular genetic data was described 40 years ago in a complex of Haitian trunk anole lizards using a small number of allozyme loci. In this example, Anolis caudalis appears to experience divergence in the color and pattern of an extensible throat fan, or dewlap, in areas of contact with closely related species at the northern and southern limits of its range. However, this case study has been largely overlooked for decades; meanwhile, explanations for geographic variation in dewlap color and pattern have focused primarily on adaptation to local signalling environments. We reinvestigate this example using amplified fragment length polymorphism (AFLP) genome scans, mtDNA sequence data, information on dewlap phenotypes and GIS data on environmental variation to test the hypothesis of RCD generated by reinforcement in Haitian trunk anoles. Together, our phenotypic and genetic results are consistent with RCD at the southern and northern limits of the range of A. caudalis. We evaluate the evidence for reinforcement as the explanation for RCD in Haitian trunk anoles, consider alternative explanations and provide suggestions for future work on the relationship between dewlap variation and speciation in Haitian trunk anoles.

Keywords: AFLP, character displacement, local adaptation, reinforcement, speciation

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Introduction

Natural selection can contribute to speciation in several ways, including divergent natural selection (i.e. ecological speciation sensu Schluter 2009; Nosil 2012), parallel natural selection (i.e. mutation order speciation; Schluter 2009), sexual selection (Lande 1981; West-Eberhard 1983) or via reinforcement (Howard 1993; Noor 1999). Until relatively recently, reinforcement—the process during which prezygotic isolating mechanisms are enhanced due to selection against maladaptive matings between species or incipient species—has attracted the most attention and debate (reviewed in Servedio & Noor 2003; Coyne & Orr 2004). Interest in reinforcement was initially driven by repeated observations of a pattern in nature known as reproductive character displacement, in which traits involved in prezygotic isolation between two species are more divergent where these species occur in sympathy than where each exists in allopatry (Brown & Wilson 1956; Howard 1993; Noor 1999). This pattern is consistent with one of the core predictions of reinforcement—that selection for species recognition should be strongest where recently diverged species come into contact.

Before proceeding, we note that the definitions we use for reproductive character displacement (as a pattern)
and reinforcement (as a process that may be responsible for this pattern) involve rejection of Butlin’s (1987) influential suggestion that these terms should instead be defined as distinct processes acting either before (reinforcement) or after (reproductive character displacement) the evolution of complete reproductive isolation (see also Coyne & Orr 2004; Pfennig & Pfennig 2012). We believe that defining these terms as processes acting at different stages of speciation is unnecessary and potentially confusing because the same process involving natural selection against maladaptive matings can lead to character displacement regardless of whether or not the populations involved are entirely reproductively isolated (see also Howard 1993; Rundle & Schluter 1998; Noor 1999; Servedio & Noor 2003; Price 2007; Hoskin & Higgs 2010; Hopkins et al. 2012; Vallin et al. 2012). Moreover, the use of terms that clearly distinguish pattern from process is critical because it is now widely recognized that patterns of reproductive character displacement can result from processes other than reinforcement (Grant 1972; Howard 1993; Noor 1995; Hoskin & Higgs 2010; Hopkins et al. 2012).

Several examples from nature provide support for reinforcement by showing that divergence in reproductive characters can evolve in situ when species come into contact, even in the face of some degree of ongoing gene flow (e.g. in Drosophila, Noor 1995; Higgie et al. 2000; Ficedula flycatchers, Saetre et al. 1997; Litoria tree frogs, Hoskin et al. 2005; and the wildflower Phlox drummondii, Hopkins et al. 2012). Although the phylogenetic scope of these detailed studies of natural populations is rather limited, comparative studies of reproductive isolation among multiple pairs of sympatrically and allopatrically distributed species subjected to laboratory hybridization experiments suggest that reinforcement may be a pervasive phenomenon in some taxa (within Drosophila, Coyne & Orr 1989, 1997; Yukilevich 2012 and the fungal genus Homobasidionymycota Le Gac & Giraud 2008), but perhaps not in others (the fungal genus Ascomycota, Le Gac & Giraud 2008). In order to quantify the prevalence of reinforcement in nature, it is first necessary to identify patterns that are consistent with its theoretical predictions, the most prominent of these being reproductive character displacement.

The pattern of reproductive character displacement has been reported in a diverse range of taxa (e.g. Saetre et al. 1997; Rundle & Schluter 1998; Marshal & Cooley 2000; Höbel & Gerhardt 2003). However, relatively few examples have been reported in squamate reptiles, a group that includes over 9000 species of lizards and snakes (but see Ferguson 1973; Gibbons 1979; Webster & Burns 1973 for potential examples from the historical literature). This absence of examples exists in spite of a diverse set of well-characterized prezygotic isolating mechanisms in squamates, including a range of auditory, visual and chemosensory cues (e.g. Fleishman et al. 1993; Hibbits et al. 2007; Whiting et al. 2009). Perhaps none of these mechanisms are more impressive than the dewlap—a colorful, extensible throat fan utilized by a range of iguanian lizards for communication (Pianka & Vitt 2003). Dewlaps are particularly diverse in anoles, a group that has repeatedly undergone adaptive radiation (reviewed in Losos 2009), and are thought to play a critical role in prezygotic isolation between anole species (Rand & Williams 1970; Crews 1975; Sigmund 1983; Losos 1985; Case 1990; Macedonia et al. 1994).

Several species of Hispaniolan trunk anoles (specifically, the brevirostris clade of the distichus species complex) exhibit patterns of geographic variation in dewlap color and display behaviour that have led to a hypothesis of reproductive character displacement driven by reinforcement (Webster & Burns 1973; Jenssen & Gladson 1984; Losos 2009). Webster & Burns (1973) reported an unusual distribution of dewlap color and pattern in trunk anoles found across Haiti’s central coast and Cul-de-Sac Plain (Fig. 1; at the time, all populations were recognized as belonging to a single species: Anolis brevirostris). Using surveys of allozymic variation, Webster & Burns (1973) found evidence that the trunk anoles along this transect could be assigned to three genetically distinct populations: (i) a northernmost population (later elevated to Anolis websteri, Arnold 1980) with uniformly bright orange dewlaps, (ii) a central population that has pale yellow dewlaps where its range abuts that of the northernmost population, but increasingly orange dewlaps as one moves southward, until the dewlap is almost entirely bright orange at the southern distribution limit of the population (later elevated to Anolis caudalis, Arnold 1980), and (iii) a disjunct population in the Cul-de-Sac plain with monochromatic, tan dewlaps (A. brevirostris).

Webster & Burns (1973) hypothesized that the pattern of dewlap variation seen in the centrally distributed population (A. caudalis) is reproductive character displacement caused by selection for enhanced species recognition at the contact zones with A. websteri in the north and A. brevirostris in the south. Aside from these dewlap color differences, the populations identified by Webster & Burns (1973) were morphologically quite similar; this observation was later corroborated by Arnold (1980), whose detailed morphological work on the group found that the populations diagnosed by Webster & Burns (1973) could only be distinguished by relatively subtle modal differences in scale counts. Nevertheless, these morphological differences in combination with the dewlap variation and genetic differences reported by Webster & Burns (1973) were viewed as sufficient to justify recognition of the three distinct
species along Webster & Burns’ (1973) central Haitian transect (Arnold 1980). Jenssen & Gladson (1984) later identified species-specific stereotypical behavioural repertoires in each of the three Haitian trunk anole species initially diagnosed by Webster & Burns (1973). Jenssen & Gladson (1984) noted that, as with dewlap color and pattern, species-specific behavioural displays appear to differ most where species come into contact. Together, the phenotypic, genetic and behavioural results reported by Webster & Burns (1973), Arnold (1980) and Jenssen & Gladson (1984) suggest reproductive character displacement (RCD) via reinforcement. Although each of the species diagnosed in these earlier studies is still recognized (Powell & Henderson 2012), nearly 30 years have passed without any additional molecular genetic work or surveys along Webster and Burns’ transect. One reason for this was the untimely passing of T. Preston Webster, who died in an automobile accident in 1975, leaving behind partially complete manuscripts on geographic genetic variation.
and reproductive isolation in trunk anoles that were ultimately published posthumously in a newsletter distributed by Webster’s advisor (Webster 1977a,b).

Meanwhile, explanations for how and why dewlaps diverge during speciation have shifted to emphasizing the role of ecological gradients (Fitch & Hillis 1984; Thorpe 2002; Thorpe & Stenson 2003; Leal & Fleishman 2004; Losos & Thorpe 2004; Thorpe & Losos 2004), including recent examples from other Hispaniolan trunk anoles in the *distichus* species complex (Ng & Glor 2011; Ng et al. 2013). This work suggests that anole populations living in different visual environments experience divergent selection on visual signals due to habitat-specific trade-offs in the efficacy of various signal designs (e.g. dewlap designs that are conspicuous in well-lit edge habitats dark forest may be inconspicuous in dark forest). This divergence could lead to the evolution of reproductive isolation among anole populations from different habitats (Boughman 2002; Leal & Fleishman 2004; Losos & Thorpe 2004; Thorpe & Losos 2004; Ng et al. 2013).

We reinvestigate Webster & Burns’ (1973) transect by resampling their original localities, quantifying geographic variation in dewlap color, sequencing mitochondrial DNA haplotypes and conducting nuclear genomic scans. Our first goal was to test whether the phenotypic pattern identified by Webster & Burns (1973) persists to the present day. Reaffirming the presence of dewlap variation along Webster & Burns’ (1973) transect was necessary given that (i) approximately 40 trunk anole generations have passed since Webster & Burns’ (1973) original study [assuming a 1-year generation time, which is likely an overestimate based on field studies of trunk anoles in the Bahamas (Schoener & Schoener 1982) and unpublished data on the life history of captive Dominican trunk anoles (A. J. Geneva & R. E. Glor, unpublished data)] and (ii) the entire transect is heavily disturbed and extends along major highways that present ample opportunities for anthropogenic dispersal. Our second goal was to use nuclear genome scans and mitochondrial sequences to test Webster & Burns’ (1973) hypothesis that three genetically distinct species exhibit phenotypic patterns consistent with reproductive character displacement, which was originally based on only six allozyme loci. Finally, we consider possible alternative explanations for the pattern of dewlap variation and provide recommendations for future study.

**Materials and methods**

**Sampling**

We collected specimens and tissue samples from a total of 137 individuals representing the four species in the *brevirostris* clade (Table S1, Supporting information; *Anolis brevirostris* n = 26, 5 localities; *Anolis websteri* n = 36, 2 localities; *Anolis caudalis* n = 63, 3 localities; *Anolis marron* n = 11, 4 localities). A single *Anolis distichus* sample was used as an out-group for phylogenetic inference. We focused on sampling *A. caudalis*, *A. websteri* and *A. brevirostris* from localities previously visited by Webster & Burns (1973), to test the hypothesis that *A. caudalis* exhibits RCD where its range meets the other two species. We obtained samples from an allopatric member of the *brevirostris* clade, *A. marron*, as well as samples of *A. brevirostris* from the Dominican Republic, in order to resolve all of the phylogenetic relationships for this small clade.

**Quantification and comparison of dewlap coloration**

Dewlaps were photographed by DLM with a digital SLR camera and a flash with uniform settings throughout the study. The dewlap was fully extended using forceps by the same investigator (REG) for all lizards. We scored dewlap coloration visually by quantifying the size of each dewlap’s basal orange patch relative to total dewlap area, using increments of 5% (individual scores are reported in Table S1, Supporting information). We tested for differences in dewlap coloration among all populations sampled from Webster’s original transect using Tukey’s honest significance test (from northwest to southeast, this transect included two populations of *A. websteri*, followed by three populations of *A. caudalis* and finally a single population of *A. brevirostris* from the Cul-de-Sac Plain of Haiti). For *A. caudalis*, the RCD hypothesis predicts that dewlap color should be less orange at the northern extent of its range than elsewhere, due to the proximity of orange-dewlapped *A. websteri*. The RCD hypothesis also predicts that *A. caudalis* dewlaps should be less pale at the southern end of its range than elsewhere, due to the proximity of pale-dewlapped *A. brevirostris*.

**Quantification of environmental variation**

To address the possibility that dewlap variation in the *brevirostris* species group is driven by environmental variation, we extracted climatic data from the WorldClim database (Hijmans et al. 2005) at a 1-km² resolution for 28 total geo-referenced localities from *A. websteri* (12 localities), *A. caudalis* (8 localities) and *A. brevirostris* (8 localities). We obtained geo-referenced localities using the HerpNet museum database (http://www.herpnet.org). We selected localities only from along Haiti’s central coast and the Cul-de-Sac Plain, but included localities that were not sampled by Webster & Burns (1973) or this study. We selected...
four abiotic variables (mean annual temperature, Bio1; annual precipitation, Bio12; temperature seasonality, Bio4, defined as the standard deviation of mean monthly temperatures * 100; and altitude) that broadly characterize variation in climate and habitat on Hispaniola and have been shown to influence dewlap variation in anoles belonging to the distinguish species group (Ng et al. 2013). Moreover, the type of climatic data included in our study is associated with variation in forest habitat and with differences in canopy cover and vegetation between mesic and xeric forest that are known to produce vastly different light environments (Endler 1993; Fleishman et al. 1997, 2009; Leal & Fleishman 2004). Nevertheless, we recognize that the climatic data are sampled at a coarser scale than that encountered by individual lizards on a day-to-day basis and that future sampling of local light environments will ultimately be required to test conclusions derived from work that relies on broadly sampled climatic variation. We plotted the data for each locality, ordered by latitude, in order to look for environmental patterns that could explain the observed dewlap variation (Fig. 4).

Mitochondrial DNA sequencing

We extracted total genomic DNA from tail tips or livers stored in 95% ethanol at -80 °C using a Wizard® SV Genomic DNA Purification System kit (Promega Corp.). We used previously published primers to amplify the mitochondrial locus NADH dehydrogenase subunit 2 [ND2, Macey et al. 1997 (Mef6.6; 5'-AAGCTT TCGGGCCCATACC-3'); Glor et al. 2003 (Asnr.REG1; 5'-AGCCGATAATGAGCCGCTGG-3')]. Total reaction volumes for each PCR were ~25 µL, including 11.4 µL diH2O, 2.5 µL each of forward and reverse primers (2 µM concentration), 2.5 µL 10× Taq reaction buffer (Mg2+ free), 2.5 µL MgSO4 (20 µM), 2.5 µL dNTP mix (5 µM), 0.125 µL DNA Taq polymerase (5 µ/µL) and 1 µL of genomic template DNA. We obtained dNTPs, Taq and 10× buffer from Bio Basic Inc. We conducted PCR using Eppendorf Mastercycler ep gradient thermocyclers using the following reaction conditions: 94 °C for 120 s followed by 30 cycles of 94 °C for 35 s, 52 °C for 35 s and 72 °C for 90 s, followed by a final extension at 72 °C for 10 min. We shipped PCR products to Beckman Coulter Genetics for purification using SPRI technology, and DNA sequencing in both directions using the Big Dye Terminator v3.1 system on an ABI PRISM 3730xl capillary sequencer. We assembled, inspected and edited sequences using Geneious v5.3 (Drummond et al. 2010). Because no insertions or deletions were detected, alignment was made by eye.

AFLP marker generation

We used a modified version of AFLP protocols from Vos et al. (1995) and Hazen et al. (2002). We obtained all restriction and ligation reagents from New England BioLabs, Inc. Specific reaction conditions for each step are found in the Supporting Information (available online). First, we digested genomic DNA using the restriction enzymes EcoRI and Msel. Second, we ligated double-stranded adaptors with single-stranded overhangs complementary to the single-stranded ends of the DNA fragments to create a set of fragments of varying size with common flanking sequences. In a subsequent set of nested PCR amplifications, we conducted preselective and selective amplification using primers complementary to the adaptors and restriction sites plus one or three additional bases, respectively. These additional bases reduced the total set of amplified fragments to yield a manageable number of fragments for scoring. We produced four nonoverlapping sets of loci by using four primer sets with different complements of additional bases.

By performing selective amplifications using fluorescently labelled primers, we were able to automate scoring of fragments via capillary electrophoresis (Table S2, Supporting Information). Selective amplification products were genotyped at the Functional Genomics Center at the University of Rochester Medical Center on an Applied Biosystems 3730 Genetic Analyzer with a LIZ500 size standard. For all analyses, we used duplexed reactions that pooled products from two primer pairs (one Hex-labelled and one FAM6-labelled).

AFLP scoring and error analysis

We first viewed and analysed AFLP electropherograms using PEAKSCANNER v1.0 (Applied Biosystems). Prior to exporting results, we employed light peak smoothing and left all other conditions at default settings. We binned and initially scored fragments using the R package RAWGENO (Arrigo et al. 2009); minimum bin size was set to 1 base pair (bp), maximum bin size to 2 bp, minimum fragment length to 50 bp and maximum fragment length equal to the maximum observed fragment length. The minimum peak height threshold was set to 100 relative florescence units (rfu) to remove low-intensity peaks resulting from instrument noise. We exported tables of raw peak heights from RAWGENO and converted the resulting tables using a custom R script (Supporting information) to produce an input format compatible with the R package AFLPScore (Whitlock et al. 2008). Using AFLPScore, we examined duplicate reactions from 14 randomly selected samples (>10% of the total number of samples, following Bonin et al. (2007)) to infer error rates via the mismatch error rate (as defined in Whitlock et al.)
2008) for each of the four primer pair combinations across a broad range of scoring parameters. We used relative phenotype-calling thresholds (a percentage of the mean peak height at a given locus, Whitlock et al. 2008) in all cases. After selecting scoring parameters for each primer pair, we used these parameters in AFLPScore to score all individuals and concatenated the results from the four primer pairs.

A recent study by Holland et al. (2008) suggests that conservative scoring parameters (i.e. those that minimize error rate) may negatively affect phylogenetic resolution for AFLP data sets because many informative characters are excluded. Overly conservative scoring parameters may also remove information about population structure (Zhang & Hare 2012). With this in mind, we used two sets of scoring parameters: one that maximized the number of loci generated while remaining under a total mismatch error rate of 10% (‘semi-strict’ scoring conditions) and another that minimized error rates in the replicated samples (‘strict’ scoring conditions; Table S4, Supporting information). The mismatch error rates from the semi-strict scoring conditions are lower than those reported by Holland et al. (2008) under their optimized scoring conditions for phylogenetic resolution, but higher than other published error rates from studies using semi-automated AFLP scoring (2–5%, although many studies do not report genotyping error, Bonin et al. 2004). Some increase in error rate is expected when using fully automated scoring, which has the benefit of eliminating the subjectivity of manual scoring (Holland et al. 2008; Arrigo et al. 2009). Additionally, our study focuses on population-level processes, and thus, a higher error rate should influence our results and interpretation to a lesser degree than individual-level analyses (e.g. parentage analyses) (Bonin et al. 2004).

Population structure analysis

We inferred population structure from the AFLP data set using the Bayesian Markov chain Monte Carlo (MCMC) clustering algorithm implemented in the program STRUCTURE v.2.3.3 (Pritchard et al. 2000). We used STRUCTURE to assign individuals to discrete genetic clusters (K), employing the admixture and correlated allele frequency models (Falush et al. 2003) and, because AFLP markers are dominant, the recessive alleles model (Falush et al. 2007). For all STRUCTURE analyses, we employed 10⁵ generations of burn-in followed by 10⁶ generations of sampling, checking model likelihoods throughout each analysis to ensure that predetermined burn-in values exceeded the number of generations required for likelihoods to reach a stationary distribution.

We used the ad hoc metric Δ(K) (Evanno et al. 2005) calculated using STRUCTURE HARVESTER (Earl & vonHoldt 2011) to identify the appropriate number of clusters. When hierarchical population structure exists in a data set, the Δ(K) method will detect the uppermost level of population structure (Evanno et al. 2005; Coulon et al. 2008). To identify hierarchical population substructure missed by the Δ(K) method, we employed iterative rounds of STRUCTURE analyses following Coulon et al. (2008). Starting with the entire data set, we sequentially analysed clusters identified using Δ(K) until likelihood values recovered an optimal K of 1 (i.e. no evidence for substructure) or until the clusters identified were no longer geographically interpretable. We assigned individuals to clusters based on a simple majority of inferred ancestry (>0.5). For these analyses, we completed five iterations at each level of K from one to five (or the maximum number of species-collection sites represented, whichever was greater). Following this, the entire data set was analysed using 100 iterations with K equal to the total number of single clusters identified using the hierarchical analyses.

We estimated genetic diversity (Hₑ) and pairwise Fₛₜ for each of the clusters identified in STRUCTURE using AFLP-SERV v1.0 (Vekemans et al. 2002). We employed 5000 iterations of the Bayesian method with a nonuniform prior distribution of allele frequencies from Zhivotovsky (1999) to estimate Fₛₜ with an assumption of Hardy–Weinberg equilibrium [tests of the HWE assumption are not possible using AFLP data alone (Yan et al. 1999)].

Phylogenetic reconstruction

We inferred phylogenetic relationships using the Metropolis-coupled MCMC algorithm implemented in MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003; Altekar et al. 2004). We inferred relationships from three data sets: (i) mtDNA sequence data alone, (ii) AFLP genotypes alone and (iii) all data combined. For combined analyses, AFLP genotypes and mtDNA sequences were analysed in separate partitions (MrBayes cannot analyse multiple data types in a single partition). For the AFLP partition, we employed the restriction site model as well as the ‘no absence sites’ parameter to correct for a bias present in AFLP data sets (no locus can have all ‘absence’ phenotypes). To determine the optimal partitioning strategy for the mtDNA data matrix, we used likelihood ratio tests to compare harmonic mean likelihoods (generated using the sump command in MrBayes) generated by three alternative partitioning strategies (Table S3, Supporting information) following the methodology of Brandley et al. (2005). To determine the best-fitting model of molecular evolution for each partition of the mtDNA data matrix, we used the Akaike Information Criteria (AIC) implemented in
Each MrBayes analysis consisted of two independent runs, each with four MCMC chains. We assessed convergence and determined appropriate burn-in for MrBayes analyses using the software TRACER v1.5 (Rambaut & Drummond 2007) and the web utility ARE WE THERE YET? (AWTY, Wilgenbusch et al. 2004). Using TRACER, we examined plots of likelihood and other parameters to assess consistency between independent runs and to determine whether our sample included sufficiently large (>100) effective sample sizes. We used the ‘cumulative’ function in AWTY to examine the posterior probability of the 20 most variable splits (bifurcations in the tree) in each run at 10 regular intervals, and defined burn-in as the point at which posterior probabilities of the splits reach stationarity. We examined graphs of the average standard deviation of split frequencies (ASDSF) between the two independent runs of each analysis to evaluate congruence of the trees generated from each independent run. Following the recommendations of the MrBayes manual, analyses were considered converged when ASDSF values reached <0.01. We also used the ‘compare’ function in AWTY to graphically compare the posterior probabilities of all splits between independent runs. In all cases, the first 25% of the samples were removed as burn-in prior to summarizing the results.

We also estimated a species tree topology directly from the AFLP data matrix using a newly developed likelihood algorithm implemented in the MCMC sampler SNAPP (Bryant et al. 2012), an add-on to the program BEAST v2.0 (Drummond et al. 2012). We used the default priors for mutation rates, ancestral population sizes and species trees. We employed an MCMC chain of 2.5 × 10⁸ generations, with parameters and trees sampled every 1000 generations. We summarized the resulting trees using the ‘consensus’ function in the R package ape (Paradis et al. 2004).

Results

Phenotypic Data

Dewlap phenotypes along Webster’s transect exhibit variation consistent with the RCD hypothesis for Anolis caudalis and its relatives (Fig. 2). The dewlaps of the northernmost population of A. caudalis exhibited very little orange—significantly less than the adjacent Anolis websteri population and the other populations of A. caudalis. These other two populations of A. caudalis were more variable in dewlap colouration, but generally had substantial orange patches in their dewlaps (Fig. 2). Dewlap colouration in both of these populations was significantly different from that of the nearby Anolis brevirostris population (the A. caudalis population closest to A. brevirostris exhibited the most orange, although not significantly more than in the middle population). The northernmost population of A. caudalis did not differ significantly from A. brevirostris in the amount of orange in the dewlap.

Genetic data

The final mtDNA sequence alignment contains 933 base pairs of the protein-coding sequence for ND2 for 137 individuals (plus 1 out-group individual). After eliminating duplicate sequences (all of which occurred within populations), we obtained a data set including 103 unique haplotypes. Our final alignment includes 373 variable characters (294 parsimony informative) and is 97.9% complete with a total of 104 ambiguous or partially ambiguous characters.

Using the semi-strict scoring conditions, we obtained a total of 821 AFLP loci from 137 individuals plus one out-group individual (Anolis distichus). We obtained 84–271 loci per primer pair (Table S4, Supporting information). For analyses in STRUCTURE, we created a second AFLP data set of 809 loci that excluded the out-group. Using the strict scoring parameters, we obtained a total of 210 loci, with 43–65 loci obtained per primer pair.
(Table S4, Supporting information), and created a data set for Structure analyses of 204 loci that excluded the out-group.

Phylogenetic reconstruction

The results of the likelihood ratio test significantly favoured three partitions for the mtDNA data set (Table S3, Supporting information). Phylogenetic relationships estimated using the 821-locus AFLP data set were largely congruent with those recovered by mtDNA alone (not shown). Because relationships among individual partitions were concordant, we present results from the combined analysis of mtDNA sequence data plus the 821 AFLP loci (Fig. 3). Each species is strongly supported as monophyletic, with strongly supported

![Phylogenetic tree diagram](image-url)

Fig. 3 Combined data (mtDNA and AFLP) phylogeny generated by MrBayes. Node labels are organized into three bins according to their Bayesian posterior probability: >95 (black), >70 (grey), <70 (white). Taxon labels include catalogue numbers for specimens housed in the Museum of Comparative Zoology (MCZ R) or ID numbers corresponding to the field series of Richard E. Glor (GLOR), as well as abbreviated species name, and a locality identifier (from 1 to the number of localities for that species). Colors indicate population clusters identified using structure and are the same as in Fig 1 of the manuscript.

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geographic substructure evident for *A. brevirostris* and *A. websteri*, but not *A. caudalis*.

The phylogenetic relationships estimated using strictly scored AFLP data set were not well resolved compared to the semi-strictly scored AFLP data set and the mtDNA data set (Fig. S2, Supporting information). The species-level relationships recovered by the semi-strictly scored AFLP data set are concordant with the results from the mtDNA sequence data alone, and sequence data from a preliminary data set that includes eight nuclear loci (Geneva et al., in prep.), suggesting that the more semi-strict scoring conditions have not led to type I error. The SNAPP analysis resulted in a semi-strict (98% majority-rule) consensus species tree topology that was identical to the species-level relationships inferred from the MRBAYES analyses of mtDNA and AFLP data (not shown).

Population structure

Our hierarchical **Structure** analyses following Coulon et al. (2008) recovered six genetically distinct clusters corresponding with each of the four species as well as geographically defined clusters within *A. brevirostris* and *A. websteri* (Fig. S1, Supporting information). This result was consistent using both the strictly and semi-strictly scored data sets, although we only present results from the semi-strictly scored 809-locus data set. The substructure we recovered was also concordant with strongly supported mtDNA clades (Fig. 1). Of the 100 **Structure** iterations performed at $K = 6$, we selected the one with the best overall lnL for visualization and interpretation of results. The clusters recovered are concordant with those identified using the hierarchical analyses (Fig. 1). Overall, we found very little evidence for gene flow between the species delimited by Arnold (1980) (Fig. 1, Table 1). Also, we observed little or no population structure within *A. caudalis* (Fig. 1, Fig. 3, Table S5, Supporting information), suggesting unimpeded gene flow between populations of *A. caudalis* that differ in dewlap color with respect to the loci sampled by our AFLP genome scans.

Environmental variation

Our investigation of environmental variation from georeferenced localities did not reveal the pattern of environmental variation expected under the hypothesis that dewlap color in the *brevirostris* clade varies in response to climatic variation that may be associated with variation in local signalling conditions rather than in response to interspecific interactions (see Discussion). In contrast, the extracted variables from the range of *A. websteri* appear more variable than those from *A. caudalis* and *A. brevirostris*, even though the dewlaps of *A. websteri* are uniformly orange (Fig. 4).

Discussion

**Reproductive character displacement and reinforcement**

Our results reveal a clear pattern of reproductive character displacement in *Anolis caudalis* where its range abuts the ranges of close relatives. This pattern is most obvious in the northernmost locality of *A. caudalis*, very close to this species’ boundary with *Anolis websteri*. Dewlap phenotypes observed in these *A. caudalis* are more distinct from the dewlaps of *A. websteri* than are dewlaps observed elsewhere in *A. caudalis’* range (Figs. 1 and 2). Dewlap phenotype patterns are also consistent with reproductive character displacement near the southern range boundary of *A. caudalis*, closest to the pale-dewlapped *Anolis brevirostris* (Figs. 1 and 2). The two more southern *A. caudalis* localities had significantly more orange in their dewlaps than the *A. brevirostris* locality, while the northernmost *A. caudalis* population did not (Fig. 2). These results confirm one of only a few instances of reproductive character displacement reported in squamate reptiles (Webster & Burns 1973).

### Table 1

<table>
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<th></th>
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We recover evidence for pervasive gene flow between all three sampled localities of *A. caudalis*, including the white-dewlapped population at the northern range limit. The maintenance of distinct phenotypes despite this gene flow implicates selection on dewlap color and pattern. Moreover, the lack of geographic genetic differentiation in *A. caudalis* stands in contrast to finer-scale structuring observed within both *A. brevirostris* and *A. websteri* over similar geographic distances. In other anoles, including other Hispaniolan trunk anoles, distinct dewlap phenotypes are often associated with distinct genetic clusters (Ng & Glor 2011; Glor & Laport 2012; Ng et al. 2013). The low genetic differentiation observed between localities of *A. caudalis* also challenges the possibility that reproductive character displacement could be leading to speciation between displaced and nondisplaced populations of *A. caudalis* (i.e. ‘RCD speciation,’ Hoskin et al. 2005; Hoskin & Higbie 2010).

Additional tests are required to determine whether the pattern of reproductive character displacement in *A. caudalis* results from reinforcement. One important prediction of reinforcement is that maladaptive hybrid matings are responsible for the evolution of RCD. At this point, we know little about the frequency of interspecific mating events in Haitian trunk anoles, but our molecular genetic data recover little or no evidence for contemporary hybridization between *A. caudalis* and *A. websteri* or *A. brevirostris*. One possibility is that such matings occurred only in the past. However, the strong cline in dewlap colouration within *A. caudalis*, despite high levels of contemporary gene flow between populations, suggests that the observed RCD is maintained by ongoing selection. Another possibility is that hybrid matings still occur, but do not result in any viable or fertile offspring (i.e. reproductive isolation is complete). If such unproductive matings occur, selection against gamete and energy wastage should occur, continuing the process of reinforcement (sensu Howard 1993; Noor 1999; Servedio & Noor 2003).

Nevertheless, it would be premature to discount the possibility of contemporary hybridization, especially given that we were unable to sample the precise points of contact of these species’ ranges (discussed below). If hybridization is ongoing, reinforcement predicts ongoing fitness deficits in hybrid offspring. Although we know little about the mechanisms that might contribute to low hybrid fitness in anoles, Webster (1977b) hypothesized intrinsic hybrid male sterility between another pair of trunk anole species after finding evidence for abnormal meiosis stemming from failure to form a trivalent between the XXY sex chromosome complements. Preliminary data from ongoing laboratory crosses with two closely related trunk anole species in the Dominican Republic recover a significantly higher proportion of infertile eggs in interspecific vs. conspecific crosses, indicating substantial intrinsic prezygotic isolation between species that are more shallowly divergent than the Haitian species investigated in the present study. On the basis of this work, it seems reasonable to predict a cost to hybridization in the Haitian trunk anoles, if indeed it still occurs. Reinforcement also predicts that the trait or traits involved in isolation are heritable. It is now known that some anole pigments are environmentally acquired (Stefan & McGraw 2007). However, recent studies have found no effect of carotenoid dietary content on dewlap color in Brown anoles (*Anolis*...
sagrei, Steffen et al. 2010), and that dewlap color and pattern are highly heritable and unaffected by carotenoid supplementation in other trunk anoles (J. Ng, unpublished data).

Alternative explanations

Although the patterns of phenotypic and genomic differentiation we observe are consistent with reproductive character displacement resulting from reinforcement, other possible explanations for these patterns must also be considered (Howard 1993; Noor 1995; Hoskin & Higgie 2010). Evidence from other geographically polymorphic anole species, including populations of another polymorphic Hispaniolan trunk anole (Anolis distichus), suggests that dewlap color and pattern evolved adaptively in response to regional variation in signalling conditions (Leal & Fleishman 2004; Ng et al. 2013). In the case of the Haitian trunk anoles, however, we consider the hypothesis that dewlap color is primarily an adaptive response to climatic conditions associated with variation in signalling environments to be unlikely. The observed distribution of dewlap variation would predict a complicated scenario involving an abrupt shift from environments favouring orange dewlaps to environments favouring yellow dewlaps associated with the transition from A. websteri to A. caudalis, an environmental cline across the range of A. caudalis and another shift from environments favouring orange dewlaps to paler dewlaps where A. brevirostris replaces A. caudalis. Sampling of climatic variables from geo-referenced museum localities of the species across the central coast of Haiti does not provide evidence for such a pattern (Fig. 4).

A second possibility is that the dewlap variation in A. caudalis is the side effect of a finer-scale shift in habitat use, with populations in the northern part of this species’ range displaying in local light microenvironments distinct from those used from southern populations, perhaps as a result of ecological character displacement between competing forms (Berglund & Schluter 1998). Testing this hypothesis requires detailed observations of the two species in sympatry. However, the type of habitat specialization that would seem necessary to drive such a drastic shift in dewlap color seems unlikely given that all of the species along our transect behave as trunk anoles throughout their ranges and are unlikely to diverge substantially from this role due to the presence of multiple other species of more-distantly related anoles occupying other microhabitats in the same region (e.g. Anolis cyphotes in the trunk-ground microhabitat and Anolis chlorocyanus in the trunk-crown microhabitat). The degree to which use of different light microhabitats within a given forest can impact signal detection in anoles remains poorly characterized, as does the degree to which species can alter their use of alternative perches to optimize signal efficiency.

Another possibility is that the reproductive character displacement observed in dewlap color may be the result of selection against interference competition between males of the species. Under this scenario, costs imposed by interspecific aggression select for improved species (in this case, ‘competitor’) recognition. This phenomenon has been termed ‘agonistic character displacement’ (Grether et al. 2009), but is included under the definition of reproductive character displacement by Hoskin & Higgie (2010). Traits can be under selection pressure from both male–male and female–male interactions (Berglund et al. 1996), and separating their effects may be difficult. Experimental evidence suggests that the anole dewlap can be important for both male–female and male–male interactions (Sigmund 1983; Losos 1985). Detailed experimental studies of the relative importance of the dewlap for mate and competitor recognition in trunk anoles are required to determine the role of interference competition in shaping dewlap divergence.

Stasis of the contact zone

It is noteworthy that the contact zones along the coast of Haiti are in the same place as they were nearly 40 years ago, given that the habitat along this transect is heavily disturbed and occurs immediately adjacent to main highways heading from the Dominican Republic to Port-au-Prince and from Port-au-Prince to northern Haiti. All of the habitats where we sampled trunk anoles were heavily disturbed by humans, and trunk anoles were frequently sampled on or in the immediate vicinity of human habitations and other artificial structures (e.g. fenceposts, courtyards), as well as on fruit trees and other human-associated vegetation. Although little is known of anole dispersal capabilities in nature, invasive species are often capable of relatively rapid expansion in the absence of ecologically similar competitors, and presumably with the aid of some degree of hitchhiking with humans; the Cuban brown anole (Anolis sagrei), for example, has expanded its range across large areas of the southeastern United States over the same time interval that trunk anole distributions along our Haitian transect appear to have remained entirely static (Campbell 1996).

Remarkable range stasis in the face of extensive habitat disturbance and pervasive opportunities for human-mediated dispersal suggests that selection may be actively maintaining the geographic positions of the existing species boundaries. One possibility is that these
ranges are maintained by niche incumbency, with each species being unable to displace established and largely ecologically identical populations of the other species. Areas of contact among similar anole species, however, are often associated with some type of ecological barrier to dispersal (Glor & Warren 2010). Although the putative areas of contact between distinct species of Haitian trunk anoles are not characterized by obvious environmental gradients, they do appear to be associated with low availability of preferred trunk anole habitat (Webster & Burns 1973). Such areas of relatively poor habitat may play an important role in maintaining the geographic position of hybrid zones, particularly when tension zones between species that produce unfit hybrids settle in areas of relatively low population density (Barton & Hewitt 1985, 1989). Testing this hypothesis and other possible explanations for the position of the current contact zones requires finer-scale studies of habitat availability and environmental variation along this transect, as well as detailed studies of reproductive isolation and the fitness of potential hybrid offspring.

Conclusions

The reproductive character displacement reported in this study adds a layer of complexity to our understanding of the factors driving dewlap divergence and speciation in anoles. Most attention on dewlap divergence and its relevance for speciation has focused on local adaptation to environmental variation. In contrast, we find a clear pattern of reproductive character displacement in the Haitian trunk anoles that is consistent with the action of reinforcement. This result is consistent with long-standing hypotheses that the anole dewlap is important for species recognition (e.g., Williams & Rand 1977). However, in the case of A. caudalis, it is clear that differences in dewlap among sampled localities do not correspond to genetic differentiation or other obvious indicators of reproductive isolation or incipient speciation. Together with results from other recent studies (Leal & Fleishman 2004; Stapley et al. 2011; Glor & Laport 2012; Ng & Glor 2011; Ng et al. 2013), our study suggests that much additional work is required to distinguish the complicated and potentially concurrent effects of interspecific interactions and ecological variation on dewlap divergence and speciation in anoles.

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References


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The AFLP data matrices, mtDNA sequence assembly and climate information extracted from geo-referenced localities are deposited in the Dryad data repository (www.datadryad.com doi:10.5061/dryad.d96q7). The R script for converting from RawGeno output to AFLP-score input, as well as phenotypic data on dewlaps, is available as part of the online supporting information.

R.E.G. and D.L.M. collected and photographed anoles in Haiti. S.M.L. conducted mtDNA sequencing and AFLP genotyping under the instruction of A.J.G., S.M.L. and A.J.G. completed population genetic and phylogenetic analyses. All authors contributed to the writing of the manuscript.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Sampling information for our study, including specimen IDs, locality information, and dewlap color information.

Table S2 Primers used for AFLP marker generation.

Table S3 Harmonic means of likelihoods and Bayes factor comparisons of mtDNA partitioning strategies.

Table S4 Scoring parameters and error rates for semi-strict and strict AFLP scoring conditions.

Table S5 Pairwise $F_{ST}$ and $H_e$ for sampled localities of A. caudalis.

Table S6 Results from Tukey's Honestly Significant Difference test comparing dewlap phenotype distributions (using % orange) from localities of A. websteri, A. caudalis, and A. brevirostris sampled by Webster & Burns (1973) and this study.

Fig. S1 Diagram summarizing results of hierarchical clustering analyses following Coulon et al. (2008).

Fig. S2 Phylogeny generated in MrBayes using dataset scored with strict AFLP scoring parameters.

Appendix S1 AFLP reaction conditions.

Appendix S2 R script for converting RawGeno output files to AFLPscore format.