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Original Article

Persistence of a Geographically-Stable Hybrid Zone in Puerto Rican Dwarf Geckos

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Abstract

Determining the mechanisms that create and maintain biodiversity is a central question in ecology and evolution. Speciation is the process that creates biodiversity. Speciation is mediated by incompatibilities that lead to reproductive isolation between divergent populations and these incompatibilities can be observed in hybrid zones. Gecko lizards are a speciose clade possessing an impressive diversity of behavioral and morphological traits. In geckos, however, our understanding of the speciation process is negligible. To address this gap, we used genetic sequence data (both mitochondrial and nuclear markers) to revisit a putative hybrid zone between *Sphaerodactylus nicholsi* and *Sphaerodactylus townsendi* in Puerto Rico, initially described in 1984. First, we addressed discrepancies in the literature on the validity of both species. Second, we sampled a 10-km-wide transect across the putative hybrid zone and tested explicit predictions about its dynamics using cline models. Third, we investigated potential causes for the hybrid zone using species distribution modeling and simulations; namely, whether unique climatic variables within the hybrid zone might elicit selection for intermediate phenotypes. We find strong support for the species-level status of each species and no evidence of movement, or unique climatic variables near the hybrid zone. We suggest that this narrow hybrid zone is geographically stable and is maintained by a combination of dispersal and selection. Thus, this work has identified an extant model system within geckos that that can be used for future investigations detailing genetic mechanisms of reproductive isolation in an understudied vertebrate group.

Subject areas: Molecular systematics and phylogenetics, Population structure and phylogeography
Keywords: geckos, hybridization, hybrid zone dynamics, speciation, *Sphaerodactylus*

Species, independently evolving metapopulation lineages that are durable through time (de Queiroz 2007; Singhal et al. 2018), are the evolutionary units of biodiversity. A sizable number of species (>10% in animals) have failed to develop sufficient prezygotic isolating barriers to prevent interspecific reproduction, or hybridization, between closely related taxa (Mallet 2005). However, species' integrity can be maintained, in these cases, by postzygotic isolating mechanisms that limit the fitness of hybrids (Coyne and Orr 2004). Fortunately, opportunities to study postzygotic isolating mechanisms occur naturally in the form of hybrid zones, geographic areas where 2 species

meet and produce interspecific offspring (Hewitt 1988). Thus, the study of hybrid zones in nature is essential to understanding the processes involved in speciation (Barton and Hewitt 1985; Harrison 1993; Butlin et al. 2012).

The establishment of hybrid zones is common in areas where previously allopatric species meet in areas of secondary contact. However, reproductive isolation is often incomplete, or "leaky", because the development of complete reproductive isolation between allopatric species is primarily due to genetic drift and not divergent selection (Vijay et al. 2016; Bay and Ruegg 2017; Mack and Nachman 2017; Ravinet et al. 2017). Moreover, hybrid zones are highly dynamic and can fluctuate geographically over both long and relatively short periods of time, especially in areas of fluctuating climate and high anthropogenic disturbance (Engebretsen et al. 2016; Leaché et al. 2017). Thus, it is important to both investigate gene flow and geographic stability when studying the complexities of postzygotic isolation within hybrid zones.

To thoroughly investigate how genomic differences among groups affect speciation, it is imperative to study hybridization in a diversity of vertebrate clades. However, some clades are better represented in the literature (e.g., birds and mammals) than others due to historical contingency and publication biases (Barton and Hewitt 1985; Harrison 1993; Vijay et al. 2016). This is because past research in birds and mammals may be biased by intrinsic genomic factors, for example, degenerated sex chromosomes, and examining the process of speciation across the diversity of organisms on Earth is essential for developing and testing theory (Coyne and Orr 1989; Lima 2014; Gerchen et al. 2018). While some animal groups have been the focus of much work, other groups have remained relatively untouched by scientific investigation, even some highly diverse groups, such as gecko lizards.

Gecko lizards (Squamata: Gekkota) comprise a highly speciose clade of lizards (>1800 species) where reports of hybridization are rare when compared to other vertebrate groups, and even other squamate groups (lizards and snakes) (Barton and Hewitt 1985; Mallet 2005; Jančúchová-Lásková et al. 2015; Uetz 2018). Although natural hybridization is suspected to have occurred in various gecko lineages, for example, *Ptyodactylus* (Werner and Sivan 1996) and *Tarentola* (Tejangkura 2012), very few cases have been confirmed using molecular genetic data. These cases include: limbless Australian geckos of the genus *Delma* (Brennan et al. 2016), numerous parthenogenetic geckos, such as *Heteronotia binoei* and *Lepidodactylus lugubris* (Fujita and Moritz 2009), and hybrid zones within 2 genera *Gekko* (Toda et al. 2001, 2006) and *Sphaerodactylus* (Murphy et al. 1984). Thus, across geckos, studies of the speciation process are generally lacking.

Dwarf geckos of the genus *Sphaerodactylus* (Gekkota: Sphaerodactylidae) are a charismatic clade of neotropical geckos that occur in high abundance throughout the Caribbean, usually in the leaf-litter of forested habitats or abandoned human dwellings (Grant 1931; Murphy et al. 1984; Thomas and Schwartz 1966). The Puerto Rican Bank and nearby islands (Mona, Monito, and Desecheo) are home to 8 currently recognized *Sphaerodactylus* species, some of which possess comparatively large geographic ranges (e.g., *Sphaerodactylus nicholsi*, *Sphaerodactylus macrolepis*, and *Sphaerodactylus klauberi*) and are at least partially sympatric with one or more species (Rivero 2006). Of these, 4 currently recognized Puerto Rican (PR) *Sphaerodactylus* species have been hypothesized to be closely related: *S. nicholsi, Sphaerodactylus townsendi, Sphaerodactylus monensis,* and *Sphaerodactylus levinsi* (Grant 1931, 1932a; Heatwole 1968). However, the taxonomic status and phylogenetic relationships between these species have been debated, without conclusion, since their description—based on both morphological and genetic data (Grant 1931; Thomas and Schwartz 1966; Murphy et al. 1984; Hass 1991; Díaz-Lameiro et al. 2013). While the taxonomic status of *S. monensis* and *S. levinsi* has remained relatively stable (occurring allopatrically on the islands of Mona and Desecheo, respectively), the relationship and taxonomic status of *S. nicholsi*, with regard to *S. townsendi*, have been somewhat contentious. This is likely because both species are sexually monochromatic, look superficially similar to one another (Grant, 1932b), occur in similar (arid and semi-arid) habitats (Pregill 1981), and, together, possess a continuous distribution along PR's southern coast with an overlapping parapatric area within the municipality of Juana Díaz (Murphy et al. 1984; Rivero 2006). In 1984, Murphy and others characterized the parapatric zone (the region where *S. nicholsi* and *S. townsendi*'s distributions overlap) as a putative hybrid zone using allozyme genetic analysis. While a large number of individuals were examined (*S. nicholsi* $N = 45$; hybrids $N = 26$; *S. townsendi* $N = 36$; total $N = 107$), Murphy et al. (1984) only confirmed hybrids from 2 localities, and a majority of the "pure" samples ($N = 68/81$) were collected from well outside the hybrid zone (e.g., Guanica or Pozuelo). Thus, provided a more complete characterization of this putative hybrid zone, *Sphaerodactylus* geckos are a potential model system to study hybridization and speciation in gecko lizards.

Broadly, there are 2 hypotheses that govern the persistence of a hybrid zone through time (Barton and Gale 1993), either dispersal-mediated selection against hybridization (Barton and Hewitt 1985) or selection for intermediate phenotypes at a zone of intermediate habitat (Moore 1977). Beyond why a hybrid zone exists to begin with, hybrid zones can be stable or dynamic (i.e., transient or fluctuating geographically) (Harrison 1993; Engebretsen et al. 2016; Leaché et al. 2017). To investigate historical processes affecting hybrid zones, cline models are an immensely effective tool to detect changes and fluctuations within a hybrid zone (Barton 1979; Barton and Gale 1993; Singhal and Moritz 2013). For instance, when a hybrid zone moves, some alleles will faithfully track this movement, while others will lag behind (Rohwer et al. 2001; Leaché et al. 2017). Those alleles that faithfully track the hybrid zone are strongly deleterious to one species, while those alleles that lag behind are likely neutral or only mildly deleterious. Indeed, cline models can be used to elucidate the selective pressures acting upon a locus within a hybrid zone, as the slope (i.e., width and shape) of the cline is a derivation of the selection acting on that locus (Barton 1979; Barton and Gale 1993). Similarly, the cline center of a locus can be used as a comparative metric to other data (e.g., unlinked loci or other characters) to elucidate differential introgression or hybrid zone movement. Ideally, measurement of hybrid zone movement requires repeated, time-structured sampling (Buggs 2007; Leaché et al. 2017). However, sampling at a single time-point can also be used to identify signatures of hybrid zone movement by resolving clines of neutral or mildly deleterious alleles (Arntzen et al. 2016; van Riemsdijk et al. 2019), especially when paired with related historical information within the study system. Thus, we utilized cline models to investigate hybrid zone dynamics in this system by testing predictions generated from a series of hypotheses.

We used 3 different approaches (below) to answer 3 research questions, 1) Do *S. nicholsi* and *S. townsendi* represent genetically distinct populations? 2) What dynamics are actively affecting this putative hybrid zone or is the hybrid zone geographically stable? 3) Is this hybrid zone maintained by dispersal and selection against

hybrids or selection for intermediate phenotypes at a zone of intermediate habitat?

First, we used genotypically "pure" individuals, sampled from across each species' range, in a species delimitation analysis to statistically demarcate these nominal taxa. Second, we sampled a transect through the municipality of Juana Díaz, PR and generated multilocus sequence data for a set of neutral markers. We then used model testing to find the best-fit cline models to represent our data and used model-testing to determine: coincidence (same center), concordance (same width and shape), and the presence or absence of exponential tails (signs of movement or introgression). Indeed, although the null hypothesis is that hybrid zones tend not to move (Barton 1979), recent work has shown that in areas of recent climatic variation or high anthropogenic disturbance hybrid zones have a higher capacity to be transient or fluctuate geographically (Engebretsen et al. 2016; Leaché et al. 2017; van Riemsdijk et al. 2019). Specifically, in PR, the rising and falling of sea levels during the Quaternary (≥6000 years ago) and agricultural boom of the early- to mid-20th century (~60-120 years ago) could both have initiated, and/or caused fluctuations in this geographic area (Heatwole and MacKenzie 1967; Murphy et al. 1984). If the hybrid zone has moved in the past ~35 years, we predicted that 1) we would not find the hybrid zone in same geographic location as Murphy et al. (1984) initially described and/or 2) we would find evidence of asymmetrical introgression (i.e., an exponential tail) leading away from locations described by Murphy et al. (1984). However, if the hybrid zone is transient or actively fluctuating, we expected to find either 3) no evidence of hybridization across this transect or 4) cline centers would be shifted between these unlinked loci. Third, we addressed whether there was evidence to favor a hypothesis of selection for intermediate phenotypes, at a zone of intermediate habitat between these two species using species distribution models and niche simulations. We predicted that if these data supported this hypothesis, then we would find 1) a unique climatic variable, or suite of variables, in the hybrid zone (e.g., sharp transition zone between preferred habitats) and 2) a low degree of fundamental niche overlap, which would provide further evidence that there is a transition in habitat use between parental species.

Materials and Methods

Data Generation

We collected and vouchered animals from across the putative hybrid zone over a 10-km-wide transect in May 2017 and "pure" individuals, for species delimitation, from allopatric populations of each species across Puerto Rico between 2012 and 2017. We identified putative hybrids via intermediate, and anomalous, color and patterning schemes across the dorsum of the animals (N = 18). Our sampling reflected the geographic extremes where we could definitively categorize individuals as "pure" *S. nicholsi* or *S. townsendi* on either side of the putative hybrid zone. A single hybrid individual (BJP016 – locality H) was sequenced from an autotomized tail (animal not captured), and thus was unidentifiable in the field (adjusted sample sizes: hybrids N = 19, *S. nicholsi* N = 19, *S. townsendi* N = 25). Uneven sampling across the putative hybrid zone is due to biologically relevant factors (uneven habitat) and land-use factors; specifically, the Military Training Center (Fort Allen) that lies in the middle of the sampling area. We extracted genomic DNA from tail or liver tissue using the Qiagen® DNeasy Blood and Tissue Kit. We generated sequence data for all 63 individuals, using PCR amplification and subsequent, single-pass Sanger sequencing in both directions (Genewiz®). All sequences were accessioned to GenBank (Supplementary Table 1), and for taxonomic assignment of hybrid animals submitted to GenBank, we standardized by using the animals' mitochondrial assignment. We amplified fragments of 7 genetic markers, 1 mitochondrial gene: NADH dehydrogenase subunit 2 (ND2); and 6 nuclear markers: Oocyte Maturation Factor mos (CMOS), Death Inducer-Obliterator 1 (DIDO), Microtubule-Actin Crosslinking Factor 1 (MACF), Microtubule Associated Protein 2 (MAP2), Protein Tyrosine Phosphatase, Non-receptor type 12 (PTPN12), and Recombination Activating Gene 1 (RAG1); primers and associated references for each marker are provided (Supplementary Table 2). Raw sequence reads were assembled using Geneious® (v10.2.2); (Kearse et al. 2012). We identified heterozygous sites using the "Find Heterozygotes" function in Geneious® and verified putative heterozygous sites by examining trace chromatograms for dual base-called sites that were equal in height and shorter than neighboring sites. We aligned DNA sequences using MUSCLE software (Edgar 2004) and refined alignments by eye. We phased allelic variants for each nuclear gene using PHASE software (Stephens et al. 2001), with default settings, implemented in DNAsp (v5.10.1) (Librado and Rozas 2009).

Species Phylogeny and Species Delimitation

To generate a species tree in a coalescent framework, we utilized a subset of our aforementioned dataset (ND2, CMOS, PTPN12, RAG1), and generated additional sequence data for mitochondrial ribosomal subunit 16S and 3 additional closely related taxa from within this clade, *S. monensis, S. levinsi,* and *S. klauberi* for all loci (Supplementary Table 2). *Sphaerodactylus klauberi* was used as an outgroup for all phylogenetic analyses. The best-partitioning scheme for each gene was determined using PartitionFinder2 (v2.1.1); (Guindon et al. 2010; Lanfear et al. 2012, 2017) on the CIPRES cluster (Miller et al. 2010) (Supplementary Table 3). Each locus consisted of a single data partition except ND2, where each codon had its own partition. Both mitochondrial loci, 16S and ND2, were combined into a single tree partition. Models of sequence evolution for each locus and partition are listed in Supplementary Table 3. We generated a species tree using the StarBEAST2 package (v0.13.5); (Ogilvie et al. 2017) in BEAST2 (v2.4.6); (Bouckaert et al. 2014), also on the CIPRES cluster (Miller et al. 2010). Each locus utilized an uncorrelated lognormal clock and a birth-death model with other priors set as default. We ran 3 independent chains of 10×10^8 mcmc iterations, with a 10% burnin, and examined likelihood values for convergence using Tracer (v1.6) (Rambaut et al. 2018). Tree files were compiled using LogCombiner and the final tree was generated in TreeAnnotator.

To further resolve the species-level relationships within this clade and perform an initial assessment of the validity of *S. townsendi* and *S. nicholsi* as distinct species, we analyzed our postburnin species trees to identify the frequency of a *S. townsendi* + *S. nicholsi* clade. We calculated the posterior probability of this hypothesis by filtering postburnin species trees that were consistent with a topology that constrained an *S. townsendi* + *S. nicholsi* clade, exclusive of *S. levinsi* and *S. monensis*, using Phylogenetic Analysis Using Parsimony (PAUP*) (v 4.0a157) (Swofford 2002).

To identify whether *S. nicholsi* and *S. townsendi* are genetically distinct populations relative to each other, we conducted statistical species delimitation under the multispecies coalescent to test for species-level divergence in this clade using STACEY (v1.2.4)

(Jones 2017). We conducted STACEY analysis using the previously described StarBEAST2 dataset, with all taxa and partitions conserved in both analyses (Supplementary Table 3). All priors were left default unless specifically stated below. In accordance with program documentation and additional specifications outlined by Barley et al. (2018), we provided an exponential distribution with a mean of 0.1 for the "popPriorScale" parameter, a lognormal distribution with a mean of 5 and a standard deviation of 2 to the "bdcGrowthRate" prior, and the "collapseWeight" was provided a uniform distribution with the lower and upper bounds set at 0 and 1, respectively (Barley et al. 2018). In addition, each partition was provided an independent strict molecular clock, with rate priors calculated from a log-normal distribution that were given a mean of 0 and standard deviation of 1 (Barley et al. 2018). We ran 3 independent chains of 5.0×10^7 mcmc repetitions, sampling every 5000 trees, and compared likelihood values from trace files using Tracer (v1.6) (Rambaut et al. 2018). We combined tree files using LogCombiner and analyzed the resulting 30,000 trees using the SpeciesDelimitationAnalyzer (SpeciesDA) (v1.8). We used a burnin of 5000 and a collapse-height of 0.0001 to calculate our final species delimitation result. To corroborate the results generated using STACEY and SpeciesDA, we analyzed a dataset of nuclear-only loci using BPP software (v4.0) (Flouri et al. 2018). We analyzed CMOS, PTPN12, and RAG1 genes for *S. nicholsi*, S. *townsendi, S. levinsi, S. monensis*, and *S. klauberi* using a specified guide tree from our StarBeast2 analysis. To examine an array of biological scenarios, we used 3 different configurations of population size (inverse-gamma θ = a, b) and divergence time (inverse-gamma τ = a, b) priors to begin our parameter estimation (Leaché and Fujita 2010). In configuration 1, we assumed "medium" N_e (θ = 3, 0.002) with "medium" divergence time (τ = 3, 0.03); in configuration 2, we assumed "small" N_e (θ = 3, 0.0002) with "recent" divergence time (τ = 3, 0.003); in configuration 3, we assumed "large" N_e (θ = 3, 0.02) with "long" divergence time (τ = 3, 0.3). This allowed us to interrogate the effects of various biological changes within the system on species delimitation analysis. We ran each mcmc chain for 5×10^5, sampling every 5, with a 10% burnin. We ran 2 independent mcmc chains for each configuration and checked log files' likelihood values for convergence using Tracer (v1.6) (Rambaut et al. 2018). In addition to statistical species delimitation methods, we generated table of uncorrected *P*-distances between lineages of the mitochondrial gene ND2 using MEGA7 (Kumar et al. 2016) to compare across other recognized gecko species (Supplementary Table 4).

Genetic Cline Analyses

To assign alleles in hybrids to their respective parental species, we constructed maximum-likelihood (ML) gene trees of phased alleles using RAxML-HPC BlackBox (v8.2.10) (Stamatakis 2014) on the CIPRES cluster (Miller et al. 2010). These gene trees were rooted using *S. klauberi*, with bootstrap support necessary for one species-specific clade (≥70) to the exclusion of the other (i.e., support for all individuals within a group excludes all members of other group). All species-specific allelic variants were condensed into binary allelic assignment, where all individuals were either "pure" *S. townsendi* (0), "pure *S. nicholsi*" (1), and/or, for nuclear markers, heterozygous (0.5) assignments.

To calculate genetic clines, we calculated the mean value of the individuals at each locality to produce a per-locality allele frequency for each locus. We plotted these values along a collapsed, 1-dimensional (longitudinal) transect using the *hzar* package (v0.2–5) (Derryberry et al. 2014) in R (R Core Team 2016). To find the best-fit cline model, we conducted a series of model-testing analyses in *hzar*. First, to identify the best-fit model for each locus we tested 4 different potential models using corrected Akaike information criterion (AICc). Model I maintained that each interval was fixed at either 0 or 1 without an exponential tail; model II maintained that each interval was free to fluctuate based on the data (was not fixed at 0 or 1), also without an exponential tail. Between these models, model I was favored, thus, models III and IV maintained fixed intervals. To test for the presence of asymmetric introgression, we model III tested for the presence of a left-side tail and model IV a right-side tail (Table 1). Further, to test for discordance between mitochondrial and nuclear markers (cline movement), we generated models V and

Table 1. (A) Corrected AICc values for each cline-fitting model tested at each locus. Score differences of >2 indicate a significantly greater fit to the model, and the best-fit model for each locus is bolded (* indicates 3 best-fit models to the data). (B) Cline center and width ranges for each locus. Cline center is respective of the longitudinal cline (i.e., along a 0–10 km axis). Cline widths are kilometer ranges irrespective of their location along the cline (i.e., distance wide)

A) *AICc*	*mtDNA*	*CMOS*	*MACF*	*PTPN12*	*RAG1*
NULL model	*60.731*	*129.450*	*131.900*	*136.889*	*130.606*
Model I (fixed, no tails)	**7.894***	**5.010**	**19.181**	**16.165**	**13.883**
Model II (free, no tails)	13.149	11.480	24.401	22.003	20.807
Model III (fixed, left tail)	12.326	9.129	23.419	20.294	18.166
Model IV (fixed, right tail)	12.526	9.159	23.385	20.469	18.167
Model V (fixed, no tails; constrained center)	**7.737***	N/A.	N/A.	N/A.	N/A.
Model VI (fixed, no tails; constrained width)	**7.734***	N/A.	N/A.	N/A.	N/A.
B) ***Cline Ranges***					
Cline centers	3.759–4.432	4.284–4.496	4.306–4.597	4.246–4.456	4.212–4.485
Cline widths	0.5969–2.794	0.4402–1.044	0.6806–1.591	0.4652–1.272	0.5311–1.505

VI, which constrained the cline center and width, respectively, of our mitochondrial cline to the average values of our nuclear data for each. For all model-testing analyses, a model with an AICc difference of >2 was considered a significantly better fit to the data than the alternate model (Leaché et al. 2017).

Species Distribution Models and Simulations

To estimate the climatic factors involved in the generation and maintenance of this hybrid zone, we constructed independent species distribution models for *S. nicholsi* and *S. townsendi* using Maxent (v3.41) (Phillips et al. 2006, 2017; Phillips and Dudík 2008). Locality information was collected by the authors (BJP, JT, JDD, and TG, personal observation), Murphy et al. (1984), and VertNet (accessed 30 January 2018) (Supplementary Table 5). GIS layers were assembled in QGIS (v3.2) (QGIS Development Team 2009). We procured the base vector layer ("Puerto Rico administrative area") from the Global Administrative Database (https://gadm.org). All 19 BioClim layers were acquired from WorldClim 2 (Fick and Hijmans 2017), current 1970–2000, at 30 arc-seconds (~1 km) (Supplementary Table 6). Landsat tree cover was parsed and stitched together to create a vegetative continuous field in QGIS (v3.2). Resolution was set to 30 m^2 per pixel. Vegetation was estimated as tree cover of horizontal wooded cover greater than 5 m in height. Map data were derived from Landsat-5 Thematic Mapper and Landsat-7 Enhanced Thematic Mapper Plus (ETM+) (Sexton et al. 2013). Finally, topographic raster group was derived from Shuttle Radar Topography Mission – February 2000 (SRTM) (Farr et al. 2007). Elevation layer was taken from SRTM at 30 m^2 resolution. Roughness, slope (using Horn (1981) algorithm [Fleming and Hoffer 1979; Ritter 1987]), aspect, hillshade, terrain position index, and terrain ruggedness index (TRI) were derived from SRTM using R package raster 2.6–7 (Hijmans and van Etten 2012). WorldClim 2 layers were trimmed and bound to Puerto Rico administrative boundaries in QGIS, then transformed to a raster in ASCII format for Maxent. Since locality cover for each species is high (locality data reasonably represent the ranges of these species) and broadly established within the putative range, no assumption was made for limiting presence data by arbitrary omission. Our niche model analysis was conducted with presence only data (Fielding 2002; Veloz 2009) to predict regions where species distributions could extend without extraneous factors (Supplementary Table 6). One hundred replicates with a random seed were conducted on *S. nicholsi* and *S. townsendi* using cross-validation to estimate robustness of prediction. A maximum number of background points were set to 10,000. Environmental variable importance used the Jackknife method in Maxent, and output format was written in the Cloglog. Once written, we determined whether or not the machine-learning "receiver operating curve" (ROC) fit the data by calculating the "area under the curve" (AUC) (Supplementary Table 6).

To understand the ecological dynamics of the hybrid zone between *S. nicholsi* and *S. townsendi*, we conducted a model-based analysis of the niche space overlap in NicheA (Qiao et al. 2016). This tests fundamental niche similarity between *S. nicholsi* and *S. townsendi* to determine how the dynamics of each niche influence the distribution of each species. By characterizing the fundamental niches between species, we can postulate the stability of a species range by the conserved factors within the environmental and geographic overlap (Stigall 2014). NicheA allows for visualization of Hutchinsonian duality (Pulliam 2000; Colwell and Rangel 2009) between environmental (E) and geographic (G) spaces across species distribution, where the niche is a multidimensional volume with a set of defined characters (E-space) influenced by the physical conditions that dictate those conditions (G-space) (Colwell and Rangel 2009). In our case, we calculated the overlap between parental species to elucidate how a hybrid zone may form based on the shared suitability of niche space between *S. nicholsi* and *S. townsendi*. Thus, we used these niche simulations to estimate 1) fundamental niche space of *S. nicholsi* and *S. townsendi* and 2) to calculate the niche overlap between the 2 species. The niche simulations employed in NicheA software use a background cloud (BC) to constrain the environmental space within the species range (Puerto Rico's geography in this study) within 3-dimensional space. Each species' niche is then simulated into a hypervolume, a set of defined characters that influence the niche of the species, within the constraints set by the BC. A minimum volume ellipsoid (MVE) is the simulated fundamental niche of a species within the constrained set of characters of the BC defined by the specific characters inherent to the species itself.

Results

Data Description

We collected 19 animals that showed evidence of hybrid ancestry at 4 localities within the hybrid zone (Figure 1); 9 of these animals possessed mitochondrial DNA inherited from *S. nicholsi* and 10 from *S. townsendi* (Supplementary Table 1). However, we found no evidence that any of these 19 animals were F_1 hybrids (i.e., heterozygous at diagnostic alleles at all 4 nuclear loci). In fact, we found that only one of the 4 hybrid-containing localities possessed any "pure" parental individuals, that is, possessed diagnostic for *S. nicholsi* alleles at all loci, among the sampled hybrids (Supplementary Table 1). One individual (TG3186 – locality I) failed to amplify any nuclear genes well-enough for sequencing, while another (JDD383 – locality H) also failed to amplify MACF and PTPN12.

Species Phylogeny and Species Delimitation

The species tree topology recovered *S. monensis* as sister species to *S. townsendi* with strong support (posterior probability = 0.96) and *S. levinsi* as the sister taxon to that *S. townsendi* + *S. monensis* clade, and *S. nicholsi* as sister to the clade of these 3 species (Figure 1a). Species tree results were concordant with a species tree generated without mtDNA (data not shown). Filtering the postburnin species trees in PAUP*, we calculated the posterior probability of an *S. nicholsi* + *S. townsendi* clade as 0.00049, or 25 out 51,003 postburnin trees included that arrangement. Species delimitation using STACEY and SpeciesDA generated high-support for all nominal taxa in our species tree as species-level lineage divergences (SpeciesDA: posterior = 0.99). Further, SpeciesDA analysis of STACEY trees was concordant with species delimitation using BPP (Supplementary Table 7). These findings are corroborated by genetic distances of ND2 between 7 and 11% (Supplementary Table 4), that are typical of species-level divergences of gekkotan ND2 sequences, for example, from ~5 to 20% (Pepper et al. 2011; Grismer et al. 2014; Pinto et al. 2019).

Genetic Cline Analyses

We conducted allelic assignment by generating gene trees of phased alleles for each locus (Supplementary Figures 1–7). We were unable to confidently assign species-specific alleles for DIDO or MAP2, as

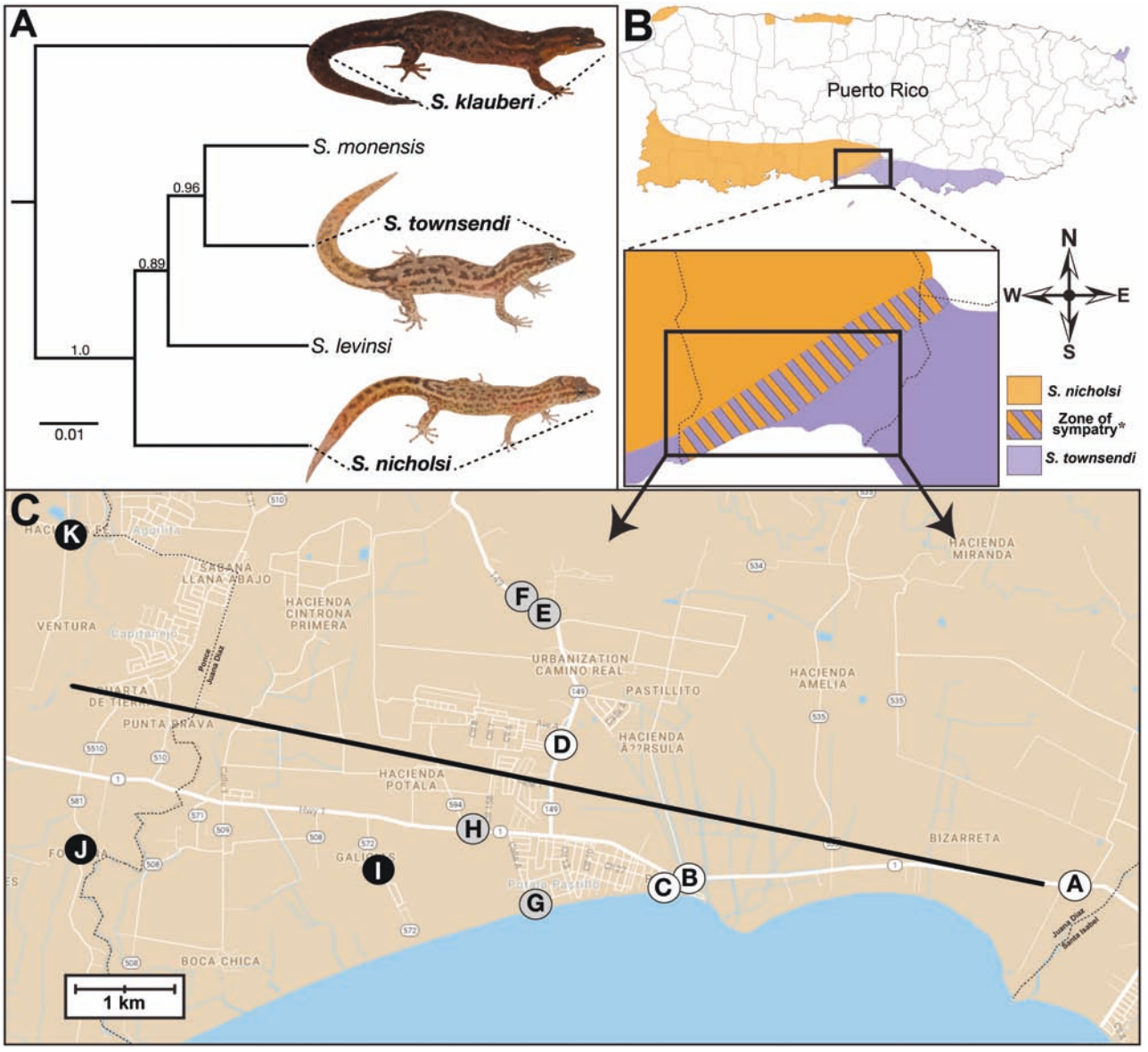

Figure 1. (**A**) StarBeast2 species tree showing phylogenetic relationships among sampled *Sphaerodactylus* species. Numbers along branches indicate posterior probability support for adjacent node. (**B**) Geographic depiction of the current distributions for *S. nicholsi* and *S. townsendi* on Puerto Rico and Caja de Muertos. (**C**) Sampling localities along the putative hybrid zone between *S. nicholsi* and *S. townsendi* in Juana Díaz. Black line indicates the collapsed one-dimensional transect utilized in genetic cline analyses. White circles (**A–D**) indicate the presence of pure *S. townsendi,* black circles (localities **I–K**) represent pure *S. nicholsi,* and gray circles (**E,H**) indicate hybrid populations overlaid on Google Maps®. *Putative contact zone adapted from Murphy et al. (1984) and modified using authors' personal observation.

gene trees showed little, or conflicting, support for species-specific clades (Supplemental Figures 3 and 5). Thus, we removed DIDO and MAP2 from all subsequent cline analyses. For the remaining 5 loci (ND2, CMOS, MACF, PTPN12, and RAG1), we identified well-supported species-specific clades for each parental species (Supplementary Figures 1, 2, 4, 6, and 7). To assess the shape and width of genetic clines, we fitted 4 different models to our data and conducted model testing to discern between different scenarios at each locus (Table 1). For all loci, the best-fit cline model was a fixed allele model with no exponential tail on either side (Table 1). All genetic clines constructed under this model possessed coincidental (same center) and concordant (width and shape) sigmoidal clines for each locus (Table 1; Figure 2). Measuring the width of these clines provided an approximate width of the total hybrid zone at ~1100 m (Table 1).

Species Distribution Models and Simulations

Our species distribution models show overlap between *S. nicholsi* and *S. townsendi* variable raster stacks over the hybrid zone, near Juana Díaz (Figure 3). Topography and elevation create a barrier from expanding north, while vegetation is not a significant factor driving distribution (Supplementary Table 6; Supplementary Figure 8). For BioClim layers, the machine-learning ROC, is well-fitted to the model—with an AUC of 0.879 for *S. nicholsi*. *S. townsendi* is also well-fitted to the model with an AUC of 0.972. *S. nicholsi* had high importance contribution from annual precipitation, precipitation of

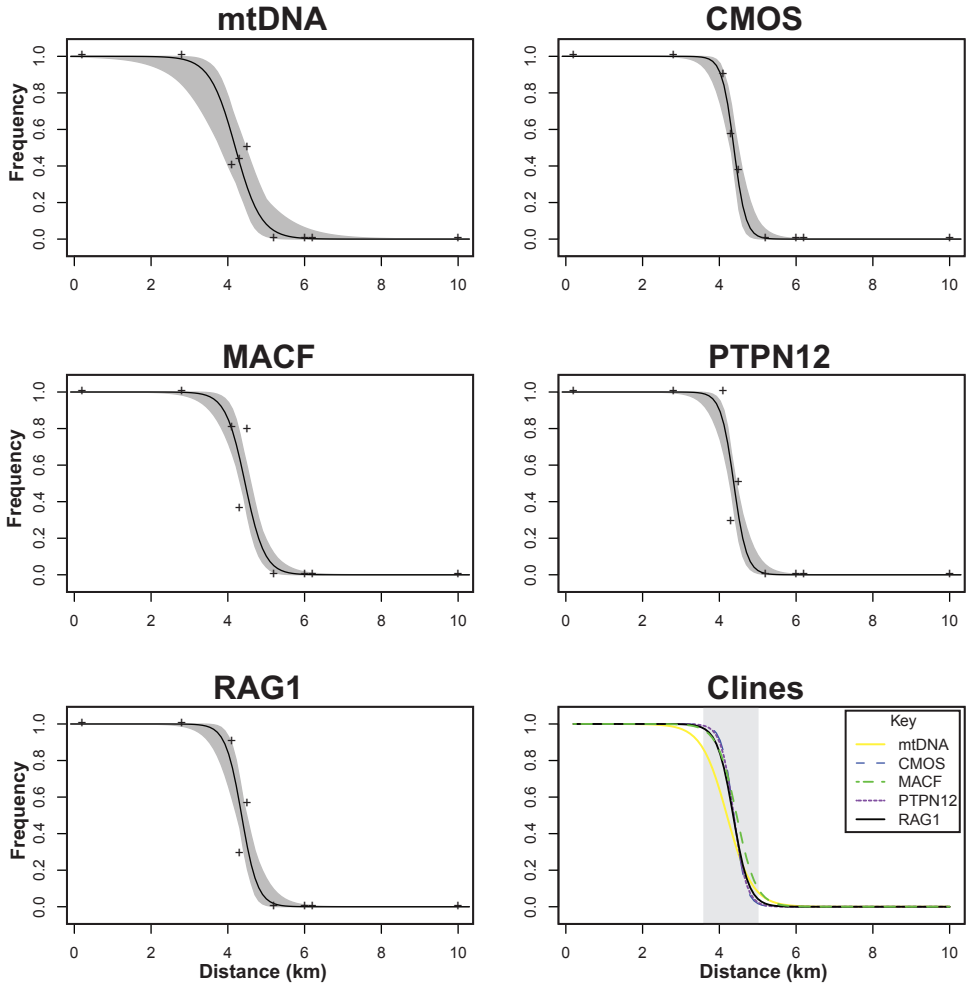

Figure 2. Genetic clines for each locus examined in this study. Frequency is the proportion of species-specific alleles found at each locality where "1" is pure *S. nicholsi* and "0" is pure *S. townsendi* with associated 95% confidence intervals (gray shading). Each plotted point (mtDNA–RAG1) is a sampled locality. Overlaid clines (bottom-right) indicate a common sigmoidal shape across all 5 unlinked markers.

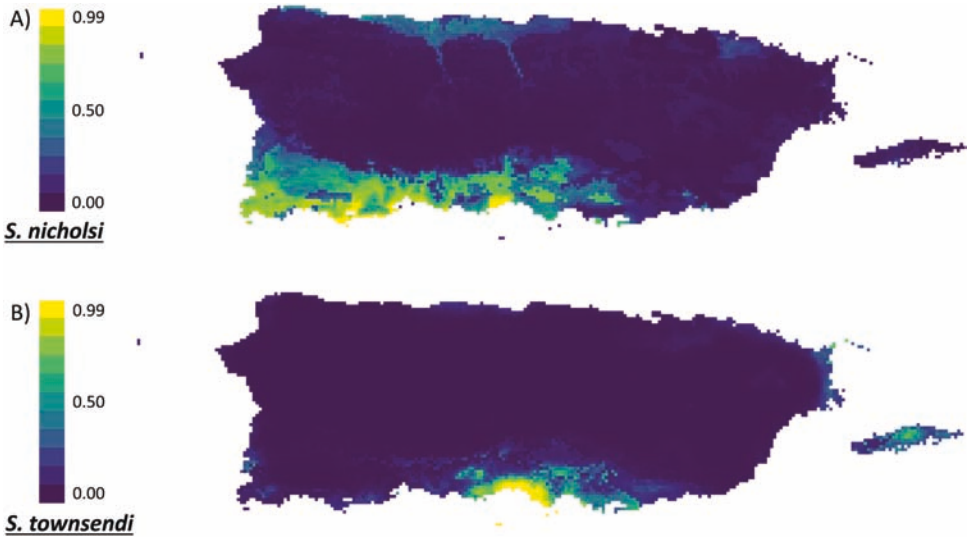

Figure 3. Niche models for (**A**) *S. nicholsi*, and (**B**) *S. townsendi*, generated in Maxent (v3.41). Warmer colors illustrate a higher percent estimation of distribution (climatic suitability) within the lineage's climatic niche space. Juana Díaz is located in the middle of the island, near the epicenter of *S. townsendi*'s predicted range.

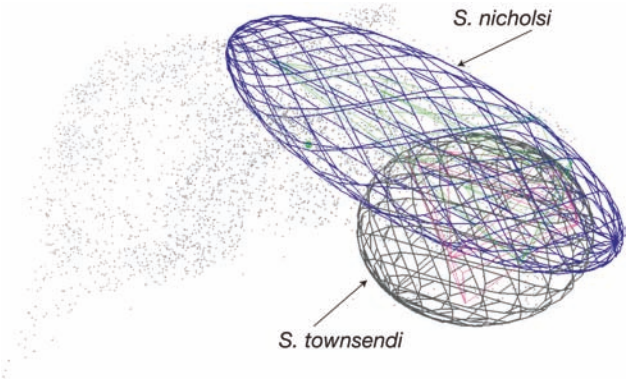

Figure 4. *Sphaerodactylus nicholsi* (dark/blue online only) and *S. townsendi* (light/gray) niche spaces plotted against one another within Puerto Rican environmental and geographic niche space (dots). By plotting 19 BioClim (2.0) layers and species distributions from Figure 3, we calculated the overlap between the Grinnellian niche space of each species, that is, their niche overlap. Percentage of the niche space of *S. nicholsi* overlapped by *S. townsendi* is 17%. Conversely, the percentage of the niche space of *S. townsendi* overlapped by *S. nicholsi* is 70%, indicating strong interspecific competition between species.

the warmest quarter, precipitation seasonality, and max temperature of the warmest month at percent contributions 38.7, 14.9, 7.5, and 7.2%, respectively (Supplementary Table 6). *Sphaerodactylus townsendi* also had similar driving variables with precipitation of the warmest quarter, precipitation seasonality, temperature seasonality, and precipitation of the driest month at 26, 17.2, 10.8, and 10.7%, respectively (Supplementary Table 6). The permutation importance in *S. nicholsi* was driven mainly by mean temperature of wettest quarter and annual precipitation at 19.3 and 14.5%. *Sphaerodactylus townsendi* had a significant driver of 54.2% for precipitation of warmest quarter (Supplementary Table 6).

The simulations of each species' fundamental niche produced niche-space hypervolumes for each species that represent their fundamental niche space. The area where ellipsoids overlap is the shared hypervolume space between each species and this overlap is measured relative to each species (i.e., as a proportion of their total hypervolume). *Sphaerodactylus nicholsi* niche hypervolume accounts for around 17.50% overlap of niche space with *S. townsendi*, while *S. townsendi* hypervolume accounts for around 70.26% overlap with *S. nicholsi* (Figure 4; Supplementary Table 6). Indeed, *S. townsendi* has a smaller fundamental niche compared to *S. nicholsi*, and, although shared niche space overlaps each species range, the only area where both species are in contact within shared niche space a hybrid zone has formed. No other area outside the hybrid zone has shared niche space and an overlapping distribution.

Discussion

To address our original 3 research questions, we conducted statistical species delimitation, genetic cline analyses, and niche modeling, respectively. We found that *S. nicholsi* and *S. townsendi* represent genetically distinct species; this hybrid zone is geographically-stable, and no evidence intermediate habitat near the hybrid zone.

Species Phylogeny and Species Delimitation

The species-level status of *S. townsendi* has been in doubt since its description (Grant 1931) insofar as it was at one point synonymized with *S. nicholsi* as a nominal subspecies (*S. nicholsi townsendi*) (Thomas and Schwartz 1966). Our phylogenetic reconstructions and species delimitation analyses confirm the hypothesis of Murphy et al. (1984) and Grant (1931) that *S. nicholsi* and *S. townsendi* are distinct species. Furthermore, *S. nicholsi* and *S. townsendi* are not sister taxa and an *S. nicholsi sensu lato* that includes a synonymized *S. townsendi* would be rendered paraphyletic by both *S. monensis* and *S. levinsi* (Figure 1a). However, these results do conflict with the only previously published molecular genetic phylogeny for this group, which found *S. townsendi* samples nested deep within *S. nicholsi* to the exclusion of both *S. monensis* and *S. levinsi* (Díaz-Lameiro et al. 2013). To resolve this discrepancy, we briefly reanalyzed Díaz-Lameiro et al.'s (2013) 16S sequence data but added several vouchered *S. townsendi* specimens we collected from populations allopatric with respect to *S. nicholsi*. This revised phylogeny indicates the 2 *S. townsendi* specimens used in Díaz-Lameiro et al. (2013) were misidentified *S. nicholsi* (Supplementary Figure 9). Thus, resolving this phylogenetic discrepancy and rejecting the hypothesis that *S. townsendi* and *S. nicholsi* form a clade to the exclusion of *S. monensis* and *S. levinsi*. These data provide us the ability to reliably distinguish these species at the molecular genetic level, which is a prerequisite of conducting subsequent hybrid zone analyses with genetic data.

Genetic Cline Analyses

Recent work has shown that, although most hybrid zones tend to stay where they first arise (Barton 1979), hybrid zones can move or widen in areas of recent climatic variation or large anthropogenic disturbance (Rohwer et al. 2001; Engebretsen et al. 2016; Leaché et al. 2017; van Riemsdijk et al. 2019). Thus, because both recent climatic fluctuations and anthropogenic disturbance have affected this region, we hypothesized that there was a strong possibility that the hybrid zone is dynamic. We broke this possibility down further into 2 core components: 1) Has the hybrid zone moved in the past ~35 years? and 2) Was this hybrid zone transient or is it geographically fluctuating?

First, we predicted that if the hybrid zone has moved, 1) we would not find the hybrid zone in the same approximate geographic location as Murphy et al. (1984) initially described and/or 2) we would find evidence of asymmetrical introgression (i.e., an exponential tail) leading away from locations described by Murphy et al. (1984). Indeed, although their detection of hybrids was convincing, their sampling around the 2 hybrid localities was somewhat scarce, which left room for alternative explanations. Here, 1) we found hybrids present both within and outside of the 2 localities where Murphy et al. (1984) documented them. Further, by comparing models that allowed asymmetrical introgression to other possible models (Table 1), 2) we found no evidence of asymmetrical introgression and, thus, no evidence of hybrid zone movement within the past ~35 generations ago (~1 generation per year).

Next, if the hybrid zone was transient or is actively fluctuating, we expect to find either no evidence of hybridization across this transect or cline centers would be shifted between these unlinked loci. In fact, we found that this hybrid zone is extant and that cline centers were concordant across all 5 unlinked loci. Indeed, *S. nicholsi* and *S. townsendi* appear to have maintained a geographically stable hybrid zone for ≥35 years. Interestingly, similarly to Murphy et al. (1984), we found no evidence of F_1 hybrids in our sampling. The fact that among 45 hybrids sampled (19 here and 26 sampled by Murphy et al. 1984) no F_1 hybrids were identified may be coincidence. It

could also be evidence of the low dispersal ability of these animals (likely due to their body size, weighing <0.5 g with an average snout-vent length (SVL) 21.7 and 24.6 mm for *S. nicholsi* and *S. townsendi*, respectively; Thomas and Schwartz 1966) and their mosaic occurrence across Juana Díaz (represented by our sampling; Figure 1c). However, this finding is also consistent with a scenario of assortative mating, as mating preference has also been shown to be a strong barrier of neutral alleles, such as those used in this study (Gavrilets and Cruzan 1998). Thus, we can conclude that this hybrid zone is not actively fluctuating over geographic space and selection against hybridization is uniform across the unlinked regions of the genome that we sampled. Overall, these concordant sigmoidal shapes of genetic clines are consistent with a hybrid zone maintained by balanced dispersal and selection or selection for hybrid phenotypes in an area of intermediate habitat (Moore 1977; Barton and Gale 1993).

Species Distribution Models and Simulations

We addressed whether there was evidence to favor a hypothesis of selection for hybridization at a zone of intermediate habitat between these two species using species distribution models and niche simulations. We predicted that if our data supported this hypothesis, then we would find 1) a unique climatic variable, or suite of variables, in the hybrid zone (e.g., sharp transition zone between preferred habitats) and 2) the low degree of fundamental niche overlap, which would provide further evidence that there is a transition in habitat use between parental species.

We used species distribution modeling to evaluate whether or not unique climatic variable(s) in the hybrid zone might favor the production of hybrids. Conversely, we found that both species found suitable habitat extending well beyond the hybrid zone (Figure 3), suggesting no zone of intermediate habitat near the hybrid zone. Next, we used niche simulations to evaluate the degree of (climatic) fundamental niche overlap, which showed strong evidence of similar habitat preference between species. Indeed, *S. nicholsi* has a much broader fundamental niche compared to *S. townsendi* and there is extensive overlap in their climatic niche spaces (Figure 4). The implications of these findings are 2-fold, 1) there is little evidence that there is a possible "zone of intermediate habitat" between these species, and 2) there is evidence for competitive effects (e.g., competitive exclusion; Gause 1934) between *S. nicholsi* and *S. townsendi*. Thus, these data taken together provide little support for the idea that hybridization occurred at a transition zone between the habitable spaces of *S. nicholsi* and *S. townsendi*. Further, the occurrence of this hybrid zone, between two species that should theoretically be in strong competition, in a zone of high anthropogenic disturbance is also interesting and may warrant further investigation.

Further Considerations and Future Directions

Biogeographic Considerations

Murphy et al. (1984) suggested 2 causes for the secondary contact of *S. nicholsi* and *S. townsendi* and, thus, the initiation of this hybrid zone, namely 1) Quaternary glacial cycling or 2) anthropogenic disturbance. Both scenarios are better-informed by our work, but remain untested. We briefly discuss the merits and flaws with relation to our data and present potential steps necessary to distinguish between these alternatives moving forward. 1) The historic availability of habitable landmass in southern Puerto Rico has been in flux for millennia. Indeed, the rising and falling of sea levels during the late-Quaternary likely provided more habitable land mass for both *S. nicholsi* and *S. townsendi* (Heatwole and MacKenzie 1967). Until as recently as 6000 years ago, southern Puerto Rico was connected to both Caja de Muertos (≥ 8.7 km south of PR) and Vieques (≥ 10 km east of PR), 2 places in which *S. townsendi* is currently distributed. This suggests that *S. townsendi* had a much wider distribution previously and has been squeezed into its current distribution by Pleistocene glacial fluctuations, certainly due to elevation tolerance (Supplementary Figure 8) but perhaps also "trapped" against strong competition with *S. nicholsi* to the north and west (Figure 1b). Further, many hybrid zones have been dated to this time period, as glacial fluctuations caused range shifts across taxa and, thus, secondary contact between previously allopatric species (Durrett et al. 2000).

Murphy et al. (1984) also suggested that 2) anthropogenic disturbance due to the sugar cane agricultural boom in the early 20th century provided *S. townsendi* with the means to invade previously inaccessible habitat. However, this hypothesis was heavily predicated on the anecdotal statements that *S. nicholsi* prefers, "interior xeric woodlands" and *S. townsendi* prefers, "more open habitats such as grassy fields, ruderal situations, and open coastal coconut palm (*Cocos*) groves." We have since shown that vegetation type is not strongly associated with either species niche space (Supplementary Figure 8) and, thus, this line of reasoning is inadequate in this system. However likely each of Murphy et al.'s (1984) hypotheses are relative to one another, the data presented here are unable effectively tease them apart. Thus, this should be a goal of future work.

Specifically teasing apart these scenarios would require range-wide, population genomic data. Moving forward, we believe that 2 lines of evidence, inference of the biogeographical history of these species (towards the present) and an estimate to the age of the hybrid zone (towards the past), will be needed to develop a complete picture of historical processes at work in this system. The main biogeographical questions in this system is, what are the spatiotemporal range dynamics of *S. nicholsi* and *S. townsendi*, and when did these species come into secondary contact? These questions can be answered by collecting *S. nicholsi* and *S. townsendi* from their entire current distributions. By examining the phylogenetic relatedness between populations and each population's historical demography, a plausible biogeographic scenario could be constructed to ascertain the approximate time since these species came into secondary contact (Díaz-Lameiro et al. 2013; Reynolds et al. 2017; Pinto et al. 2019). The second line of evidence would attempt to corroborate the proposed date of secondary contact and the specific age of the hybrid zone by using calculations of linkage disequilibrium to estimate how many generations have passed since the establishment of the hybrid zone (Schumer et al. 2014). In essence, if the estimates of secondary contact and the establishment of the hybrid zone coincide, the evidence would favor an "ancient" hybrid zone. However, if these estimates are disparate (i.e., the hybrid zone being much younger than the time since secondary contact), this would provide strong evidence of a recent, possibly anthropogenically mediated, hybrid zone. Thus, these contrasting scenarios provide a spectrum of biogeographical and population genetic predictions to test when sufficient range-wide, genomic data are available for this system.

Behavioral and Morphological Considerations

The occurrence of interspecific reproduction is mediated by a combination of morphological and behavioral factors (Barton and Hewitt 1985; Harrison 1993). When 2 species with homologous morphologies and courting behaviors meet, they are at higher risk for

reproductive infidelity than species that are highly divergent at these traits. *Sphaerodactylus* geckos are ancestrally sexually dichromatic, and most species maintain these differentiations in male and female color and patterning (Grant 1931; Thomas and Schwartz 1966, and citations herein). However, dichromatism has been lost independently multiple times across the genus (Schwartz and Henderson 1991; Regalado 2015), as it was in the ancestor of *S. nicholsi* and *S. townsendi* (Figure 1a). The loss of distinct visual cues in mate recognition may have led to a divergence in courtship and mating strategies between sexually dichromatic species and their monomorphic cousins (Regalado 2012). The Lost Recognition Cue (LRC) model has proposed these divergences in monochromatic versus dichromatic behaviors (Regalado 2015). This model predicts that in the absence of sexual dichromaticism to visually distinguish potential conspecific mates, a behavioral "court–threaten" approach will be utilized when an animal encounters a sexually ambiguous conspecific until the sex of the counterpart becomes clear (Regalado 2003, 2012, 2015). Indeed, this model is conceptually similar to those of sexually dichromatic fishes that lose visual acuity due to water turbidity, thus, losing the ability to distinguish color differences between sexes of different species (Seehausen et al. 1997). Under this LRC model, we can extrapolate that sexually monochromatic species that share a common mate-choice strategy may be more susceptible to reproductive infidelity, such as the case with *S. nicholsi* and *S. townsendi*. However, more promising work has begun to elucidate alternative mate-choice factors, such as chemosensing in reproductive isolation in geckos (Zozaya et al. 2019). Indeed, these findings are also consistent with a hypothesis of assortative mating in this system, which, without a behavioral or morphological barrier to reproduction, chemical signaling may play a role as well. Thus, further investigation into what factors influence prezygotic reproductive barriers in geckos may help uncover why hybridization is underreported in this group.

Conclusions

Overall, we conducted a 3-part study to characterize a putative hybrid zone between *S. nicholsi* and *S. townsendi*. First, we statistically demarcated these nominal taxa as valid species (Figure 1a). Second, we sampled a transect across the municipality of Juana Díaz, PR and identified the best-fit cline models. Third, we attempted to elucidate whether or not this hybrid zone was maintained by dispersal and selection or selection for hybridization at a zone of intermediate habitat and found that these animals overlap so extensively in their habitat. Indeed, we found that *S. nicholsi* and *S. townsendi* represent genetically distinct populations; this hybrid zone is geographically stable; no evidence intermediate habitat near the hybrid zone.

These data provide important findings, both within geckos and across vertebrates more broadly. Indeed, most hybrid zone investigations have taken place within a few select vertebrate clades, while other groups have remained relatively untouched, such as geckos. Studying hybrid zones in a diversity of organisms is essential for understanding the processes that create and maintain biodiversity. However, as reports of hybridization are rare in gecko lizards compared to other groups, the prior knowledge of hybridization in this system provided by Murphy et al. (1984) gave us both a starting line for this investigation and important temporal snapshot of this system. This first example of a geographically stable hybrid zone in geckos provides a natural experiment for further investigation of the evolutionary patterns of postzygotic isolation in a "new" vertebrate clade. Future directions should focus on both the biogeographic and demographic history of these 2 species and their hybrid zone and identifying postzygotic isolating barriers present in this system.

Supplementary Material

Supplementary data are available at *Journal of Heredity* online.

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Author Contributions

B.J.P. designed study, conducted fieldwork, performed laboratory work, analyzed the genetic data, and drafted the manuscript. J.T. conducted fieldwork, species distribution analyses, and wrote accompanying sections. J.D.D. conducted fieldwork, contributed to specimen identification, and provided feedback on early versions of the manuscript. T.G. assisted in design of study and provided feedback on early versions of the manuscript. All authors read and approved the final manuscript prior to submission.

Data Availability

Sequence data is available on GenBank, accession numbers and sample metadata are available in Supplemental Table 1. R code and data used for cline analyses and model testing is available on Figshare (Pinto BJ, doi:10.6084/m9.figshare.7571807.v1).

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