

Sea level, topography and island diversity: phylogeography of the Puerto Rican Red-eyed Coquí, *Eleutherodactylus antillensis*

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Abstract

Quaternary climatic oscillations caused changes in sea level that altered the size, number and degree of isolation of islands, particularly in land-bridge archipelagoes. Elucidating the demographic effects of these oscillations increases our understanding of the role of climate change in shaping evolutionary processes in archipelagoes. The Puerto Rican Bank (PRB) (Puerto Rico and the Eastern Islands, which comprise Vieques, Culebra, the Virgin Islands and associated islets) in the eastern Caribbean Sea periodically coalesced during glaciations and fragmented during interglacial periods of the quaternary. To explore population-level consequences of sea level changes, we studied the phylogeography of the frog *Eleutherodactylus antillensis* across the archipelago. We tested hypotheses encompassing vicariance and dispersal narratives by sequencing mtDNA (c. 552 bp) of 285 individuals from 58 localities, and four nuDNA introns (totalling c. 1633 bp) from 173 of these individuals. We found low support for a hypothesis of divergence of the Eastern Islands populations prior to the start of the penultimate interglacial c. 250 kya, and higher support for a hypothesis of colonization of the Eastern Islands from sources in eastern Puerto Rico during the penultimate and last glacial period, when a land bridge united the PRB. The Río Grande de Loíza Basin in eastern Puerto Rico delineates a phylogeographic break. Haplotypes shared between the PRB and St. Croix (an island c. 105 km south-east of this archipelago) likely represent human-mediated introductions. Our findings illustrate how varying degrees of connectivity and isolation influence the evolution of tropical island organisms.

Keywords: Caribbean Sea, gene flow, human-mediated introductions, island biogeography, isolation, phylogeography, Puerto Rican Bank, quaternary, West Indies

Received 15 September 2011; revision received 26 July 2012; accepted 31 July 2012

Introduction

Ecological and evolutionary studies of island biotas provide fundamental perspectives on speciation, adaptive radiation, community assembly and biogeography

(Losos & Ricklefs 2009). Factors such as isolation, topography, island area and island age contribute to biogeographic patterns (MacArthur & Wilson 1967; Triantis *et al.* 2008; Whittaker *et al.* 2008), with historical fluctuations of sea level hypothesized to play an important role in shaping insular biomes (e.g. Heatwole & MacKenzie 1967; Carine 2005). Quaternary (2.6 mya—present) climatic oscillations changed eustatic sea levels more than 100 m (Waelbroeck *et al.* 2002; Bintanja *et al.* 2005; van Daele *et al.* 2011), altering the size, number

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and degree of isolation of islands worldwide. During interglacial periods, rising sea levels fragmented and reduced the size of terrestrial habitats, leading to population isolation and smaller population sizes, which facilitated evolutionary divergence or local extirpation (Pregill & Olson 1981). As sea levels lowered during glacial periods, terrestrial areas expanded, potentially connecting previously isolated populations and allowing colonization of new areas. Although some studies detected high levels of gene flow (e.g. Malone *et al.* 2003; Heaney *et al.* 2005) and low levels of endemism (e.g. Heatwole & MacKenzie 1967; Rand 1969) in tropical archipelagoes with a history of land-bridge connections, others inferred absence of gene flow between populations and high endemism, despite opportunities for dispersal (e.g. Esselstyn & Brown 2009; Lim *et al.* 2011). This contrast implies that the role of fluctuating sea levels on diversification in tropical insular systems is complex.

We explore the genetic consequences of cyclical sea level fluctuations in the Puerto Rican Bank (hereafter PRB), an archipelago in the eastern Caribbean Sea (Fig. 1a, b) comprised of the main island of Puerto Rico (8768 km²; maximum elevation 1338 m) and the 'Eastern Islands' (east of 65.6°W; c. 340 km²; maximum elevation 500 m), which include Vieques, Culebra, the Virgin Islands (St. Thomas, St. John, Tortola, Virgin Gorda and Anegada) and more than 180 associated small cays. Whereas low sea level during glacial periods led to a land bridge between Puerto Rico and the Eastern Islands, higher sea level during interglacial periods separated the Eastern Islands from each other and from Puerto Rico (Heatwole & MacKenzie 1967; Renken *et al.* 2002). Indeed, Puerto Rico and the Eastern

Islands form distinctive biogeographic clusters (Hedges 1999), suggesting limited gene flow between these regions, despite the periodic emergence of a land-bridge connection.

Distinctive phylogeographic patterns inferred for species in Puerto Rico and the Eastern Islands suggest disparate evolutionary histories. Previous mtDNA studies inferred long-term persistence and diversification in topographically diverse Puerto Rico (e.g. Velo-Antón *et al.* 2007; Rodríguez-Robles *et al.* 2008, 2010; Jezkova *et al.* 2009; Barker *et al.* 2011), with sea level fluctuations promoting speciation in the Eastern Islands (Brandley & de Queiroz 2004; Oneal *et al.* 2010). However, those studies did not examine population-level responses across the archipelago. We herein use multiple loci to examine demographic phenomena (Edwards & Beerli 2000; Hudson & Turelli 2003) and assess the effect of inundations on PRB populations of the Red-eyed Coquí, *Eleutherodactylus antillensis* (Anura: Eleutherodactylidae) Reinhardt and Lütken 1863. This widespread (sea level to 1220 m), predominantly arboreal frog occurs in most larger islands of the PRB (except for Anegada; Henderson & Powell 1999) and is ideal for testing alternative biogeographic scenarios. Because anurans are intolerant of saltwater (Balinsky 1981; Duellman & Trueb 1994), dispersal of *E. antillensis* among islands during high sea level stands has probably been limited, although there are reported instances of frogs crossing salt-water barriers via rafting (Measey *et al.* 2007).

We formulate and test two competing hypotheses to elucidate the relative roles of persistence and dispersal in structuring genetic diversity in a widespread species across a climatically dynamic tropical archipelago. The 'Ancient Eastern Islands Isolation Hypothesis' proposes

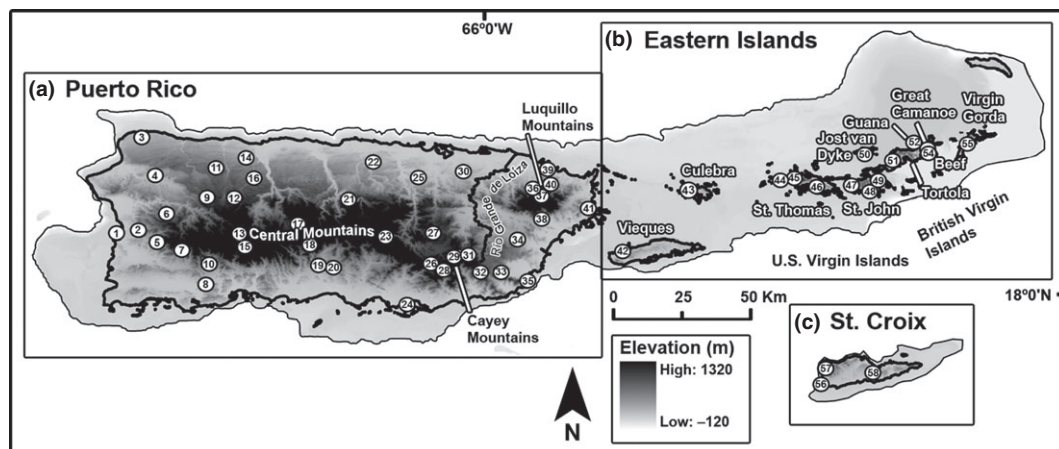


Fig. 1 Map of the Puerto Rican Bank (a and b) and St. Croix (c) illustrating the topography of the islands and the approximate geographic location of the sampling localities of *Eleutherodactylus antillensis* (see Table S1, Supporting Information, for specific locality data). The outermost line in a, b and c indicates the approximate land configuration at maximum sea level (−120 m; Siddall *et al.* 2003) lowering during the Last Glacial Maximum (c. 26.5–19 kya), and the thicker line depicts the current extent of land area.

that *E. antillensis* persisted in isolation in the Eastern Islands since before the start of the penultimate interglacial c. 250 kya (Dutton *et al.* 2009), despite the emergence of a land-bridge connection uniting the PRB during glacial periods between the penultimate interglacial (190–245 kya; Dutton *et al.* 2009), last interglacial (119–130 kya; Siddall *et al.* 2003; Hearty *et al.* 2007; van Daele *et al.* 2011) and Holocene interglacial (c. 0–12 kya; Waelbroeck *et al.* 2002; Siddall *et al.* 2003; van Daele *et al.* 2011). This hypothesis, which predicts deep divergence between populations in the Eastern Islands and Puerto Rico, is based on previous work suggesting that vicariance due to Pliocene or Pleistocene changes in sea level promoted speciation between Puerto Rican and Eastern Islands taxa (Brandley & de Queiroz 2004; Oneal *et al.* 2010). In contrast, the 'Eastern Dispersal Hypothesis' proposes that *E. antillensis* colonized the Eastern Islands from sources in eastern Puerto Rico subsequent to the penultimate interglacial via a land bridge. This hypothesis is based on the premise that in Caribbean land-bridge archipelagoes, topographically complex islands may have lower extinction rates than smaller, lower-elevation islands and act as sources of migrants to these areas during periods of low sea level (Rand 1969; Losos 1996). The Eastern Dispersal Hypothesis predicts relatively recent gene flow (shallow divergence) from populations in eastern Puerto Rico (the putative source area) towards the Eastern Islands populations, with Eastern Islands' samples nesting within clades from eastern Puerto Rico. Prevailing east-to-west ocean currents in the PRB region would have hindered eastward dispersal to the Eastern Islands during brief periods (c. 11–18 kyrs; Hearty *et al.* 2007; Dutton *et al.* 2009) of high sea level during at least three interglacial periods since c. 250 kya, but comparatively long periods of low sea level (Siddall *et al.* 2003; Dutton *et al.* 2009; Rohling *et al.* 2009) produced a land bridge between eastern Puerto Rico and the Eastern Islands that would have facilitated eastward dispersal. We focus on how sea level fluctuations during the last two glacial-interglacial periods shaped genetic diversity in *E. antillensis*, because these events may have produced changes in the size, configuration and isolation of terrestrial habitats (Siddall *et al.* 2003; Dutton *et al.* 2009; van Daele *et al.* 2011) that impacted the connectivity of populations in the PRB (Heatwole & MacKenzie 1967).

We also assess the presence of *E. antillensis* on St. Croix, an island c. 105 km southeast of the PRB (Fig. 1c) that has never had a direct land connection with this archipelago (Gill *et al.* 1989). Although St. Croix has a relatively high proportion of endemics (Heatwole & MacKenzie 1967; Brandley & de Queiroz 2004), several species on this island became established following unintentional human-mediated introductions from

sources in the PRB (Platenberg 2007). We test the hypothesis that the *E. antillensis* population on St. Croix originated from a recent human-mediated introduction to this island (Grant & Beatty 1944). Because the Ancient Eastern Islands Isolation Hypothesis and the Eastern Dispersal Hypothesis address natural historical processes in the PRB, understanding the extent to which human transport may have shaped the distribution of *E. antillensis* is critical.

Materials and methods

Sampling, DNA sequencing, haplotype phasing and sequence alignment

We sampled 285 individuals from 58 localities (avg. 4.6 individuals per locality, range 2–5; Fig. 1a–c; Table S1, Supporting Information) at elevations of 0–981 m from 12 of the 13 islands where *Eleutherodactylus antillensis* occurs (the exception being Great Thatch). A subset of specimens at each locality (except for Virgin Islands National Park, St. John) was euthanized and deposited at the Museum of Southwestern Biology (MSB), University of New Mexico, Albuquerque, or the Museum of Vertebrate Zoology (MVZ), University of California, Berkeley. Tissue samples (liver, thigh muscle and/or toe clips) are from the Division of Genomic Resources, MSB, and the Rodríguez-Robles laboratory at the University of Nevada, Las Vegas.

We included 285 *E. antillensis* in analyses of the mitochondrial (mtDNA) control region (CR) (c. 552 bp). Nucleotide sequences from the nuclear DNA (nuDNA) intron-spanning loci β -crystallin (intron 1; CRYBA, c. 192 bp), myosin heavy chain (putative flanking intron of exon 36; MYH, 463 bp), rhodopsin (intron 1; RH1, 596 bp) and ribosomal protein L9 (intron 4; RPL9int4, 382 bp) were obtained from 173 specimens representing three randomly chosen individuals from each of the 58 localities (except for Great Camanoe, from which only two samples were available). DNA was extracted in a total volume of 30 μ L using a modified CTAB/PVP (cetyl trimethylammonium bromide/polyvinylpyrrolidone)—chloroform/isoamyl alcohol DNA extraction technique (Stewart & Via 1993; M. Perdue, unpublished data). Polymerase chain reactions (PCRs) contained c. 50 ng of template DNA, 10 μ M of each primer, 10 mM dNTP, 25 mM MgCl₂, 2 μ L of 10 $\times$ polymerase reaction buffer and 0.20 units of AmpliTaq Gold *Taq* polymerase (Applied Biosystems, Foster City, CA, USA) and were adjusted to a final volume of 15 μ L with ddH₂O. Sequencing reactions were conducted using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and cleaned with ethanol precipitation. Primer descriptions and PCR

conditions are provided in the Supporting Information (Table S2, Supporting Information).

We edited sequences using SEQUENCHER 4.5 (GeneCodes) and deposited them in GenBank (accession numbers JN385299–JN38696; Table S1, Supporting Information). For heterozygote individuals polymorphic at more than one position, the gametic phase was inferred using PHASE 2.1 (Stephens *et al.* 2001; Stephens & Donnelly 2003). We collapsed multi-base insertion–deletions (indels) in RPL9int4 into single-base polymorphisms for phasing analyses. Five independent runs from different starting seeds were run for 1000 iterations, with a single thinning interval and 100 burn-in iterations. When PHASE could not reconstruct haplotypes with a posterior probability of ≥ 0.85 , PCR fragments were cloned for a minimum of five clones per sample using the pGEM-T-Easy Vector Systems kit (Promega). Finally, we repeated the PHASE analyses using known haplotypes. We aligned sequences in MAFFT 6 (Katoh *et al.* 2002, <http://mafft.cbrc.jp/alignment/server/>). To avoid disproportionate weighting of multi-base indels, we collapsed multi-base indels in the RPL9int4 alignment into single-base polymorphisms for analyses in which indels were included (see below). Alignments were deposited in DRYAD (doi:10.5061/dryad.hc257).

Neutrality, recombination and genetic diversity in *Eleutherodactylus antillensis*

We assessed selective neutrality of each locus with the Hudson–Kreitman–Aguade (HKA) test (Hudson *et al.* 1987), with 10 000 coalescent simulations in HKA (J. Hey, <http://genfaculty.rutgers.edu/hey/software/>), and by using Fu's F_S test (Fu 1997) with 10 000 coalescent simulations in ARLEQUIN 3.1 (Excoffier *et al.* 2005). To investigate recombination in nuDNA loci, we conducted RDP (Martin *et al.* 2005), Bootscan (Salminen *et al.* 1995; Martin *et al.* 2005), Geneconv (Padidam *et al.* 1999), Chimera (Posada & Crandall 2001), MaxChi (Maynard Smith 1992) and SiScan (Gibbs *et al.* 2000) tests in RDP 3 (Martin *et al.* 2010). Recombination was also inferred by calculating the pairwise homoplasy index [Φ_w] (Bruen *et al.* 2006) in SPLITSTREE 4.11.3 (Huson & Bryant 2006). We estimated percentage of fragment length containing segregating sites [$S(\%)$] and nucleotide (π) diversity for each locus in ARLEQUIN. Indels were included in these analyses.

Phylogenetic and network analyses

To test predictions of group structure in Puerto Rico and the Eastern Islands, we performed maximum parsimony, maximum likelihood and Bayesian analyses of CR haplotypes and each nuDNA locus separately.

Phylogenies for CR, CRYBA and MYH were rooted with the closely related *Eleutherodactylus brittoni* and *E. cochranae* (Hedges *et al.* 2008). We lacked RH1 and RPL9int4 sequences for *E. brittoni*, and therefore used *Eleutherodactylus portoricensis* as the second outgroup for these data sets. Because CR evolves rapidly and divergent outgroups can confound phylogenetic analysis due to homoplasy (Brandley *et al.* 2009), we also used mid-point rooting (Hess & De Moraes Russo 2007) for this locus. We constructed parsimony trees and generated nonparametric bootstrap values using PHYLIP 3.68 (Felsenstein 1993). Nodal support was evaluated using 1000 bootstrap replicates, and we used CONSENSE (Felsenstein 1993) to obtain a 50% majority rule consensus tree. Phylogenetic trees were constructed with MEGA 5.05 (Tamura *et al.* 2011) using the maximum likelihood method with 1000 bootstrap replicates and applying the model of nucleotide substitution (CR–GTR + Γ ; CRYBA–HKY; MYH–HKY; RH1–HKY; RPL9int4–HKY + I + Γ) for the data that encompasses the model chosen by AIC in MODELTEST 3.7 (Posada & Crandall 1998). We employed a Bayesian approach to phylogenetic reconstruction implemented in MRBAYES 3.1.2 (Huelsenbeck & Ronquist 2001) using these same models of nucleotide substitution. We ran four chains for 20 000 000 generations each, with default parameters as starting values and sampling every 1000 generations, and repeated the analysis twice to ensure consistency. We discarded the first 5000 trees as burn-in, and checked for stationarity and convergence of the chains with the software TRACER 1.5 (Rambaut & Drummond 2007). Bayesian posterior probabilities (BPP) were obtained from the 50% majority rule consensus of the remaining trees. Indels were included in the maximum parsimony analysis, but excluded in the maximum likelihood and Bayesian analyses. We generated a maximum parsimony network for CR haplotypes with NETWORK 4.2 (<http://www.fluxus-technology.com>) and created geographic visualizations of nuDNA haplotypes using PHYLOGEOVIZ 2.4.4 (Tsai 2011). Indels were included in the NETWORK and PHYLOGEOVIZ analyses.

Genetic structure

To assess genetic structuring in *E. antillensis*, we estimated the number of populations (K) and incorporated sampling locality data in a spatially explicit framework to infer the location of genetic discontinuities among those populations. MtDNA has a higher mutation rate and faster coalescence time than nuDNA introns, and thus we analysed these two marker classes separately to compare patterns of spatial structure inferred from each. To define groups for additional hypothesis testing procedures using MIGRATE and DIYABC (see below),

we analysed the five loci together. Indels were included in these analyses. To explore spatial genetic structure in CR, we conducted a spatial analysis of molecular variance (SAMOVA) in SAMOVA 1.0, which defines groups of populations that are geographically homogenous and maximally differentiated from each other (Dupanloup *et al.* 2002). Using GENELAND 3.2.4, we inferred groups of *E. antillensis* based on nuDNA, and on mtDNA and nuDNA combined, with and without a spatially explicit model (Guillot *et al.* 2005b, 2008), and thus conducted four different analyses. GENELAND infers the number of populations using individual multilocus genotypes and, when provided with coordinates, the spatial location of genetic discontinuities among those populations (Guillot *et al.* 2005a). For nuDNA, each variable site was encoded as an allele. Including mtDNA in GENELAND analyses violates assumptions of Hardy–Weinberg equilibrium within populations and linkage equilibrium between loci, but encoding each mtDNA sequence as a single haplotype is not statistically problematic (G. Guillot, personal communication). For each of the four GENELAND analyses, we examined $K = 1$ –10 distinct groups (five replicates each), with 250 000 Metropolis-coupled Markov chain Monte Carlo iterations recorded every hundredth step after an initial 10% burn-in, and applied the uncorrelated allele frequency model. We accepted the modal K value from the posterior distribution as the most probable number of groups (Guillot *et al.* 2005b). To assess diversity across the range of *E. antillensis*, we used spatial group assignments from GENELAND (see Results; Fig. 2) and estimated the number of haplotypes (h), haplotype diversity (H_d), nucleotide diversity (π) and Fu's F_s (Fu 1997) with 10 000 coalescent simulations for each group using ARLEQUIN. Indels were included in these analyses.

Testing alternative models of gene flow

We constructed gene flow models in accordance with predictions of a priori hypotheses and assessed the models using Bayesian inference in MIGRATE 3.2.7 (Beerli & Felsenstein 2001; Beerli 2006). The model selection procedure inferred parameters using coalescent theory (Kingman 1982), and then ordered models by their Bayes factors (Beerli & Palczewski 2010). We evaluated two models that differed in migration rates between populations in Puerto Rico and the Eastern Islands (Table 1). The Ancient Eastern Islands Isolation Hypothesis predicts an absence of gene flow between populations in Puerto Rico and the Eastern Islands (Model 1), whereas the Eastern Dispersal Hypothesis predicts gene flow from populations in eastern Puerto Rico towards Eastern Islands populations (Model 2). The sampling sites from the PRB were pooled into three groups (western Puerto Rico, eastern Puerto Rico and Eastern Islands) based on results of the GENELAND analyses of all loci (see Results; Fig. 2). We allowed for gene flow in either direction between western and eastern Puerto Rico in the two models. Indels were excluded from these analyses.

Settings in MIGRATE were specified to promote convergence and efficiently sample search space. We applied Felsenstein's (1984) model of nucleotide substitution, with mutation rates estimated from the data and allowed to vary. To promote convergence, we applied uniform prior distributions for Θ and M and used slice sampling. Prior distributions of Θ (mean = 0.08 and range 0–0.8) and M (mean = 50 000 and range 0–100 000) were selected after conducting preliminary runs with broad prior distributions (P. Beerli, personal communication), and then modifying the priors in subsequent runs if posterior distributions were not

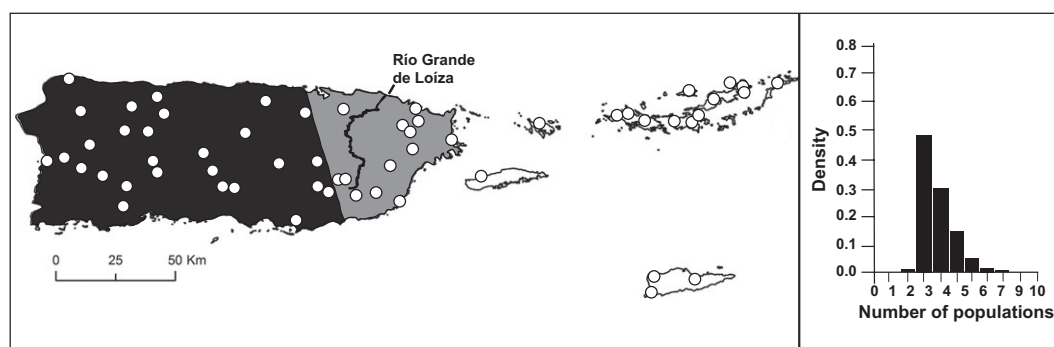


Fig. 2 Results of the GENELAND analysis of *Eleutherodactylus antillensis* based on all loci (mtDNA control region and four nuDNA introns). A map of estimated group membership, indicated by different shades of grey (left panel), and a histogram of posterior probability values for each value of K (number of populations; right panel) is presented. Groups correspond to those indicated in Fig. 3 and Tables 1–5 as follows: western Puerto Rico (black), eastern Puerto Rico (grey) and Eastern Islands (white). The location of the Río Grande de Loíza in eastern Puerto Rico is indicated.

Table 1 Models of gene flow used for testing the Ancient Eastern Islands Isolation Hypothesis and the Eastern Dispersal Hypothesis in MIGRATE 3.2.7

	Ancient Eastern Islands Isolation Hypothesis Model 1		Eastern Dispersal Hypothesis Model 2	
				
All samples				
Bézier-corrected log marginal likelihood	–5180		–4986	
Log Bayes factor	–194		0	
Rank of model	2		1	
Excluding potentially introduced samples				
Bézier-corrected log marginal likelihood	–4932		–4773	
Log Bayes factor	–159		0	
Rank of model	2		1	

The Bézier-corrected log marginal likelihood, log Bayes factor and rank of each model are indicated. WPR, western Puerto Rico, EPR, eastern Puerto Rico, EIS, Eastern Islands.

contained within the bounds of the prior distribution. After a burn-in period of 100 000 generations, we sampled every 100 steps from one long chain (50 000 steps). To calculate Bézier-corrected log marginal likelihoods, we applied thermodynamic integration under a static heating scheme (Beerli & Felsenstein 2001) of four chains (temperatures 1.0, 1.5, 3.0, 10⁴). To efficiently sample search space when calculating parameters, we applied the randomtree index, set the replicate index to 20 and combined chains from all replicates. This simulation was repeated five times to ensure consistency. In subsequent runs, we changed the random number seed and used Bayesian estimates of Θ and M from the previous run as starting values (Beerli & Felsenstein 2001). We calculated the natural log Bayes factor following Beerli & Palczewski (2010).

Testing alternative models of divergence

Using DIYABC 1.0.4.46 (Cornuet *et al.* 2010), we performed coalescent simulations (Hudson 1990) in an approximate Bayesian computation (ABC) framework to explore the history of divergence in populations of *E. antillensis* in the PRB. We generated a divergence model for each hypothesis. Both models of divergence included eastern and western Puerto Rican groups (see GENELAND Results; Fig. 2), and only differed in the timing of divergence of the Eastern Islands group (Fig. 3). Indels were excluded from these analyses.

The priors for all demographic parameters had uniform distributions that were bound by specified minimum and maximum values (Table 2). Each model incorporated an ancestral effective population size (N_a , prior distribution: 10–1 000 000) and an effective

population size for populations in western Puerto Rico (N_1 , prior distribution: 10–6 000 000), eastern Puerto Rico (N_2 , prior distribution: 10–5 000 000) and the Eastern Islands (N_3 and N_4). We set the prior distribution for the effective population size of Eastern Islands populations lower in the divergence model for the Eastern Dispersal Hypothesis (N_4 , prior distribution: 10–2 000 000) than in the divergence model for the Ancient Eastern Islands Isolation Hypothesis (N_3 , prior distribution: 0–4 000 000), because spatial expansion into the Eastern Islands by a small number of founder individuals may have lowered the effective population size there. We adjusted the upper boundary for the prior distribution for effective population size parameters to the geographic size of eastern Puerto Rico and the Eastern Islands relative to western Puerto Rico because genetic variation may be lower in smaller populations (Frankham 1996), and the values are reasonable with respect to the estimated density of adult male *E. antillensis* per hectare on Guana Island (Ovaska 2005) and the large effective population sizes attained by other eleutherodactyline frogs (Crawford 2003a; Barker *et al.* 2011). The divergence model for the Ancient Eastern Islands Isolation Hypothesis depicts the Eastern Islands group diverging from western and eastern Puerto Rican groups between the start of the penultimate interglacial and the early middle Pleistocene (t_1 , prior distribution: 250 000–500 000 generations), and the western and eastern Puerto Rican groups diverging between the Holocene interglacial and the early middle Pleistocene (t_2 , prior distribution: 1–500 000 generations; Fig. 3). We used the ‘set condition’ option to impose the rule that $t_1 > t_2$ because the Ancient Eastern Islands Hypothesis predicts deep divergence between Puerto Rican and

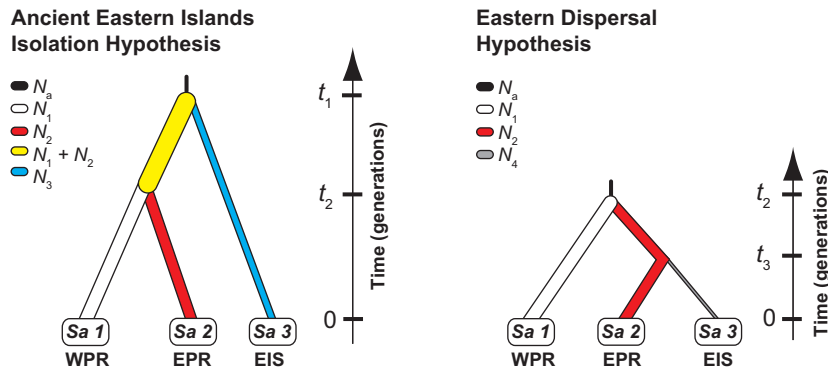


Fig. 3 The two models of divergence for *Eleutherodactylus antillensis* tested in DIYABC 1.0.4.46 differ in the estimated divergence time for Puerto Rican and Eastern Islands populations and the effective population size of the Eastern Islands populations. The divergence model for the Ancient Eastern Islands Isolation Hypothesis depicts the Eastern Islands group diverging from western and eastern Puerto Rican groups between the start of the penultimate interglacial and the early middle Pleistocene (t_1 prior distribution = 250 000–500 000 generations), despite the emergence of a land-bridge connection between this region and Puerto Rico during the penultimate and last glacial periods. The divergence model for the Eastern Dispersal Hypothesis depicts relatively shallow divergence (t_3 prior distribution: 1–250 000 generations) between Eastern Islands and Puerto Rican groups due to colonization of the Eastern Islands from populations in eastern Puerto Rico (the putative source area) during the penultimate and last glacial periods, which resulted in a small effective population size in the Eastern Islands. In both models, the eastern and western Puerto Rican groups began diverging between the Holocene interglacial and the early middle Pleistocene (t_2 prior distribution: 1–500 000 generations). Time is number of generations, with a generation length of 1 year. Sa 1 comprises samples from western Puerto Rico (WPR); Sa 2 comprises samples from eastern Puerto Rico (EPR); and Sa 3 comprises samples from the Eastern Islands (EIS), based on the GENE-LAND analysis. The ancestral effective population size (N_a) and effective population size for WPR (N_1), EPR (N_2) and EIS (N_3 and N_4) groups are indicated. Times and population sizes are not to scale.

Eastern Islands populations. In contrast, the divergence model for the Eastern Dispersal Hypothesis depicts the western Puerto Rican group diverging from the eastern Puerto Rican group between the Holocene interglacial and the early middle Pleistocene (t_2), and the Eastern Islands and eastern Puerto Rican groups diverging after *E. antillensis* colonized the Eastern Islands via a land bridge between the Holocene interglacial and the penultimate interglacial (t_3 , prior distribution: 1–250 000 generations). We imposed the rule that $t_2 > t_3$ for this model because the Eastern Dispersal Hypothesis predicts shallow divergence between eastern Puerto Rican and Eastern Islands populations. Time was number of generations, with a generation length of 1 year, based on observations that *E. antillensis* may reach reproductive maturity in the subsequent wet season after hatching (Ovaska 2005), and on studies of the closely related Puerto Rican Coquí, *Eleutherodactylus coqui* (Stewart & Woolbright 1996). We conducted preliminary runs with broad prior distributions and modified the priors in subsequent runs if posterior distributions were not contained within the bounds of the prior distribution.

Obtaining meaningful estimates of historical demographic parameters (e.g. gene flow, divergence times, effective population size) depends in part on the accuracy of estimates of mutation rates for individual loci (Hey & Nielson 2004). The lower boundary for the CR mutation

rate was set to $\mu = 0.96 \times 10^{-8}$ nucleotide substitutions per site per year (sub/s/y), based on estimates for nucleotide substitution rates for mtDNA in eleutherodactyline frogs (Crawford *et al.* 2007). Because this rate was estimated from coding regions of mtDNA, which may evolve more slowly than CR, we estimated an upper boundary for CR by inferring its rate relative to a coding mtDNA locus, cytochrome *b* (cyt *b*), using sequences collected from *E. portoricensis* (Barker *et al.* 2011) (cyt *b* accession numbers—HM229960–HM229995; CR accession numbers—HM229817, HM229820, HM229826, HM229828, HM229832, HM229838, HM229845, HM229846, HM229849, HM229857, HM229860, HM229862, HM229870, HM229871, HM229876, HM229881, HM229884, HM229885, HM229896, HM229898, HM229899, HM229910, HM229911, HM229919, HM229923, HM229926, HM229931–HM229933, HM229935, HM229938, HM229940, HM229941, HM229944, HM229945, HM229955). Mutation rates of a particular marker can vary across species (Nabholz *et al.* 2009; Lanfear *et al.* 2010), but we did not collect a comprehensive cyt *b* data set for *E. antillensis* because preliminary work indicated that CR had higher levels of variation. We compared the overall mean mtDNA CR and cyt *b* genetic distances (*p*-distances) among individuals of *E. portoricensis* using MEGA 5.05 and determined that the nucleotide substitution rate of mtDNA CR was *c.* 4.2 times that of cyt *b*. Based on an estimated coding mtDNA

Table 2 Prior distributions used for the coalescent simulations (performed in DIYABC 1.0.4.46) of models of genetic divergence for *Eleutherodactylus antillensis* groups in western Puerto Rico (WPR), eastern Puerto Rico (EPR) and the Eastern Islands (EIS)

Symbol	Description	Prior distribution
Demographic parameters		
N_a	Ancestral effective population size	Uniform ($10, 1 \times 10^6$)
N_1	Effective population size of WPR group	Uniform ($10, 6 \times 10^6$)
N_2	Effective population size of EPR group	Uniform ($10, 5 \times 10^6$)
N_3	Effective population size of EIS group in divergence model for Ancient Eastern Islands Isolation Hypothesis	Uniform ($10, 4 \times 10^6$)
N_4	Effective population size of EIS group in divergence model for Eastern Dispersal Hypothesis	Uniform ($10, 2 \times 10^6$)
t_1	Divergence time between the start of the penultimate interglacial and the early middle Pleistocene	Uniform (250.0, 500.0)
t_2	Divergence time between the Holocene interglacial and the early middle Pleistocene	Uniform (0.001, 500.0)
t_3	Divergence time between the Holocene interglacial and the penultimate interglacial	Uniform (0.001, 250.0)
Mutation rate parameters		
μ , mitochondrial DNA	Mean mutation rate (per site per generation)	Uniform ($0.96 \times 10^{-8}, 4.1 \times 10^{-8}$)
	Individual locus mutation rate (per site per generation)	Gamma ($0.96 \times 10^{-8}, 4.1 \times 10^{-8}$) Hasegawa, Kishino and Yano (HKY) model, % invariant sites = 83, shape of gamma = 0.73
μ , autosomal diploid DNA	Mean mutation rate (per site per generation)	Uniform ($6.0 \times 10^{-10}, 4.1 \times 10^{-9}$)
	Individual locus mutation rate (per site per generation)	Gamma ($6.0 \times 10^{-10}, 4.1 \times 10^{-9}$) Hasegawa, Kishino and Yano (HKY) model, % invariant sites = 0, shape of gamma = 2

We used uniform prior distributions for demographic parameters. Divergence time is in thousands of generations. We used the 'set condition' option to impose the rule that $t_1 > t_2$ for the model of genetic divergence based on the Ancient Eastern Islands Isolation Hypothesis, because this hypothesis predicts deep divergence between Puerto Rican and Eastern Islands populations. In contrast, we imposed the rule that $t_2 > t_3$ for the model of genetic divergence for the Eastern Dispersal Hypothesis, because this hypothesis predicts shallow divergence between eastern Puerto Rican and Eastern Islands populations. Prior distributions for the mutation rate (μ) were drawn from a uniform distribution across loci, and individual locus mutation rates were then drawn from a gamma distribution to allow for rate heterogeneity across sites. Models of nucleotide substitution are provided.

rate of 0.96×10^{-8} sub/s/y, we estimated that CR evolves at a rate of 4.1×10^{-8} sub/s/y per lineage. This estimate is refined from an earlier study of genetic diversification in *E. portoricensis* (Barker *et al.* 2011). In two *E. antillensis* individuals for which both CR and cyt *b* sequences were available, CR had approximately three times as much variation as cyt *b*, and therefore we believe that a rate of 4.1×10^{-8} sub/s/y for CR is appropriate for this species. The minimum (6.0×10^{-10} sub/s/y) and maximum (4.1×10^{-9} sub/s/y) values of μ for the nuDNA introns were based on a lower boundary estimate of anuran nuDNA (Crawford 2003a), and a tenfold slower rate than our upper boundary for mtDNA (Sheldon *et al.* 2000; Crawford 2003b), respectively.

We simulated 1 000 000 data sets for the Ancient Eastern Islands Isolation Hypothesis and the Eastern Dispersal Hypothesis and used the number of haplotypes, number of segregating sites, average number of pairwise differences and Tajima's *D* as summary statistics to

compare observed and simulated data sets. We selected these summary statistics based on their performance in previous ABC studies (Hickerson *et al.* 2006; Beaumont 2008). The 1000 and 10 000 simulated data sets with summary statistics most similar to the observed data were identified through the direct and logistic regression rejection steps of the ABC algorithm and used for ABC estimation of parameters.

We evaluated the appropriateness of each model of genetic divergence and its associated parameters and performed model-checking computations as empirical verifications of the performance of the ABC procedure. To check that at least one combination of and priors produced simulated data sets similar to our observed data, we used the 'Pre-evaluate scenario-prior combinations' option in DIYABC that involved a principal component analysis (PCA) of the first 100 000 simulated data sets (Cornuet *et al.* 2010). As part of the model-checking procedure, we conducted PCA on test quantities

obtained with the model-posterior combination from the most strongly supported model, together with 10 000 pseudo-observed data sets (Cornuet *et al.* 2010). We evaluated the 'goodness-of-fit' of each model by using the 'Confidence in scenario choice' option in DIYABC and simulated 500 data sets per model with parameter values drawn from the same prior distributions as the models. From these results, we calculated the proportion of times that the most strongly supported model did not have the highest posterior probability when it was the true model (type I error), as well as the proportion of times that the least supported model had the highest posterior probability when it was not the true model (type II error). Because these error rates are computed from Bayesian posterior probabilities, they are not strict type I and type II errors in a classical frequentist framework, whereby the null hypothesis is only completely rejected or completely supported (Bertorelle *et al.* 2010).

Results

Neutrality, recombination and genetic diversity in Eleutherodactylus antillensis

Nucleotide variation in the five loci was consistent with neutral expectations (HKA test, $\chi^2 = 6.64$, d.f. = 4, $P = 0.16$), but significantly negative values of Fu's F_S for CR ($F_S = -25.66$, $P = 0.0002$) and RPL9int4 ($F_S = -23.16$, $P = 0.0002$) indicate selection or recent population expansion (Fu 1997). We did not detect recombination in the nuDNA data sets. The mtDNA CR data set contained 98 haplotypes with 49 variable positions; seven were single-base pair indels. The four nuDNA loci totalled c. 1633 bp and exhibited varying levels of polymorphism (Table 3), with CRYBA the least variable (five variable positions; one a single-base pair indel), and RPL9int4 the most variable (22 variable positions; two single-base pair indels and one five-base pair indel). MYH and RH1 did not contain indels.

Phylogenetic and network analyses

Phylogenetic analyses of CR revealed a shallow, largely unresolved topology. We present the results of the phylogeny rooted with outgroup taxa (Fig. S1, Supporting Information) because it was not appreciably different from the mid-point rooted phylogeny. Only a single clade (which contained three haplotypes) was well supported across maximum parsimony, maximum likelihood and Bayesian analyses, and therefore most CR haplotypes form a basal polytomy. Phylogenetic analyses of nuDNA loci showed varying degrees of resolution, but each indicated that most genetic structuring

Table 3 Descriptive statistics for the five loci used in this study

Locus	L (bp)	S (%)	π	Western Puerto Rico				Eastern Puerto Rico				Eastern Islands						
				N	<i>h</i> (# priv)	H_d	π	Fu's F_S	N	<i>h</i> (# priv)	H_d	π	Fu's F_S	N	<i>h</i> (# priv)	H_d	π	Fu's F_S
mtDNA																		
CR	552	49 (8.9)	0.0065	140	51 (45)	0.933	0.0055	-26.379*	63	27 (19)	0.939	0.0063	-16.183*	67	30 (27)	0.953	0.0060	-21.721*
nuDNA																		
CRYBA	192	5 (2.6)	0.0019	84	4 (1)	0.146	0.0020	-0.774	39	4 (1)	0.376	0.0031	-0.145	41	3 (1)	0.180	0.0010	-1.318
MYH	463	9 (1.9)	0.0017	84	5 (2)	0.115	0.0004	-4.033*	39	6 (2)	0.594	0.0018	-1.711	41	5 (2)	0.653	0.0023	0.522
RH1	596	8 (1.3)	0.0018	84	4 (1)	0.623	0.0021	2.684	39	6 (3)	0.503	0.0013	-1.490	41	4 (1)	0.534	0.0010	-0.090
RPL9int4	382	22 (5.8)	0.0065	84	13 (7)	0.500	0.0034	-4.564	39	29 (20)	0.944	0.0096	-16.302*	41	9 (3)	0.499	0.0042	-1.309

Sequence length (L, including alignment gaps; bp, base pairs), number of segregating sites (S), with percentage of sequence length in parentheses, and nucleotide diversity (π) are shown. The following statistics are reported for western Puerto Rican, eastern Puerto Rican and Eastern Islands populations of *Eleutherodactylus antillensis*: sample size (N), number of haplotypes (*h*), with number of private haplotypes in parentheses, haplotype diversity (H_d), nucleotide diversity (π) and Fu's F_S . Significant F_S values at $P < 0.02$ are indicated with an asterisk.

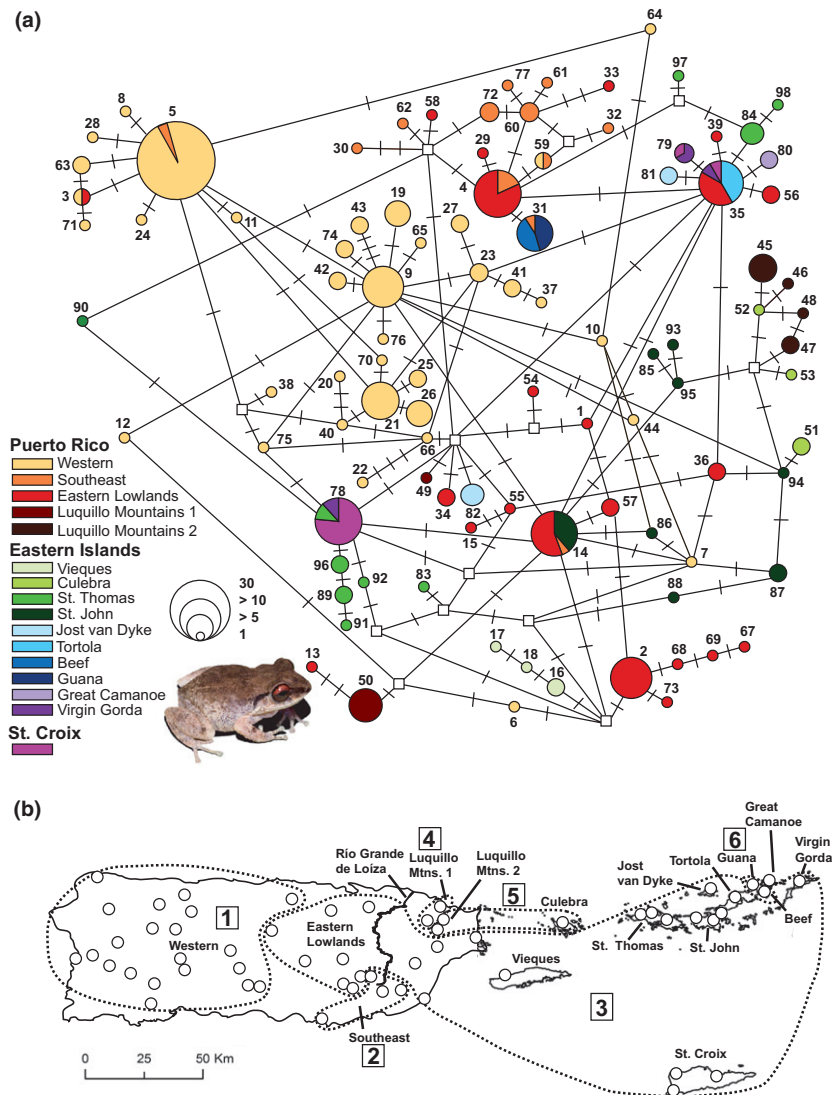


Fig. 4 (a) Maximum parsimony network representing the relationships among haplotypes of the mtDNA control region (CR) of 285 individuals of *Eleutherodactylus antillensis* (inset image; photograph by David Dennis). Indels were included in this analysis. Circles represent unique haplotypes; hatch marks depict single mutations; empty squares indicate missing (i.e. extant unsampled or extinct ancestral) haplotypes. Circle size is proportional to haplotype frequency, and the colour of each circle indicates the individual island from which the haplotype was sampled, except for Puerto Rico, where circles are coloured according to SAMOVA results. Numbers designating haplotypes correspond to those in Table S1 and Fig. S1 (Supporting Information). (b) Genetic partitions of populations of *E. antillensis* identified by the SAMOVA analysis of the mtDNA CR for $K = 6$, where K is the number of predefined groups. Groups are depicted with dashed lines. When $K = 6$, the first group comprises all but 10 localities west of the Río Grande de Loíza in Puerto Rico (Western); the second group contains four localities in south-eastern Puerto Rico (Southeast); the third group consists of 12 eastern Puerto Rican localities (Eastern Lowlands), St. Croix and all Eastern Islands, except Culebra, Beef and Guana; the fourth group is made up of two localities in the Luquillo Mountains region (Luquillo Mountains 1); the fifth group comprises two localities in the Luquillo Mountains region (Luquillo Mountains 2) and Culebra; and the sixth group contains populations from Beef and Guana.

in *E. antillensis* occurs within Puerto Rico (Fig. S2, Supporting Information). Most Eastern Islands samples fall within an unresolved polytomy (CRYBA) and/or within clades that also contain Puerto Rican samples (MYH, RH1, RPL9int4), although most of these clades were not well supported across all three analyses. The

MYH and RH1 phylogenies recovered a well-supported clade containing three or fewer Eastern Islands samples across all three analyses.

The maximum parsimony network revealed that three CR haplotypes are shared between Puerto Rico and the Eastern Islands (H14, H31, H35; Fig. 4a). We

ran a MIGRATE and DIYABC analysis that excluded samples with these CR haplotypes to account for the possibility of human transport of *E. antillensis* in the PRB (see Discussion). Most Eastern Islands populations contain shallowly differentiated private CR haplotypes. All three CR haplotypes in St. Croix (H35, H78, and H79) are shared with populations from Virgin Gorda, Puerto Rico, St. Thomas, and/or Tortola. Three CRYBA haplotypes, five MYH haplotypes, four RH1 haplotypes and nine RPL9int4 haplotypes occur in the Eastern Islands, but fewer than half of those are endemic to the region. Endemic nuDNA haplotypes are usually restricted to a single-island population (e.g. CRYBA, MYH, RH1; Fig. S3, Supporting Information) and occur at low frequencies. There are no unique nuDNA haplotypes in St. Croix.

Genetic structure

Spatial genetic structure inferred from CR, nuDNA loci and all five loci combined identified a distinctive group of *E. antillensis* in western Puerto Rico, but varied with respect to eastern Puerto Rico and the Eastern Islands. SAMOVA analyses of CR did not identify the precise number of populations (K) displaying the highest differentiation among groups (F_{CT}), because F_{CT} values increased as K increased. Some groups contained a single population when $K \geq 7$, indicating that group structure was declining, and thus we present results for $K = 6$ (c.f. Heuertz *et al.* 2004; Fig. 4b). GENELAND analyses with and without a spatially explicit model inferred three groups ($K = 3$) for nuDNA and for the loci combined. The spatial location of genetic discontinuities among populations was identical in analyses of nuDNA and the combined five loci and depicted one group located west of the Río Grande de Loíza in Puerto Rico, a second one comprising all but three sites east of the Río Grande de Loíza, and a

third one occurring in the Eastern Islands and St. Croix (Fig. 2).

Genetic parameters for populations in western Puerto Rico, eastern Puerto Rico and the Eastern Islands varied across loci. For CR, populations from eastern Puerto Rico had fewer private haplotypes than those from western Puerto Rico and the Eastern Islands (Table 3). Populations from all three groups had significantly negative values of F_u 's F_S for CR. For the four nuDNA loci, individuals from the Eastern Islands had fewer haplotypes than those from eastern Puerto Rico (Table 3). Populations in eastern Puerto Rico had significantly negative values of F_u 's F_S for RPL9int4, whereas populations in western Puerto Rico had significantly negative values of F_u 's F_S for MYH.

Assessing alternative models of gene flow and divergence

MIGRATE produced consistent parameter estimates across replicates and provided stronger support for the Eastern Dispersal Hypothesis (Model 2) than for the Ancient Eastern Islands Isolation Hypothesis (Model 1; Table 1). The migration rate (M) of *E. antillensis* from populations in eastern Puerto Rico into Eastern Islands populations was significantly larger than zero, and the scaled effective population size (Θ) of the eastern Puerto Rican group was significantly larger than that of the western Puerto Rican and Eastern Islands groups (Table 4). Overlapping 95% confidence intervals (CIs) indicated that differences in the median M of the groups were not significant. Analyses excluding potentially introduced samples produced similar results.

The DIYABC analysis supported the Eastern Dispersal Hypothesis (posterior probability = 0.97; 95% CI: 0.95–0.98), with estimated divergence dates of 52.4 kya (95% CI: 2.7–207.0 kya) between eastern Puerto Rican and Eastern Islands groups, and of 89.4 kya (95% CI:

Table 4 Scaled effective population sizes (Θ) and scaled migration rates (M) estimated from the Bayesian MIGRATE 3.2.7 analysis (Model 2) for all loci of western Puerto Rican (WPR), eastern Puerto Rican (EPR) and Eastern Islands (EIS) populations of *Eleutherodactylus antillensis*

	Θ			M		
	WPR	EPR	EIS	EPR to WPR	WPR to EPR	EPR to EIS
All samples	0.0061 (0–0.0112)	0.0500 (0.0204–0.0754)	0.0077 (0.0008–0.0142)	383 (0–1100)	983 (0–1867)	1583 (300–2833)
Excluding potentially introduced samples	0.0059 (0–0.0108)	0.0707 (0.0372–0.0828)	0.0143 (0.0044–0.0254)	417 (0–1200)	2217 (633–3933)	1283 (133–2300)

Values are presented as the median of 20 replicate MIGRATE runs per data set, with the 95% confidence intervals in parentheses.

$\Theta = N_e\mu$ for mtDNA and $4N_e\mu$ for nuclear DNA, where N_e = effective population size, and μ = mutation rate per site per generation. $M = m/\mu$, where m = immigration rate.

Table 5 Posterior probabilities (*PP* [95% CI]) and parameter estimates of models of genetic divergence for *Eleutherodactylus antillensis* in Puerto Rico and the Eastern Islands tested in *DIYABC* 1.0.4.46

Hypothesis	PP	Demographic parameters						Mutation parameters			
		N_a	N_1	N_2	N_3	N_4	t_1	t_2	t_3	Mitochondrial DNA μ	Autosomal diploid DNA μ
Ancient Eastern Islands Isolation											
All data	0.03 (0.02–0.05)	4.3×10^5 (1.3×10^5)	2.8×10^6 (1.3×10^6)	3.4×10^6 (1.6×10^6)	2.2×10^6 (1.0×10^6)		270.0 (251.0–428.0)	208.0 (13.0–418.0)		1.1×10^{-8} (9.7×10^{-9})	7.4×10^{-10} (6.0×10^{-10})
		-8.8×10^5	-4.9×10^6	-4.8×10^6	-3.6×10^6					-2.2×10^{-8}	-2.9×10^{-9}
Excluding potentially introduced samples	0.10 (0.06–0.13)	4.0×10^5 (8.9×10^4)	1.2×10^6 (4.6×10^5)	1.2×10^6 (4.0×10^5)	4.4×10^5 (1.6×10^5)		347.0 (254.0–489.0)	282.0 (31.4–461.0)		1.1×10^{-8} (9.7×10^{-9})	1.5×10^{-9} (6.0×10^{-10})
		-8.8×10^5	-2.6×10^6	-3.2×10^6	-1.4×10^6					-2.4×10^{-8}	-4.0×10^{-9}
Eastern Dispersal											
All data	0.97 (0.95–0.98)	2.5×10^5 (7.4×10^4)	2.5×10^6 (9.2×10^5)	3.6×10^6 (1.7×10^6)		1.3×10^6 (4.8×10^5)		89.4 (19.2–311.0)	52.4 (2.7–207.0)	2.3×10^{-8} (9.7×10^{-9})	2.1×10^{-9} (6.0×10^{-10})
		-7.2×10^5	-5.4×10^6	-4.9×10^6		-2.0×10^6				-3.8×10^{-8}	-4.0×10^{-9}
Excluding potentially introduced samples	0.90 (0.87–0.94)	3.5×10^5 (1.0×10^5)	2.1×10^6 (7.7×10^5)	2.0×10^6 (7.2×10^5)		4.7×10^5 (9.8×10^4)		220.0 (30.4–473.0)	114.0 (0.8–241.0)	1.3×10^{-8} (9.7×10^{-9})	1.4×10^{-9} (6.0×10^{-10})
		-8.4×10^5	-5.3×10^6	-4.7×10^6		-1.7×10^6				-2.9×10^{-8}	-3.9×10^{-9}

Median parameter estimates (95% CI) for the following demographic parameters are given below for each model: ancestral effective population size (N_a); effective population size for western Puerto Rican (N_1), eastern Puerto Rican (N_2) and Eastern Islands (N_3 and N_4) groups; t_1 = divergence time (in thousands of generations) between the start of the penultimate interglacial and early middle Pleistocene; t_2 = divergence time (in thousands of generations) between the Holocene interglacial and the early middle Pleistocene; and t_3 = divergence time (in thousands of generations) between the Holocene interglacial and the penultimate interglacial. Median parameter estimates for mutation rate (μ) parameters are also provided.

19.2–311.0 kya) for the split between western and eastern Puerto Rican groups (Table 5). The eastern Puerto Rican group had a larger estimated effective population size (N_2) than those in western Puerto Rico (N_1) and the Eastern Islands (N_4), but the overlapping 95% CIs indicated that these differences were not significant. The Ancient Eastern Islands Isolation Hypothesis had much lower support (posterior probability = 0.03; 95% CI: 0.02–0.05). Pre-evaluation of models showed that the observed data set was surrounded by simulated data sets for each model, indicating that the simulations produced data sets similar to the observed one (Fig. S4, Supporting Information). Additionally, the observed data set was surrounded by many simulated pseudo-observed data sets of the Eastern Dispersal Hypothesis model (Fig. S5, Supporting Information), reflecting a good fit of the model-posterior combination to the pseudo-observed data set (Cornuet *et al.* 2010). In assessing the goodness-of-fit of models, the type I error rate was 0.24, and the type II error rate was 0.26. Analyses excluding potentially introduced samples had higher support for the Eastern Dispersal Hypothesis (posterior probability = 0.90; 95% CI: 0.87–0.94) than for the Ancient Eastern Islands Isolation Hypothesis (posterior probability = 0.10; 95% CI: 0.06–0.13) and produced estimated divergence dates deeper than those inferred in the analysis with all samples (Table 5). When we analysed the Eastern Dispersal Hypothesis model with a larger upper boundary for the prior distribution of divergence time parameters (10–1 000 000 generations), the estimated divergence between eastern Puerto Rican and Eastern Islands groups was 48.4 kya (results not shown). This finding demonstrated that prior distribution of divergence time parameters used for hypothesis testing was sufficiently large, and provided further support for a scenario in which *E. antillensis* populations in the Eastern Islands began diverging from those in eastern Puerto Rico during the last glacial period.

Discussion

Sea level changes and colonization of the Eastern Islands by Eleutherodactylus antillensis

The dynamic history of the PRB left an indelible mark on its biota (Heatwole & MacKenzie 1967). Sea level changes significantly altered the size and degree of isolation of terrestrial habitats in this archipelago (Heatwole & MacKenzie 1967; Renken *et al.* 2002). Brief periods (*c.* 11–18 kyr) of high sea level stands fragmented the Eastern Islands region to an extent similar to its current configuration (Fig. 1b) at least three times in the past 250 000 years (Dutton *et al.* 2009; Muhs *et al.* 2011).

Longer periods of low sea level stands predominated during the past five glacial periods (Rohling *et al.* 2009), uniting the PRB into a single landmass. The hypotheses that motivated this study represent competing models for population-level responses to sea level changes. The Ancient Eastern Islands Isolation Hypothesis predicts deep divergence between populations of *E. antillensis* in the Eastern Islands and Puerto Rico, because effectively no migration occurred between these regions when a land bridge united the PRB during the penultimate and last glacial periods. In contrast, the Eastern Dispersal Hypothesis predicts relatively shallow divergence between populations in eastern Puerto Rico (the putative source area) and those in the Eastern Islands, because individuals from Puerto Rico colonized the Eastern Islands via a land-bridge connection between these regions during the penultimate and last glacial periods.

Our multilocus phylogeographic analysis of *E. antillensis* revealed genetic signatures largely supporting the Eastern Dispersal Hypothesis. These signatures include: (i) Eastern Islands populations share some CR haplotypes with those in eastern Puerto Rico (Fig. 4a); (ii) Eastern Islands populations group with several eastern Puerto Rican populations in SAMOVA analyses of CR (Fig. 4b); (iii) Eastern Islands populations share most nuDNA haplotypes with Puerto Rican populations (Table 3 and Fig. S3, Supporting Information); (iv) highest support for a model where gene flow occurs from populations in eastern Puerto Rico towards Eastern Islands populations (Table 1); and (v) highest support for a model in which eastern Puerto Rican and Eastern Islands populations diverged subsequent to the start of the penultimate interglacial *c.* 250 kya (Table 5). Absence of a divergent Eastern Islands clade in phylogenies inferred from mtDNA and nuDNA loci (Figs S1 and S2, Supporting Information), low support for a model in which gene flow does not occur between eastern Puerto Rico and Eastern Islands populations of *E. antillensis* and low support for a model in which eastern Puerto Rican and Eastern Islands populations diverged prior to the start of the penultimate interglacial do not support the Ancient Eastern Islands Isolation Hypothesis. However, we cannot rule out the possibility that *E. antillensis* was present in the Eastern Islands prior to the penultimate interglacial. These populations may have become extinct, and the region recolonized by founders from eastern Puerto Rico. Alternatively, Eastern Islands populations may have persisted in isolation prior to the penultimate interglacial, and experienced gene flow with eastern Puerto Rican populations across the land bridge during the last two glacial periods. We did not find strong evidence for admixture in Eastern Islands populations, such as elevated genetic variation and strong differentiation (Kolbe *et al.* 2004). However, inferring admixture events for

weakly differentiated populations by relying on a limited marker set can be challenging (Corander & Marttinen 2006).

The phylogenies constructed from mtDNA and nuDNA lacked detailed resolution, but did not conform to predictions of the Eastern Dispersal Hypothesis. Low levels of sequence divergence probably explain why most CR haplotypes and individual samples for each nuDNA locus form a basal polytomy. In phylogenies inferred from MYH, RH1 and RPL9int4, many Eastern Islands samples nested in Puerto Rican clades, but those clades were not well supported across all three analyses and they contained individuals from both eastern and western Puerto Rico (Fig. S2, Supporting Information). This lack of resolution likely indicates shallow levels of isolation across the PRB.

Our estimated divergence date of 52.4 kya (95% CI: 2.7–207.0 kya) between eastern Puerto Rican and Eastern Islands populations suggests that isolation occurred during the last glacial period. Lower sea level during the last glacial period *c.* 118–12 kya (Waelbroeck *et al.* 2002; Siddall *et al.* 2003; van Daele *et al.* 2011) united Puerto Rico and the Eastern Islands. However, increasingly xeric environments in the coastal lowlands (Renken *et al.* 2002) could have promoted isolation of *E. antillensis* following establishment in the Eastern Islands. Small, arid islands in the PRB do not support populations of *E. antillensis*, and drought is associated with lower population densities of these frogs (Ovaska 2005). Sediment records from lowland Guatemala provide evidence for a moist climate *c.* 200–85 kya (Mueller *et al.* 2010) that shifted towards drier conditions *c.* 48 ± 23 kya (Hodell *et al.* 2008), and a high-resolution pollen record from north-eastern Venezuela suggests that dry forests and savanna predominated in Caribbean lowlands *c.* 28–68 kya (González *et al.* 2008; Hessler *et al.* 2010). Data for the PRB are lacking for these time periods, but an analysis of eolianite deposits from the lowlands of the PRB indicates aridity during the Last Glacial Maximum *c.* 26.5–19 kya (Renken *et al.* 2002). In the Greater Antilles (Cuba, Hispaniola, Jamaica, Puerto Rico) and in the PRB in general (Higuer-Gundy *et al.* 1999; Renken *et al.* 2002; Hodell *et al.* 2008), increasing rainfall following the Last Glacial Maximum led to an expansion of mesic forests that may have increased population connectivity, although a concomitant rise in sea level (Peltier 2002) would have reduced dispersal opportunities in the PRB. Large confidence intervals surrounding the estimated divergence between eastern Puerto Rican and Eastern Islands groups prevent us from inferring whether xeric conditions during glacial periods, or salt-water barriers during interglacials, were primarily responsible for initiating population divergence in *E. antillensis*.

Spatial expansion of *E. antillensis* into the Eastern Islands may have left a signature of lower genetic diversity and a smaller effective population size in the founder populations, but estimates of these parameters were either inconsistent across loci, or the differences between them were not significant. For the four nuDNA markers, individuals from the Eastern Islands had fewer haplotypes than those from eastern Puerto Rico, but a similar result was not inferred for CR. A higher mutation rate and faster coalescence time of mtDNA, combined with increased isolation of populations in the Eastern Islands since sea levels rose *c.* 14–11 kya (Heatwole & MacKenzie 1967), may explain the large number of CR haplotypes in this region. The establishment of populations from independent source populations (Kolbe *et al.* 2004) and/or a high population growth rate following a founder event(s) may also limit the loss of genetic diversity following spatial expansion (Austerlitz *et al.* 1997). Eastern Islands populations of *E. antillensis* have a smaller scaled effective population size (Θ) than populations in eastern Puerto Rico (Table 4), a finding that is consistent with a history of spatial expansion. DIYABC estimated the effective population size of the Eastern Islands group (N_4) to be smaller than that of the eastern Puerto Rican group (N_2), but the slightly overlapping 95% CIs suggest that these differences may not be significant (Table 5). Our ability to distinguish between complex population genetic models (e.g. incorporating admixture or changes in population size), and to reduce error rates associated with measuring confidence in scenario choice, and with the variance of divergence times, effective population size and migration rates may improve with additional independent loci (Beerli & Palczewski 2010; Cornuet *et al.* 2010; Robert *et al.* 2011).

In the GENELAND analyses of nuDNA and of all loci, populations of *E. antillensis* in the Eastern Islands formed a distinctive group (Fig. 2), a finding consistent with previous work showing that species in this region form distinctive biogeographic clusters (Hedges 1999). Nevertheless, levels of differentiation between Puerto Rican and Eastern Islands groups of *E. antillensis* are shallower than in the codistributed lizard *Anolis cristatellus* (Brandley & de Queiroz 2004) and in species of *Amphiacusta* ground crickets (Oneal *et al.* 2010). The latter likely persisted in isolation in the Eastern Islands over several glacial–interglacial cycles. Indeed, endemic species of plants (e.g. *Solanum conocarpum*; Acevedo-Rodríguez 1996), frogs (*Eleutherodactylus lentus* and *E. schwartzi*; Henderson & Powell 1999), lizards (e.g. *Anolis ernstwilliamsi*, *A. roosevelti*, *Sphaerodactylus parthenopion* and *Mabuya macleani*; Lazell 1983; Henderson & Powell 1999; Mayer & Lazell 2000), amphisbaenians (e.g. *Amphisbaena fenestrata*; Henderson & Powell

1999) and snakes (e.g. *Typhlops richardii*; Henderson & Powell 1999) survived in the Eastern Islands throughout multiple glacial–interglacial cycles. Despite this high endemism in the Eastern Islands region, most species occur on multiple islands, and therefore *single-island* endemism is low (Heatwole & MacKenzie 1967).

Diversification of Eleutherodactylus antillensis within Puerto Rico

Larger patches of suitable habitat, greater topographic and ecological diversity (Ewel & Whitmore 1973), and less pronounced inundations potentially lowered extinction rates in Puerto Rico relative to the Eastern Islands, and/or promoted diversification in multiple isolated populations on this large island (Rand 1969; Losos 1996). In fact, Puerto Rico contains the highest species richness (Figuerola Colón 1996) and largest proportion of endemics in the PRB (Heatwole & MacKenzie 1967; Hedges 1999). Mountainous regions in Puerto Rico harbour distinctive phylogroups of frogs and lizards (Velo-Antón *et al.* 2007; Rodríguez-Robles *et al.* 2010; Barker *et al.* 2011), and likely shaped genetic diversity in *E. antillensis* by providing mesic refugia during particularly arid glacial periods. Most well-supported clades in the *E. antillensis* nuDNA phylogenies are comprised of individuals from Puerto Rico (Fig. S2, Supporting Information), where group structure is highest (Figs 2 and 4b).

Our analyses of group structure suggest that a historical dispersal barrier for *E. antillensis* occurs in the lowlands west of the Río Grande de Loíza in Puerto Rico (Figs 2 and 4b). Eastern Puerto Rican and western Puerto Rican populations began diverging 89.4 kya (95% CI: 19.2–311.0 kya), coinciding with a shift in Caribbean climate to drier hydrologic conditions and the onset of wet–dry oscillations (Hodell *et al.* 2008; Mueller *et al.* 2010). In addition to promoting isolation between eastern Puerto Rican and Eastern Islands populations, xerophytic vegetation in the lowlands of the PRB during at least some portions of the last glacial period may have also inhibited dispersal between populations in eastern and western Puerto Rico. First proposed as a barrier to westward dispersal of the mesophilic Locust Coquí, *Eleutherodactylus locustus* (Rivero & Mayorga 1963), and the Golden Coquí, *Eleutherodactylus jasperi* (Drewry & Jones 1976), the Río Grande de Loíza Basin marks genetic discontinuities in *Eleutherodactylus coqui* (Velo-Antón *et al.* 2007) and the Mountain Garden Lizard, *Anolis krugi* (Rodríguez-Robles *et al.* 2010). In combination with strong, cool winds during the last glacial period (Hodell *et al.* 2008), increased aridity in the highlands connecting the Cayey Mountains (in the south-eastern region of Puerto Rico) to the Central

Mountains (Fig. 1a) could have limited dispersal of *E. antillensis* through higher elevation areas as well. The lowlands of western Puerto Rico receive less rainfall than those in eastern Puerto Rico (Daly *et al.* 2003) and may have been particularly xeric during the last glacial period (Renken *et al.* 2002), which may explain why scaled effective population size of *E. antillensis* is significantly smaller in western Puerto Rico than in the eastern part of the island.

Human-mediated introductions of Eleutherodactylus antillensis

Failure to identify a single unique *E. antillensis* haplotype on St. Croix, an island that has never had a direct land connection with the PRB (Gill *et al.* 1989), supports anecdotal evidence of a recent (1937) human-mediated introduction to St. Croix (Grant & Beatty 1944). The GENE-LAND analysis revealed that St. Croix populations most likely originated from Eastern Islands sources (Fig. 2). Although our data support the natural occurrence of *E. antillensis* in the Eastern Islands, three CR haplotypes (H14, H31, H35; Fig. 4a), all occurring in eastern Puerto Rico, are shared with distant Eastern Islands (St. John, Tortola, Guana, Beef and Virgin Gorda). Incomplete lineage sorting resulting from recent divergence and rapid spatial expansion from eastern Puerto Rico may account for this observation, but these particular shared haplotypes occur within 11 km of a shipping port in Puerto Rico. A nearly ubiquitous presence of *E. antillensis* in residential gardens and plant nurseries, and frequent transport of horticultural materials in the PRB (Platenberg 2007), supports possible accidental transport of individuals and/or eggs in potted plants between islands. Multiple introductions of *E. coqui* and the Cuban Tree Frog, *Osteopilus septentrionalis*, to various Eastern Islands (MacLean 1982; Owen *et al.* 2005) suggest the possibility of human transport of *E. antillensis* in the PRB. The estimated divergence dates between eastern Puerto Rican and Eastern Islands groups, and between western and eastern Puerto Rican groups, are deeper when potentially introduced samples are excluded (Table 5), raising the possibility that human transport of *E. antillensis* has complicated our historical inferences.

In conclusion, environmental change over the past two glacial–interglacial cycles shaped genetic diversity in *E. antillensis* by providing opportunities for colonization via land-bridge connections during periods of low sea level, as well as by creating habitat barriers that promoted isolation between populations in eastern Puerto Rico and the Eastern Islands, and between populations in eastern and western Puerto Rico. Our results support anecdotal evidence of a human-mediated introduction of *E. antillensis* to St. Croix, and therefore also

illustrate the role of recent events in shaping phylogeographic patterns in this species. Collectively, our findings are consistent with previous studies indicating that glacial land connections promoted dispersal in other Caribbean land-bridge archipelagoes, including the Great Bahama Bank (Malone *et al.* 2003) and the Turks and Caicos Banks (Reynolds *et al.* 2011). Sea level rise of up to 2 m by 2100 (Pfeffer *et al.* 2008) may reduce populations in small, low-elevation islands, increasing their risk of extirpation through demographic stochasticity, environmental stochasticity and catastrophes, loss of genetic heterozygosity and rare alleles, edge effects, and/or human disturbance (Burkey 1995). Because colonization success by over-water dispersal is low for terrestrial species in the Caribbean (Losos 1996), exploring the geographic distribution of distinct lineages in these dynamic insular systems contributes fundamental information for conservation management strategies.

Acknowledgements

We thank F. Bird-Picó, T. Figueroa, W. Falcón-Linero, J. Fumero, S. Lazell, C.D. Ortiz, G. Perry, C. Petrovic, R. Platenberg, M.J. Quiñones, A. Ríos-Franceschi, Y. Rodríguez, J.R. Snider and J.A. Stoken for assistance in the field, M. Farrah and M. Osborne for help in the laboratory, and the Department of Natural and Environmental Resources of Puerto Rico, U.S. Forest Service, the U.S. Virgin Islands Division of Fish and Wildlife, and the National Park Service for granting collecting permits. We acknowledge technical support from the University of New Mexico (UNM) Department of Biology's Molecular Biology Facility, which is supported by National Institutes of Health Grant P20RR18754 from the Institute Development Award Program of the National Center for Research Resources, and from the University of Alaska, Fairbanks Life Science Informatics, a core research resource supported by grant RR016466 from the National Center for Research Resources. T. Giermakowski provided field supplies and curatorial support, and M.P. Heinicke, M.B. Hickman, J. Hollis, C. Metzger, J. Richter, M.J. Ryan, J.W. Streicher, F. Torres-Pérez, T. Turner, D. Warnock and the Cuervo Lab Group at UNM reviewed earlier drafts of this manuscript. This study was partly funded by grants from the National Science Foundation (DBI-0001975, DEB-0327415) and the American Museum of Natural History to J.A.R.-R., the Undergraduate Opportunities (UnO) program at the Museum of Southwestern Biology (National Science Foundation DEB-0731350) to J.A.C., and awards from the Falconwood Foundation, Society for the Study of Amphibians and Reptiles, the UNM Biology Department, Office of Graduate Studies, and Graduate and Professional Student Association to B.S.B. Fieldwork was partially supported by Long Term Ecological Research Grants (National Science Foundation DEB-0218039 and DEB-0620910) to R.B.W.

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This study forms part of B.S.B.’s doctoral research, which focused on integrating molecular genetics, ecological niche modelling and GIS-based landscape analyses to understand how anurans responded to past climate change in the PRB. J.A.R.-R. works on the biogeography and behavioural and evolutionary ecology of reptiles and anurans from the PRB. V.S.A. completed her undergraduate Honours Thesis on aspects of the phylogeography of *Eleutherodactylus antillensis*. A.M. was an UnO undergraduate scholar and contributed to data collection and analyses. R.B.W. has a long-standing interest in long-term population dynamics of tropical organisms. J.A.C. is interested in biotic conservation, historical biogeography, evolution and island biology.

Data Accessibility

Sample locations and PCR conditions are uploaded as online Supporting Information. GenBank accession numbers for *Eleutherodactylus antillensis* and outgroup (*Eleutherodactylus brittoni*, *E. cochranae*, *Eleutherodactylus portoricensis*) mtDNA and nuDNA sequences: JN385299–JN38696; JX142403–JX142406; HQ823678; JX157960–JX157962. Final DNA sequence assembly DRYAD (doi:10.5061/dryad.hc257).

Supporting information

Additional Supporting Information may be found in the online version of this article.

Fig. S1 Maximum likelihood tree for 98 unique mtDNA control region haplotypes of *Eleutherodactylus antillensis*.

Fig. S2 Maximum likelihood trees recovered from individual nuclear intron-spanning loci β -crystallin (CRYBA), myosin heavy chain (MYH), rhodopsin (RH1) and ribosomal protein L9 (RPL9int4) for *Eleutherodactylus antillensis*.

Fig. S3 Georeferenced pie diagrams for each sampling locality of *Eleutherodactylus antillensis*.

Fig. S4 Results of a principal component analysis (PCA), performed in DIYABC 1.0.4.46, of the first 100 000 simulated data sets of two divergence models for *Eleutherodactylus antillensis*.

Fig S5 Results of a principal component analysis (PCA), performed in DIYABC 1.0.4.46, of the test quantities obtained with the Eastern Dispersal Hypothesis model-posterior combination, together with 10 000 pseudo-observed data sets.

Table S1 Catalogue numbers for traditional and photographic voucher specimens deposited in the Museum of Southwestern Biology (MSB), University of New Mexico, Albuquerque, and the Museum of Vertebrate Zoology (MVZ), University of California, Berkeley.

Table S2 Primers used for amplification (amp) and sequencing (seq) of fragments of the mtDNA control region (CR), nuclear intron-spanning loci β -crystallin (CRYBA), myosin heavy chain (MYH), rhodopsin (RH1) and ribosomal protein L9 (RPL9int4) in *Eleutherodactylus antillensis* and outgroup taxa.