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Climate as a driver of tropical insular diversity: comparative phylogeography of two ecologically distinctive frogs in Puerto Rico

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Abstract

The effects of late Quaternary climate on distributions and evolutionary dynamics of insular species are poorly understood in most tropical archipelagoes. We used ecological niche models under past and current climate to derive hypotheses regarding how stable climatic conditions shaped genetic diversity in two ecologically distinctive frogs in Puerto Rico. Whereas the Mountain Coquí, *Eleutherodactylus portoricensis*, is restricted to montane forest in the Cayey and Luquillo Mountains, the Red-eyed Coquí, *E. antillensis*, is a habitat generalist distributed across the entire Puerto Rican Bank (Puerto Rico and the Virgin Islands, excluding St. Croix). To test our hypotheses, we conducted phylogeographic and population genetic analyses based on mitochondrial and nuclear loci of each species across their range in Puerto Rico. Patterns of population differentiation in *E. portoricensis*, but not in *E. antillensis*, supported our hypotheses. For *E. portoricensis*, these patterns include: individuals isolated by long-term unsuitable climate in the Río Grande de Loíza Basin in eastern Puerto Rico belong to different genetic clusters; past and current climate strongly predicted genetic differentiation; and Cayey and Luquillo Mountains populations split prior to the last interglacial. For *E. antillensis*, these patterns include: genetic clusters did not fully correspond to predicted long-term unsuitable climate; and past and current climate weakly predicted patterns of genetic differentiation. Genetic signatures in *E. antillensis* are consistent with a recent range expansion into western Puerto Rico, possibly resulting from climate change and anthropogenic influences. As predicted, regions with a large area of long-term suitable climate were associated with higher genetic diversity in both species, suggesting larger and more stable populations. Finally, we discussed the implications of our findings for developing evidence-based management decisions for *E. portoricensis*, a taxon of special concern. Our findings

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Supplementary material (Appendix EXXXXX at www.oikosoffice.lu.se/appendix). Appendix 1-5.

illustrate the role of persistent suitable climatic conditions in promoting the persistence and diversification of tropical island organisms.

Introduction

Contemporary climatic change is affecting species distributions, with important consequences for the long-term persistence and evolutionary potential of biodiversity (Hoffman and Sgrò 2011, Parmesan 2006). Many tropical species are shifting upward in elevational distribution in response to changing climate, which may lead to smaller populations or extinction (e.g. Feeley et al. 2011, Freeman and Freeman 2014). Endemics on small, isolated islands are particularly vulnerable to environmental change (Fordham and Brook 2010, Gillespie et al. 2008). Exploring the historical processes of persistence of island biotas provides insight into potential responses of endemic biodiversity to changing conditions.

The large size and topographic complexity of the Greater Antilles (Cuba, Jamaica, Hispaniola, Puerto Rico, in the eastern Caribbean Sea) result in a range of environmental conditions that likely accommodated distributional shifts during climatic extremes of the Quaternary. Paleoclimates of the Greater Antilles fluctuated between dry glacial and wetter interglacial climates, oscillations that were associated with changes in vegetative cover (Higuera-Gundy et al. 1999, McFarlane et al. 2002). Local extinctions of many Greater Antillean vertebrate species have been attributed to vegetation shifts during the last glacial maximum (LGM) to Holocene transition c. 18–12 kya (reviewed in Pregill and Olson 1981). However, radiometric dating of fossils indicates that many extinctions occurred later in the Holocene (MacPhee 2009, McFarlane 1999), suggesting that climate-driven distributional shifts were less extensive than previously hypothesized. Indeed, continuous records of some species through the Late Quaternary fossil record (Goodfriend and Mitterer 1993), high species richness, and high regional endemism (Figueroa Colón 1996, Hedges 1999) imply that Greater Antillean species persisted and diversified throughout the Quaternary.

Puerto Rico, the smallest and most easterly of the Greater Antilles, provides an excellent system for testing hypotheses concerning population persistence and diversification. The Central Mountains and the Luquillo Mountains (Fig. 1) have relatively high species richness and species endemism (Figueroa Colón 1996, Hedges 1999), and harbor distinctive phylogroups of *Eleutherodactylus* frogs and *Anolis* lizards (Barker et al. 2011, Rodríguez-Robles et al. 2010, Velo-Antón et al. 2007). The Río Grande de Loíza Basin is a warmer, more arid landscape (mean annual temperature and precipitation of c. 24–25 °C and 1500–2250 mm, respectively; Daly *et al.*, 2003) that physiographically separates the Central and Luquillo Mountains (Fig. 1). These mountains support highly divergent representatives of a montane forest frog specialist (*Eleutherodactylus portoricensis*), which suggests that late Quaternary shifts did not effectively reconnect montane populations across the Río Grande de Loíza Basin (Barker et al. 2011). On the other hand, some widespread species exhibit differentiation across this basin despite continuous contemporary distributions (Barker et al. 2012, Rodríguez-Robles et al. 2010, Velo-Antón et al. 2007), implying a history of habitat isolation. To date, molecular studies of Puerto Rican taxa generally were based on a single

mitochondrial (mtDNA) locus, or did not explicitly incorporate climatic data. Ecological niche models (ENMs), which characterize the climatic space that a species occupies under past and current conditions, can be used to generate spatially explicit hypotheses of population history that can be tested with multi-locus datasets to examine responses to past environmental change (e.g., Richards et al. 2007).

Here we combine ENMs with genetic data from several mtDNA and nuclear loci to explore how the extent and presumed connectivity of suitable habitats during the late Quaternary structured genetic diversity in two ecologically distinctive frogs in Puerto Rico.

Eleutherodactylus portoricensis, the Mountain Coquí, is restricted to mesic montane forests above 180 m.a.s.l. in the Cayey Mountains, a southeastern extension of the Central Mountains, and the Luquillo Mountains in northeastern Puerto Rico (Schwartz and Henderson 1991). In contrast, *E. antillensis*, the Red-eyed Coquí, is a habitat generalist distributed across the entire Puerto Rican Bank (Puerto Rico and the Virgin Islands, excluding St. Croix) from 0–1219 m.a.s.l. (Henderson and Powell 1999). The influence of past environmental change on these two frogs is expected to differ based on their climatic associations and physiological tolerances. Whereas *E. portoricensis* is restricted to the understory of cool, moist rainforest, *E. antillensis* is most common at low elevations, where it inhabits wooded habitats, open pastures and mesic savannas (Schwartz and Henderson 1991). Due to its high tolerance to dehydration at warmer temperatures (Beuchat et al. 1984), the Río Grande de Loíza Basin probably is a less effective barrier to gene flow for *E. antillensis* than for *E. portoricensis*.

We used ENMs under last interglacial (LIG; ~130–116 thousand years ago [kya]), LGM (~21 kya), mid-Holocene (~6 kya), and current climatic conditions to derive hypotheses regarding spatio-temporal patterns of differentiation between conspecific populations of *E. portoricensis* and *E. antillensis*. For *E. portoricensis*, the Cayey–Luquillo Refugia Hypothesis predicts deep differentiation between populations in the Cayey and Luquillo Mountains, with the basic divergence dating to the LIG or earlier, and being caused by geographic isolation of the montane areas by unsuitable climate in the Río Grande de Loíza Basin and surrounding lowlands during the four time periods. For *E. antillensis*, the partial isolation hypothesis predicts shallow differentiation between populations east and west of the Río Grande de Loíza in eastern Puerto Rico, and between populations north and south of the Central Mountains, due to their partial isolation caused by unsuitable climate in these regions over the aforementioned time periods.

We next explored how relative size of areas of suitable climate under LIG, LGM, mid-Holocene, and current climatic conditions influenced levels of genetic diversity. Genetic diversity is expected to increase when suitable climate was persistent (Carnaval et al. 2009) because larger, stable populations tend to experience smaller fluctuations in allelic frequencies (Frankham 1996), and a larger number of individuals leads to the accumulation of mutations. Accordingly, we predicted that (1) *E. portoricensis* populations in the Luquillo Mountains have higher genetic diversity than those in the Cayey Mountains, and that (2) *E. antillensis* populations in eastern Puerto Rico have higher genetic diversity than those in the western part of the island, due to a greater area of persistent suitable climate in the former region. We also examined evidence for recent range expansion of *E. antillensis* into western

Puerto Rico from sources in the eastern part of the island, where a greater area of persistent climate was predicted by our ENMs. Finally, we briefly considered the implications of our findings for developing evidence-based management decisions for *E. portoricensis*, a taxon of special conservation concern. This new perspective on the role of late Quaternary climate in shaping endemism in Puerto Rico can improve our understanding of diversification and speciation dynamics in tropical islands.

Materials and Methods

Ecological niche modeling

We created ENMs for *E. portoricensis* and *E. antillensis* using georeferenced records from field surveys and online databases. Occurrence records (<15 m uncertainty) were collected with a GPS unit during surveys in Puerto Rico between 2001 and 2008. Because *E. portoricensis* was synonymous with *E. coqui* prior to 1966 (Thomas 1966), and georeferenced records were not available for most museum specimens, we used recent (1981–2008) location records for *E. portoricensis* from the PRGAP project (Gould et al. 2008) and amphibian monitoring surveys in the Luquillo Mountains (Woolbright 1997). Additional occurrence records for *E. antillensis* were compiled from GBIF (<http://www.gbif.org>; accessed 26 July 2011) and HerpNet (<http://www.herpNet.org>; accessed 19 October 2013). To correct for potential bias in sampling effort, we removed localities <1 km apart, and verified that remaining localities were uniformly distributed across each species range, which resulted in 18 occurrence records for *E. portoricensis* (Fig. 2a) and 77 for *E. antillensis* (Fig. 2b).

Eleutherodactylus antillensis is naturally absent from primary montane forest (Schwartz and Henderson 1991), and its abundance declines with forest age (Herrera-Montes and Brokaw 2010). These observations suggest that *E. antillensis* was probably more restricted to the lowlands prior to the 19th and 20th centuries, during which humans removed more than 90% of Puerto Rico's original forests (Birdsey and Weaver 1987). To account for potential occurrence bias introduced by anthropogenic deforestation, we removed localities that were 5 km from montane forest, identified from a land cover map created using recent (1999–2003) satellite imagery (Helmer et al. 2002).

We constructed ENMs using current (mean climatology from 1950 to 2000), mid-Holocene (Braconnot et al. 2007), and LIG (Otto-Bliesner et al. 2006) bioclimatic data at a 30" (c. 1 × 1 km) resolution obtained from the WorldClim database (Hijmans et al. 2005, <http://worldclim.org>). Climate data for the LGM were derived from simulation runs using the Community Climate System Model (CCSM3) (version 3; (Collins et al. 2006) and the Model for Interdisciplinary Research on Climate (MIROC) (version 3.2; (Hasumi and Emori 2004), and downscaled to a 2.5' (4 × 4 km) resolution (Waltari et al. 2007). We applied to each dataset a mask that encompassed the Puerto Rican Bank and St. Croix (an island c. 105 km southeast of Puerto Rico) to ensure that the study region contained areas that have been potentially accessible to *E. portoricensis* and *E. antillensis* via dispersal during time periods relevant to our study (Merow et al. 2013).

To avoid under-predicting suitable climatic conditions for the two frogs (Merow et al. 2013), we measured correlations between 19 climate variables in ENMTools 1.3 (Warren et al. 2010), and removed those with a Pearson's correlation coefficient (r) >0.75 . The remaining four variables, which are relevant to *Eleutherodactylus* species in Puerto Rico (Beuchat et al. 1984, Stewart and Woolbright 1996), are annual mean temperature (bio 1), temperature annual range (bio 7), annual precipitation (bio 12), and precipitation seasonality (bio 15). We used Maxent 3.3.3 (Phillips et al. 2006, Phillips et al. 2004) to develop logistical ENMs for the two species under past and current climatic conditions. Statistical significance of ENMs for *E. portoricensis* and *E. antillensis* was tested against a null model derived from random localities (sensu Raes and ter Steege 2007) in ENMTools 1.3 (Warren and Seifert 2011). Details on the settings in Maxent and ENMTools are given in the Supplementary Material (Appendix 1).

Generating hypotheses from ecological niche models

To generate hypotheses concerning the connectivity and size of persistent suitable climates under past and current climatic conditions, we derived long-term climatic suitability surfaces, which represent climatic suitability over the LIG, LGM, mid-Holocene, and the present. We summed the pixels of ENMs derived under climatic conditions of these four time periods (sensu Graham et al. 2006), which resulted in a surface where each pixel had a value that ranged from 0 (never predicted to be suitable) to 4 (predicted to be suitable across all four time periods). Using ArcGIS 10.2 (ESRI, Redlands, CA, USA), we generated two long-term climatic suitability surfaces for *E. portoricensis* and *E. antillensis* to accommodate different scenarios of LGM climatic conditions (CCSM3 and MIROC). To visualize the location and size of regions with persistent suitable climate under all climatic conditions, we reclassified long-term climatic suitability surfaces to show the highest 50% of suitability values. The connectivity of long-term suitable climate for each species provided the basis for the Cayey–Luquillo Refugia Hypothesis (*E. portoricensis*) and the partial isolation hypothesis (*E. antillensis*). The relative size of areas with long-term suitable climate were assessed to make predictions regarding levels of genetic diversity in each species.

DNA sequence data

Analyses of population structure and genetic diversity relied on previously published mtDNA control-region (CR) sequences (Barker et al. 2012, Barker et al. 2011) of 144 *E. portoricensis* from 16 localities in the Cayey Mountains ($N = 73$) and Luquillo Mountains ($N = 71$), and 203 *E. antillensis* from 41 localities across the species' range in Puerto Rico (Fig. 1; see Supplementary material Appendix 2 for GenBank accession numbers). To gain an additional perspective on population history, we analyzed sequences of four nuclear loci (nuDNA): intron-spanning loci β -crystallin (intron 1; CRYBA), myosin heavy chain (putative flanking intron of exon 36; MYH), rhodopsin (intron 1; RH1), and ribosomal protein L9 (intron 4; RPL9int4) from three randomly chosen individuals from each locality (Supplementary material Appendix 2). All CRYBA, MYH, and RPL9int4 sequences of *E. portoricensis* were generated for this study; the remaining nuDNA sequence data were previously reported (Barker et al. 2012, Barker et al. 2011). Finally, we included previously published mtDNA cytochrome *b* sequences (Barker et al. 2011) for *E. portoricensis* from the

same subset of individuals from which we collected CRYBA, MYH, RH1, and RPL9int4 nuclear sequences (Supplementary material Appendix 2).

Tissue samples are from the Division of Genomic Resources, Museum of Southwestern Biology (MSB), University of New Mexico, Albuquerque, and the Rodríguez-Robles laboratory at the University of Nevada, Las Vegas. Voucher specimens of *E. antillensis* are archived at MSB or the Museum of Vertebrate Zoology (MVZ), University of California, Berkeley. Due to the sensitive conservation status of *E. portoricensis*, we did not collect voucher specimens of this species. Details of DNA extraction methods, PCR conditions, sequencing reactions, sequence editing, haplotype reconstruction, and primer descriptions are described elsewhere (Barker et al. 2012, Barker et al. 2011). We aligned sequences in MAFFT 6 (Katoh et al. 2002, <http://mafft.cbrc.jp/alignment/server>), with the exception of CR, cyt *b*, and RH1 sequences for *E. portoricensis*, which were aligned in MUSCLE 3.7 (Edgar 2004). To create a ‘combined nuDNA’ dataset for testing models of genetic differentiation and exploring genetic diversity among regions (see below), we concatenated sequences for the four nuDNA loci in Sequence Matrix (Vaidya et al. 2011), which resulted in a 1392 bp alignment for *E. portoricensis* and a 1630 bp alignment for *E. antillensis*.

Recombination and neutrality

We tested for recombination in the new nuDNA datasets (CRYBA, MYH, and RPL9int4) for *E. portoricensis* using RDP 3 (Martin et al. 2010). For each species, 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, <https://bio.cst.temple.edu/~hey/software/software.htm>). We used Arlequin 3.5 (Excoffier and Lischer 2010) to test for departures from neutrality by comparing Tajima's D (Tajima 1989) and Fu's F_S (Fu 1997) statistics to 10,000 coalescent simulations of a large, neutrally evolving population of constant size.

Population structure

To test hypotheses regarding differentiation between conspecific populations of *E. portoricensis* and *E. antillensis*, we quantified population structure in STRUCTURE 2.3.3 (Pritchard et al. 2000) using the four nuDNA intron loci (CRYBA, MYH, RH1, and RPL9int4). Each variable site was encoded as an allele. We explored population structure under a model of correlated (Falush et al. 2003) and uncorrelated allele frequencies, and under admixture and no-admixture models, for a total of four analyses for each species. For each analysis, we conducted two independent runs for all values of K (number of genetic clusters) between 1 and 8 using an MCMC length of 100,000 generations following a burn-in of the same length. We chose the best K by examining the log probability of the data [$\ln \text{Pr}(X|K)$] and plots of ΔK (sensu Evanno et al. 2005) produced by STRUCTURE HARVESTER (Earl and vonHoldt 2011). Mean membership fractions of individuals at each sampling locality assigned to a genetic cluster were visualized in ArcGIS 10.2. We also created bar plots showing membership fractions estimated for each individual with DISTRUCT 1.1 (Rosenberg 2004). To visualize relationships among haplotypes of new nuDNA (CRYBA, MYH, and RPL9int4) sequences in *E. portoricensis*, we generated maximum parsimony networks with Network 4.2 (<http://www.fluxus-technology.com>).

Isolation by distance and isolation by resistance as predictors of genetic differentiation

Areas with persistent suitable climate are expected to facilitate migration among populations. We tested whether our estimated long-term climatic suitability surface is a reliable predictor of genetic differentiation between conspecific populations of *E. portoricensis* and *E. antillensis*. Using Circuitscape 3.5.8 (McRae 2006, McRae and Beier 2007), we converted pixel values in the long-term climatic suitability surfaces into conductance values, which represent an index of the amount of dispersal into a pixel. Thus, lower conductance values were assigned to pixels that have greater resistance to dispersal, and higher conductance values to pixels that have less resistance to dispersal. Circuitscape analyses were conducted twice for each species to accommodate different long-term stability surfaces resulting from using different (CCSM3 and MIROC) scenarios of LGM climatic conditions.

For each species, we tested whether an ‘isolation by resistance’ (IBR) model can more accurately predict observed patterns of genetic differentiation between population pairs than a null model of isolation by distance (IBD; Wright 1943). Under an IBR model, genetic differentiation is expected to increase linearly with resistance to movement (i.e. lower conductance values) between populations. When the effects of resistance are stronger than geographic distance in influencing gene flow, a model of IBR should retain a significant relationship with genetic differentiation after partialling out the effects of IBD. Conversely, factoring out the effect of IBR should result in a nonsignificant relationship between IBD and genetic differentiation. To quantify genetic distance, we measured Slatkin's linearized F_{ST} (Slatkin 1995) in Arlequin 3.5. We calculated genetic distances separately for CR and the combined nuDNA to account for potentially different genetic structure resulting from the distinctive mode of inheritance of these markers. We treated indels as a fifth state, coding each as a single mutational event. Euclidean distances between sampling localities were calculated in ArcGIS and the resulting values log-transformed (Rousset 1997). To evaluate models of IBR and IBD, we calculated the correlation between genetic distance and resistance and geographic distance, and evaluated expectations for IBR and IBD models with partial Mantel tests. Mantel and partial Mantel tests were performed with 10,000 permutations using the Ecodist package (Goslee and Urban 2007) in R 3.0.2 (R Development Core Team 2013). Two models of IBR, $IBR_{LGM-CCSM3}$ and $IBR_{LGM-MIROC}$ were evaluated to accommodate the different scenarios of LGM climatic conditions.

Coalescent-based estimates of migration and divergence for *E. portoricensis*

We conducted coalescent-based analyses of divergence in IMA2 (Hey 2010) to test predictions of the Cayey–Luquillo Refugia Hypothesis regarding the timing of divergence of *E. portoricensis* populations from these two mountain ranges. This analysis was not conducted for *E. antillensis* because of the shallow population genetic structure in this species (see ‘Results’). To reduce computation times, we only included individuals for which sequence data from all loci (CR, *cyt b*, CRYBA, MYH, RH1, and RPL9int4) were available ($N = 36$). We optimized priors, heating parameters, number of chains, and burn-in length through several preliminary Markov Chain Monte Carlo (MCMC) runs. We used uniform prior distributions for all demographic parameters, and applied the Hasegawa-Kishino-Yano (HKY) model of nucleotide substitution (Hasegawa et al. 1985) to each data

set, which was the model chosen by AIC in MODELTEST 3.7 (Posada and Crandall 1998). The upper bounds for the priors of divergence time (t), maximum migration (m), and maximum population size ($4N_e\mu$), where N_e is the effective population size and μ is the mutation rate per generation, were set to 12.0, 1.0, and 20.0, respectively. We also conducted an IM_{A2} analysis in which the upper bound for the t prior was set to 1.8, because this parameter showed a bimodal distribution despite good mixing, suggesting that two alternative demographic histories are similarly probable. Final runs included 150 chains, had a 96 hour burn-in (approximately 1.7×10^8 steps), and were performed for 3.0×10^7 steps. We conducted two runs, started with different seeds, to ensure that parameter estimates were consistent. To calculate demographic parameters, we used a per locus μ of 2.32×10^{-5} for CR (Barker et al. 2012), 6.17×10^{-6} for cyt *b* (Crawford et al. 2007), and 3.07×10^{-6} for RH1. Details of the methods for estimating μ for RH1 are given in the Supplementary Material (Appendix 3).

Genetic diversity

We explored how the area of long-term suitable climate for each frog species influenced genetic diversity. We used a Mann-Whitney-Wilcoxon test (one-sided) in R (version 3.0.2; R Development Core Team, 2013) to test the predictions that *E. portoricensis* populations in the Luquillo Mountains have higher genetic diversity than those in the Cayey Mountains (Fig. 3a), and that *E. antillensis* populations in eastern Puerto Rico have higher genetic diversity than in the western part of the island (Fig. 3b). Genetic diversity based on CR and the combined nuDNA dataset was estimated at each locality using the population mutation rate obtained from the observed number of segregating sites (θ_S), where $\theta = 4N_e\mu$ (Watterson 1975), and nucleotide diversity (π) using Arlequin 3.5. We explored genetic diversity in populations grouped by region by calculating haplotype diversity (H_d), number of haplotypes (h), number of private (i.e. unique) haplotypes, θ_S , and π for each locus in Arlequin 3.5.

We explored evidence for recent range expansion of *E. antillensis* into western Puerto Rico from sources in the eastern part of the island, where a greater area of persistent climate was predicted by our ENMs. A steady decrease in genetic diversity along the range owing to a series of consecutive founder effects is a signature of a range expansion (Austerlitz et al. 1997). We tested for a decrease in genetic diversity with increasing geographic distance from eastern Puerto Rico by regressing θ_S and π on longitude using R (version 3.0.2).

Results

Ecological niche modeling

The mean area under the receiving operating characteristic curve (AUC) for the test data was 0.97 (SD = 0.015) for *E. portoricensis* and 0.67 (SD = 0.042) for *E. antillensis*. Low AUC values for *E. antillensis* probably result from the commonness of this species and the small extent of the study region (i.e. the Puerto Rican Bank and St. Croix; Lobo et al. 2008, Merow et al. 2013). AUC values of ENMs were significantly higher than null model AUCs for both species.

ENMs for *E. portoricensis* under past and current climatic conditions predicted highest climatic suitability in the Cayey and Luquillo Mountains (Fig. 2a), with one exception. The ENM under the CCSM3 scenario of LGM climatic conditions predicted low suitability in the Luquillo Mountains. However, the Multivariate Environmental Similarity Surfaces (MESS) analysis indicated that precipitation seasonality (bio 15) had values outside the range of current climatic conditions in the Luquillo Mountains, indicating that predictions of the latter scenario should be treated with caution (Elith et al. 2010). Relatively unsuitable climate was predicted in the Río Grande de Loíza Basin and coastal lowlands for all four times periods, suggesting that populations in the Cayey and Luquillo Mountains have been isolated since the LIG. The lowest 50% of long-term climatic suitability values occurred in the vicinity of the Río Grande de Loíza Basin and the surrounding coastal lowlands, and completely isolate long-term suitable climate (highest 50% of values) in the Cayey and Luquillo Mountains (Fig. 3a). The area of long-term suitable habitat is larger in the Luquillo Mountains than in the Cayey Mountains (Fig. 3a).

ENMs for *E. antillensis* under past and current climate predicted highest climatic suitability in the northern and eastern coastal lowlands, and in an area west of the Río Grande de Loíza (Fig. 2b). The lowest 50% of long-term climatic suitability values occurred in the Central Mountains, the Luquillo Mountains, the lowlands of southwestern Puerto Rico, and in an area east of the Río Grande de Loíza. Long-term suitable climate was only partially isolated (east-west of the Río Grande de Loíza in eastern Puerto Rico, and between populations on north and south sides of the Central Mountains; Fig. 3b). The area of long-term suitable habitat is larger in eastern Puerto Rico than in the western part of the island (Fig. 3b). ENMs estimated from different scenarios of LGM climatic conditions did not produce appreciably different long-term climatic suitability surfaces for either *E. portoricensis* or *E. antillensis*.

Recombination and neutrality

We did not detect recombination in the new nuDNA data sets (CRYBA, MYH, RPL9int4) for *E. portoricensis*. Single-base pair indels were present in CRYBA and RPL9int4; multi-base pair indels in RPL9int4 ranged in size from 3 to 46 base pairs. Nucleotide variation in all loci is consistent with neutral expectations according to the HKA test in *E. portoricensis* ($\chi^2 = 3.30$, d.f. = 4, $p = 0.51$) and *E. antillensis* ($\chi^2 = 6.64$, d.f. = 4, $p = 0.16$). Significant negative values of Tajima's D and/or Fu's F_S for CR ($F_S = -23.95$, $p = 0.001$), cyt b ($D = -1.51$, $p = 0.04$; $F_S = -11.93$, $p = 0.001$), and MYH ($F_S = -4.41$, $p = 0.01$) in *E. portoricensis*, and for CR ($D = -1.53$, $p = 0.031$; $F_S = -25.66$, $p < 0.001$) and RPL9int4 ($F_S = -25.02$, $p < 0.001$) in *E. antillensis*, indicate selection or recent population expansion (Fu 1997, Tajima 1989).

Population structure

STRUCTURE analyses of nuDNA of *E. portoricensis* revealed a strong genetic discontinuity between individuals from the Cayey and Luquillo Mountains (Fig. 4a). In all four analyses, the mean log probability of the data and K were greatest at $K = 2$, and individuals in each mountain range were assigned to a distinctive genetic cluster with high probability. Maximum parsimony networks revealed that only ca. 22% of the haplotypes of

CRYBA (5/16), MYH (2/9), and RPL9int4 (4/20) are shared between Cayey and Luquillo Mountains (Supplementary material Appendix 4).

STRUCTURE analyses of nuDNA of *E. antillensis* revealed differentiation between populations in eastern and western Puerto Rico (Fig. 4b, c). In three of the four analyses, the mean log probability of the data and K were greatest at $K = 2$. However, the analysis under the admixture and correlated allele frequencies models indicated that the mean log probability of the data was greatest at $K = 3$. In all analyses, mean membership fractions of individuals to any single genetic cluster were highest in easternmost and westernmost populations, whereas most individuals in central Puerto Rico exhibited mixed ancestry. When $K = 3$, one cluster occurred predominantly in eastern Puerto Rico, a second one was predominantly located in western Puerto Rico, and a third one occurred across the entire island (Fig. 4b). When $K = 2$, all individuals east of the Río Grande de Loíza River were assigned to a distinctive cluster with high probability, most individuals in central Puerto Rico exhibited mixed ancestry, and most individuals in western part of the island were assigned to a second cluster with high probability (Fig. 4c).

Isolation by distance and isolation by resistance as predictors of genetic differentiation

Long-term climatic suitability was a strong predictor of observed genetic differentiation among populations of *E. portoricensis*. Both resistance and geographic distance exhibited a high correlation with linearized F_{ST} in CR ($r = 0.84, 0.91$, and 0.91 for the IBD, $IBR_{LGM-CCSM3}$, and $IBR_{LGM-MIROC}$ models, respectively, $p < 0.001$), and the combined nuDNA ($r = 0.82, 0.88$, and 0.87 for the IBD, $IBR_{LGM-CCSM3}$, and $IBR_{LGM-MIROC}$ models, respectively, $p < 0.001$; Table 1). The IBR models retained a significant correlation with linearized F_{ST} after partialling out the effects of IBD, indicating that IBR is a better predictor of genetic differentiation than IBD. Factoring out the effect of IBR resulted in nonsignificant correlations between geographic distance and linearized F_{ST} , providing further support for IBR.

Neither geographic distance nor long-term climatic suitability were strong predictors of observed genetic differentiation among *E. antillensis* populations. Resistance and geographic distance exhibited low correlations with linearized F_{ST} in CR ($r = 0.17, 0.30$, and 0.25 for the IBD, $IBR_{LGM-CCSM3}$, and $IBR_{LGM-MIROC}$ models, respectively, $p < 0.001$), and the combined nuDNA ($r = 0.23, 0.22$, and 0.21 for the IBD, $IBR_{LGM-CCSM3}$, and $IBR_{LGM-MIROC}$ models, respectively, $p < 0.001$; Table 1). After partialling out effects of IBD, IBR models retained a significant correlation with linearized F_{ST} in CR, but not in the combined nuDNA dataset. Factoring out the effect of IBR resulted in nonsignificant correlations between geographic distance and linearized F_{ST} .

Coalescent-based estimates of migration and divergence for *E. portoricensis*

According to the IM_a2 analysis with a higher upper bound for the t prior, Cayey and Luquillo Mountains populations of *E. portoricensis* diverged 1.5 mya [95% highest posterior density interval (HPD) = 162.0 kya–3.2 mya], and after their initial separation gene flow between them occurred in a westward direction, from the Luquillo to the Cayey Mountains (Table 2). The analysis with a lower upper bound for the t prior indicated that these

populations diverged 182.3 kya [95% highest posterior density interval (HPD = 102.4–308.8 kya) and did not experience gene flow after their initial separation. In both analyses, posterior probability distributions of $4N$ and m had unambiguous peaks bounded within the prior values. In the analysis with a lower upper bound for the t prior, the posterior distribution of t was bimodal. Examination of convergence diagnostics [e.g. low autocorrelations between variables, high swap rates, high effective sample sizes (all >200), trend plots, high acceptance ratios] indicated good mixing over the course of each run.

Genetic diversity

The prediction that *E. portoricensis* populations in the Luquillo Mountains have higher genetic diversity than those in the Cayey Mountains was largely supported. Luquillo Mountain populations had higher CR θ_S and π than those in the Cayey Mountains, although the difference was significant only for θ_S (Table 3). Combined nuDNA θ_S and π were both significantly higher in Luquillo than in Cayey populations (Table 3). With the exception of RH1, all loci had higher θ_S , π , Hd, and more private haplotypes in Luquillo Mountains populations (Supplementary material Appendix 5), indicating congruence across most loci.

The prediction that *E. antillensis* populations in eastern Puerto Rico have higher genetic diversity than those in western Puerto Rico was largely supported. CR θ_S and π were higher in eastern Puerto Rican populations, but the difference was not significant (Table 3). Combined nuDNA θ_S and π were both significantly higher in eastern populations than in western demes (Table 3). With the exception of RH1, all loci had higher estimates of θ_S , π , Hd, and more private haplotypes in eastern Puerto Rico (Supplementary material Appendix 5), indicating congruence across most loci.

The prediction of a recent range expansion of *E. antillensis* into western Puerto Rico was partially supported. Longitude exhibited a significant negative relationship with combined nuDNA θ_S ($R^2 = 0.57$, $F = 49.6$, d.f. = 38, $p < 0.001$) and π ($R^2 = 0.58$, $F = 53.3$, d.f. = 38, $p < 0.001$), but not with CR θ_S ($R^2 = 0.004$, $F = 0.16$, d.f. = 38, $p = 0.69$) or π ($R^2 = 0.008$, $F = 0.33$, d.f. = 38, $p = 0.57$).

Discussion

Population structure

Spatio-temporal patterns of differentiation in *E. portoricensis* populations largely support the Cayey–Luquillo Refugia Hypothesis. These patterns include: (i) individuals from the Cayey and Luquillo Mountains belong to different genetic clusters (Fig. 4a); (ii) long-term climatic suitability surfaces predicted observed genetic differentiation more accurately than a null model of IBD (Table 1); and (iii) divergence of the Cayey and Luquillo Mountains populations began prior to the LIG (Table 2). Collectively, these results indicate that montane forest in the Cayey and Luquillo Mountains persisted over the late Quaternary, a scenario that may have allowed *E. portoricensis* to diversify in each of the two areas. Additionally, our findings suggest that persistent unsuitable climate in the vicinity of the Río Grande de Loíza Basin (Fig. 3a) is a strong barrier to gene flow between populations in the Cayey and Luquillo Mountains.

Our two IMA2 analyses for *E. portoricensis* supported a pre-LIG divergence time for Cayey and Luquillo Mountains populations, but they estimated different divergence times and population migration rates. According to the analysis with a higher upper bound for the t prior, these populations diverged 1.5 mya (HPD = 162.0 kya–3.2 mya), and unidirectional migration occurred from Luquillo into the Cayey Mountains. In contrast, divergence times estimated from the analysis with a lower upper bound for the t prior supported a split that occurred later in the Quaternary (182.3 kya; 95% HPD = 102.4–308.8 kya), with no subsequent gene flow. Vicariance resulting from the gradual erosion of mountains which once connected the Cayey and Luquillo Mountains at the eastern edge of the Río Grande de Loíza Basin throughout the Pliocene and Pleistocene (Meyerhoff 1933) may have initiated allopatric divergence in *E. portoricensis*. This frog is patchily distributed and highly philopatric (Joglar 1998, Stewart and Woolbright 1996), which suggests that populations in the Cayey and Luquillo Mountains were at least partially isolated prior to the disappearance of montane connections between these regions. Thus, a demographic history characterized by isolation with migration seems more plausible than one characterized by abrupt isolation with no subsequent gene flow, especially given that montane connections eroded over thousands of years (Meyerhoff 1933). On the other hand, recent physiographic data suggest that there may not have been a high-elevation connection between the Cayey and Luquillo Mountains (Brocard et al. pers. comm.). If confirmed, this new finding suggests that the initial divergence between *E. portoricensis* populations from these two mountain ranges was not caused by vicariance due to eroding high-elevation connections, but instead by the uplift of the Cayey and Luquillo Mountains. Upslope contractions of *E. portoricensis* by *c.* 200 m in the Luquillo Mountains during the past 30 years, which are partly in response to climate change (Longo and Burrowes 2010, Woolbright 1997), suggest that past climate-driven elevational range shifts brought populations in each the Cayey and Luquillo Mountains into closer geographic proximity. However, the shallow and wide Río Grande de Loíza Basin, which reaches a low point of 0 m in the Caguas Basin, probably became an increasingly strong barrier to dispersal between disjunct montane populations as it eroded throughout the Quaternary. These data are consistent with an absence of post-LIG gene flow inferred by both IMA2 analyses.

The inferred population structure in *E. antillensis* is not consistent with both predictions of the partial isolation hypothesis, which is probably in part due to a recent range expansion of this species into western Puerto Rico. The partial isolation hypothesis predicts shallow differentiation between populations east and west of the Río Grande de Loíza in eastern Puerto Rico, and between populations north and south of the Central Mountains, due to their partial isolation caused by unsuitable climate in these regions over the aforementioned time periods (Fig. 3b). STRUCTURE analyses revealed shallow differentiation between populations east and west of the Río Grande de Loíza Basin, but not between populations north and south of the Central Mountains (Fig. 4b, c). These results suggest that only long-term unsuitable climate east of the Río Grande de Loíza acted as a historical dispersal barrier to dispersal for *E. antillensis*. A recent range expansion of *E. antillensis* into western Puerto Rico, which was supported by analyses of the combined nuDNA, would result in an absence of differentiation between populations north and south of the Central Mountains, because there has been insufficient time for genetic differences to accumulate between populations.

A recent range expansion could also explain why both geographic distance and long-term climatic suitability surfaces were weak predictors of genetic differentiation in *E. antillensis* (Table 1).

The exclusion of factors that may influence *E. antillensis*' present-day distribution from the parameters used to construct ENMs can be another reason why predictions of the partial isolation hypothesis were not fully supported by our data. This species occurs in forest-edges in montane regions, but not within primary forest, indicating that land-cover is an important factor that directly or indirectly influences the distribution of *E. antillensis* (Gould et al. 2008, Herrera-Montes and Brokaw 2010). Unfortunately, land cover datasets are lacking for time periods other than the present. Nevertheless, data from pollen, fossils, and eolianite deposits suggest that xeric, shrubby vegetation, and savannas predominated in the lowlands of Puerto Rico (Renken et al. 2002) during the LGM (Higuera-Gundy et al. 1999, McFarlane et al. 2002, Pregill and Olson 1981). *Eleutherodactylus antillensis* is absent from very xeric habitats, and drought is associated with lower densities of this frog (Ovaska 2005), suggesting that its distribution in the lowlands of Puerto Rico during the LGM may have been more restricted than our ENMs predicted. Thus, including past land-cover data in our ENM analyses may produce more accurate modeled distributions, if such data were available.

Genetic diversity

Genetic diversity in *E. portoricensis* and *E. antillensis* was higher in regions with relatively extensive areas of long-term suitable climate, suggesting that those areas harbored larger and more stable populations of the two frogs. Both mtDNA and combined nuDNA θ_S and π were highest in Luquillo Mountains populations of *E. portoricensis*, and in eastern Puerto Rican populations of *E. antillensis* (Table 3). With the exception of RH1, all loci had higher θ_S , π , Hd, and more private haplotypes in these populations (Supplementary material Appendix 5). A lack of statistical significance for higher values of mtDNA θ_S and π is probably a result of individuals having variable levels of mtDNA genetic diversity in each region. Overall, these results are consistent with a previous study that suggested that temporally stable climatic conditions promote the accumulation of genetic diversity in the Puerto Rican lizard *Anolis krugi* (Rodríguez-Robles et al. 2010).

Our analyses of genetic diversity of combined nuDNA, but not of CR, revealed evidence for a recent range expansion of *E. antillensis* into western Puerto Rico from sources in the eastern part of the island, which may be explained by different evolutionary dynamics of mtDNA and nuDNA, population growth, and/or sex-biased dispersal. Our inability to detect a range expansion based on CR may be due to its faster mutation rate and smaller N_e compared to nuDNA, which results in a more rapid loss of low frequency haplotypes and accumulation of closely related, private haplotypes. Conversely, the relatively slow mutation rate and larger N_e of nuDNA should increase persistence of alleles following a range expansion. A rapid population growth following a founder event(s) may limit the loss of genetic diversity following range expansion (Austerlitz et al. 1997). We calculated significant negative values of Tajima's D and/or Fu's F_S for CR, which supports the possibility of rapid population growth. Finally, our inability to detect a range expansion

based on CR may reflect lower dispersal in females than males, because it is only possible to detect that a population has gone through a large range expansion if current populations exchange a large number of migrants per generation (Excoffier et al. 2009).

An increase in mesic conditions (Renken et al. 2002) and anthropogenic deforestation (Burney and Burney 1994) in Puerto Rico during the Holocene may have facilitated *E. antillensis*' expansion into the western region of the island. Deforestation was particularly extensive in Puerto Rico's Central Mountains during the 19th and 20th centuries (Birdsey and Weaver 1987), and led to replacement of primary montane forest with disturbed habitats (e.g. road-side edges, pastures, city gardens, agricultural areas) that this species can occupy (Herrera-Montes and Brokaw 2010, Joglar 1998). The Central Mountains comprise a large area of western Puerto Rico (Fig. 1), which suggests that many *E. antillensis* populations in this region became established in the past 200 years.

Conservation implications

Eleutherodactylus portoricensis is classified as “endangered” by the International Union for Conservation of Nature (Angulo 2008), and as “vulnerable” by the Department of Natural and Environmental Resources of Puerto Rico (Departamento de Recursos Naturales y Ambientales 2004). Populations appear to be extirpated from the western portion of the Central Mountains (Barker and Ríos-Franceschi in press), and they have been contracting to elevations above 600 m in the Luquillo Mountains over the past three decades (Longo and Burrowes 2010, Woolbright 1997). Population declines and extirpations in the Luquillo Mountains were attributed to extended periods of drought and a synergistic interaction between climate and infection with *Batrachochytrium dendrobatidis*, a chytrid fungus that causes the disease chytridiomycosis (Burrowes et al. 2004, Longo and Burrowes 2010, Woolbright 1997). We suggest that *E. portoricensis* populations in the Cayey and Luquillo Mountains should each be minimally designated as a “distinct population segment” (Federal Register, February 7, 1996, 61:4722–4725), or more appropriately, as “endangered” (Barker and Ríos-Franceschi in press), due to their contracting range and deep temporal divergence, which suggests that these demes are on independent evolutionary trajectories. Lower genetic diversity in Cayey Mountains populations, compared to Luquillo Mountains demes, suggests smaller effective population sizes in the former, which may increase their risk of extinction (Frankham 1996). Although *E. portoricensis* has persisted in narrow ranges through past warming events, the simultaneous occurrence of several threats to Puerto Rican montane species (increasing temperature, longer dry seasons, increased drought frequency, and upward shifts in the cloud base; Cashman et al. 2010, Comarazamy and González 2011, Jennings et al. 2014) places the long term survival of this frog in grave danger.

Conclusion

We documented how connectivity and suitable climate shaped genetic differentiation and genetic diversity in two tropical insular species. Patterns of population differentiation in *E. portoricensis*, but not in *E. antillensis*, were consistent with the connectivity of past and current suitable climates predicted by ENMs. Unsuitable climate in the vicinity of the Río Grande de Loíza Basin is a strong barrier to gene flow between *E. portoricensis* populations in the Cayey and Luquillo Mountains, and a weak barrier for *E. antillensis*. Regions with a

large area of long-term suitable climate are associated with higher genetic diversity in both species, suggesting that they fostered larger and more stable populations. Understanding the relative roles of landscape features, dispersal capabilities, and climate characteristics that enable population persistence will improve predictions of future environmental change on biotas in Puerto Rico and other tropical insular systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Map of Puerto Rico showing the topography of the island and the geographic origins of the *Eleutherodactylus portoricensis* (triangles) and *E. antillensis* (circles) samples included in our genetic analyses. The Central Mountains (=Cordillera Central), the Cayey Mountains (=Sierra de Cayey; a southeastern extension of the Central Mountains), the Río Grande de Loíza, and the Luquillo Mountains (=Sierra de Luquillo) are indicated. The dotted line depicts the approximate distribution of the Río Grande de Loíza Basin.

Figure 2.

Logistic output of ecological niche models (ENMs) for (a) *Eleutherodactylus portoricensis* and (b) *E. antillensis* in Puerto Rico under last interglacial (LIG), last glacial maximum [under CCSM3 [LGM (CCSM3)] and MIROC [LGM (MIROC) scenarios], mid-Holocene, and current climatic conditions. Warmer colours represent areas of higher climatic suitability. Geographic locations used for ecological niche modeling are depicted in ENMs under current climate. The inset on Puerto Rico's map (top left) shows the study area for *E. portoricensis*.

Figure 3.

Map showing the long-term climatic suitability surfaces for (a) *Eleutherodactylus portoricensis* (b) and *E. antillensis*, calculated as the sum of climatic suitability pixels under last interglacial (LIG), last glacial maximum (LGM), mid-Holocene, and current climatic conditions. Two surfaces for each species are shown to illustrate CCSM3 (top) and MIROC (bottom) scenarios of LGM climatic conditions. Long-term climatic suitability surface values are presented in four groups of equal size (i.e. quartiles). The inset on Puerto Rico's map (top left) shows the study area for *E. portoricensis*. Dotted lines represent the division used to define regional populations (western and eastern Puerto Rico) of *E. antillensis* for testing the hypothesis that genetic diversity is higher in areas with a larger region of suitable climate over the LIG, LGM, mid-Holocene, and present-day periods. The Central Mountains (=Cordillera Central), the Cayey Mountains (=Sierra de Cayey; a southeastern extension of the Central Mountains), the Río Grande de Loíza, and the Luquillo Mountains (=Sierra de Luquillo), and the geographic origins of the *E. portoricensis* and *E. antillensis* samples included in our genetic analyses are indicated.

Figure 4.

Maps of the study areas for (a) *Eleutherodactylus portoricensis* and (b, c) *E. antillensis* in Puerto Rico, illustrating the results of STRUCTURE analyses of four nuDNA intron loci (CRYBA, MYH, RH1, and RPL9int4) under a model of correlated allele frequencies, and the conductance surfaces estimated from ecological niche models under last interglacial, last glacial maximum (CCSM3 scenario), mid-Holocene, and current climatic conditions. The conductance surface for *E. antillensis* is shown twice to illustrate results from a STRUCTURE analysis based on the models of (b) admixture and (c) no-admixture. Pie charts depict the mean membership fractions of individuals at each sampling locality in relation to a genetic cluster inferred by STRUCTURE analyses. To prevent overlap of pie charts, we used black leader lines to indicate the geographic location of nearby sampling localities. Bar plots are shown for the best estimate of K, where K is the number of genetic clusters. Each vertical bar shows the proportional representation of the estimated cluster membership for a single individual, and is sorted by the latitude and longitude of its locality. The location of the Río Grande de Loíza is indicated.

Table 1

Models of gene flow tested for *Eleutherodactylus portoricensis* and *E. antillensis*. Pearson's correlation coefficients (r) and associated p values are reported for simple Mantel tests correlating Slatkin's linearized F_{ST} and one geographic factor (either log geographic distance or resistance), and for partial Mantel tests correlating Slatkin's linearized F_{ST} and one geographic factor after removing the effect of the other. Slatkin's linearized F_{ST} was calculated from the mitochondrial control region (CR) and the combined nuclear DNA loci (CRYBA, MYH, RH1, and RPL9int4) separately. Two results for analyses of resistance are reported to accommodate CCSM3 and MIROC scenarios of last glacial maximum climatic conditions.

Model	<i>E. portoricensis</i>				<i>E. antillensis</i>			
	mtDNA		combined		mtDNA		combined	
	CR r	p value	mtDNA r	p value	CR r	p value	mtDNA r	p value
<i>Simple Mantel</i>								
Log geographic distance	0.84	<0.001	0.82	<0.001	0.17	<0.001	0.23	<0.001
Resistance (CCSM3)	0.91	<0.001	0.88	<0.001	0.30	<0.001	0.22	0.001
Resistance (MIROC)	0.91	<0.001	0.87	<0.001	0.25	<0.001	0.21	0.001
<i>Partial Mantel</i>								
Log geographic distance and resistance (CCSM3)	0.03	0.38	0.09	0.23	-0.15	0.95	0.05	0.24
Log geographic distance and resistance (MIROC)	0.02	0.43	0.14	0.12	-0.05	0.72	0.07	0.17
Resistance (CCSM3) and log geographic distance	0.66	<0.001	0.56	0.002	0.29	<0.003	0.09	0.19
Resistance (MIROC) and log geographic distance	0.66	<0.001	0.50	0.001	0.19	0.05	0.08	0.22

Table 2

Estimated divergence time (t), ancestral population sizes (N_A), current population sizes (N_{e1} and N_{e2}), and population migration rates ($2N_m$) for isolation with migration models for *Eleutherodactylus portoricensis*. The peak value and 95% highest posterior density (HPD) values are indicated for each demographic parameter. Two separate analyses were carried out with different upper bounds for the t prior to explore alternative demographic histories (see text for details). Asterisks indicate that the population migration rate is significantly different from zero ($p < 0.01$) according to the Nielsen and Wakeley (2001) test.

Population 1 × population 2	t (years)	N_A	N_{e1}	N_{e2}	$2N_{m1 \rightarrow 2}$	$2N_{m2 \rightarrow 1}$
Cayey Mountains × Luquillo Mountains						
“Low” upper bound for t prior						
Peak value	182,315	486,644	209,239	271,011	1.487	1.163
Lower 95% HPD	102,418	283,794	129,318	101,954		
Upper 95% HPD	308,797	718,975	296,152	473,579		
“High” upper bound for t prior						
Peak value	1,512,771	665,894	331,826	352,812	6.578**	2.427
Lower 95% HPD	162,014	422,480	207,068	0		
Upper 95% HPD	3,183,846	942,806	466,473	1,012,587		

Table 3

Results of Mann-Whitney-Wilcoxon tests (one-sided) comparing the mean value of the observed number of segregating sites (θ_S) and nucleotide diversity (π) of the mitochondrial control region (CR) and the combined nuclear DNA loci (CRYBA, MYH, RHI, and RPL9int4) in *Eleutherodactylus portoricensis* populations in the Cayey and Luquillo Mountains, and in *E. antillensis* populations from western and eastern Puerto Rico. Population groups correspond to those indicated in Figure 3.

		<i>E. portoricensis</i>		<i>E. antillensis</i>	
		Cayey Mountains	Luquillo Mountains	western Puerto Rico	eastern Puerto Rico
				p value	p value
mtDNA CR	θ_S	4.60	7.43	0.008	1.50
	π	0.0087	0.0122	0.41	0.0027
Combined nuDNA					
	θ_S	8.25	9.71	0.02	3.77
	π	0.0057	0.0067	0.01	0.0024