

Genetic structuring in the threatened “Lagartijo del Bosque Seco” (*Anolis cooki*) from Puerto Rico

Javier A. Rodríguez-Robles^{a,*}, Tereza Jezkova^a, Manuel Leal^b

^a School of Life Sciences, University of Nevada, Las Vegas, 4505 Maryland Parkway, Las Vegas, NV 89154-4004, USA

^b Department of Biology, Duke University, Durham, NC 27708-0338, USA

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Abstract

Species with restricted geographic distributions consisting of isolated populations are particularly susceptible to extinction because these demes face an increased risk of disappearing due to environmental, demographic, and genetic stochasticity. We used partial DNA sequences of the cytochrome *b* (1083 bp) and ND2 (1041 bp) mitochondrial genes to investigate the phylogeography and population genetics of *Anolis cooki*, a threatened lizard endemic to the southwestern coast of the Caribbean Island of Puerto Rico. Maximum likelihood and Bayesian methods revealed relatively shallow genetic differentiation among 27 unique haplotypes (from 52 individuals) from the known extant populations of *A. cooki* in mainland Puerto Rico. Despite this pattern, specimens from the same geographic area tended to nest together. The most basal division within *A. cooki* is between haplotypes from the three westernmost populations (Punta Águila, Morrillos, Playa Santa) and the remainder demes (Bahía Ballena, La Cueva, Punta Verraco). The three westernmost populations of *A. cooki* are separated from their conspecific demes by the Guánica Bay and the Loco River drainage system, which together may represent a physiographic barrier for *A. cooki*. Each population of *A. cooki* only has private haplotypes; in other words, there are no shared mitochondrial types between populations. Because the number of private haplotypes can be used as an indirect measure of gene flow, this finding suggests that currently there is no migration among demes, and that each is an independent demographic unit, despite the relatively short distances (ca. 2 km) that separate some of them. Pairwise F_{ST} values and spatial analyses of molecular variation confirmed the existence of distinct groups of genetically defined sampling areas, and of significant molecular variation among populations within groups and within populations. The conservation status of the populations of *A. cooki* varies greatly. The demes from Punta Águila, Morrillos, and Bahía Ballena inhabit protected areas, and are larger, genetically diverse, and seemingly stable. The population from Playa Santa showed a high level of genetic diversity, but it occurs in an area that has been intensively developed for residential and touristic purposes, and its long-term survival is uncertain. *A. cooki* is also known from Caja de Muertos, an island off the southcentral coast of Puerto Rico. Surveys conducted on September 2006 and March 2007 did not produce any specimens, and a thorough assessment of Caja de Muertos is needed to determine the present status of *A. cooki* on the island.

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1. Introduction

[*Anolis cooki*] clearly survives as of now. But is this a stable situation? It is easy to see climatic change wiping *cooki* out. But even without climatic change is

cooki holding its own? It is not possible to answer this question on any current evidence, but the existence of *cooki* may already be marginal. It may be the very model of a species about to submerge (Williams, 1972, p. 83).

Species with restricted geographic distributions consisting of small, isolated populations are particularly susceptible to extinction because these demes face an increased risk of disappearing due to environmental, demographic, and

* Corresponding author. Fax: +1 702 895 3956.

E-mail addresses: javier.rodriguez@unlv.edu (J.A. Rodríguez-Robles), jezkovat@unlv.nevada.edu (T. Jezkova), mleal@duke.edu (M. Leal).

genetic stochasticity. Environmental stochasticity is random, unpredictable variation in environmental factors, such as rainfall and food supply. Demographic stochasticity is variation in birth and death rates and sex ratios due to chance alone. Genetic stochasticity encompasses the deleterious impact of inbreeding, loss of genetic diversity, and mutational accumulation on populations (Fischer and Matthies, 1998; Shrestha et al., 2002; Johansson et al., 2007). Small, disjunct populations are also more likely to be decimated by catastrophes (extreme forms of environmental fluctuation such as hurricanes, floods, landslides, severe cold, and forest fires), disease outbreaks, and destruction or degradation of their habitat due to anthropogenic activities (Young, 1994; Lande, 1999). For these reasons, island populations are more prone to extinction than mainland populations. Indeed, island endemics have higher extinction risks than nonendemic species (Frankham, 1998).

Genetic variation within a species is considered to be of great importance for its long-term survival (Lande, 1999). Without an appropriate amount of genetic diversity, species have reduced potential to adapt to environmental change and to cope with evolving predators, competitors, and parasites (Hudson, 1996; Kirkpatrick, 1996). Small

population size can lead to random changes in gene frequencies (genetic drift), which results on average in a loss of genetic variance from a population (Lande, 1999; Frankham et al., 2002). Because future evolutionary adaptation depends on the existence of genetic diversity, loss of variation increases the possibility of extinction. The primary objective of conservation biology is thus to preserve both evolutionary processes and the ecological viability of populations by maintaining as many distinctive genetic “units” as possible within a species (Moritz, 2002; Forest et al., 2007; Lankau and Strauss, 2007).

Anolis cooki Grant (“Lagartijo del Bosque Seco,” Dry Forest Anole) is a moderate-size, sexually dimorphic lizard (snout-to-vent length up to 70 mm in males, up to 59 mm in females; Schwartz and Henderson, 1991) endemic to the southwestern coast of Puerto Rico, the smallest and easternmost of the Greater Antilles, in the Caribbean Sea. The species has a discontinuous distribution on Caja de Muertos Island (located off the municipality of Juana Díaz in southcentral Puerto Rico) and between the municipalities of Cabo Rojo and Guayanilla on mainland Puerto Rico (Gorman et al., 1968; Jenssen, 1990; Schwartz and Henderson, 1991; Rivero, 1998; Fig. 1). Currently, there are only a few known populations of *A. cooki*, and all

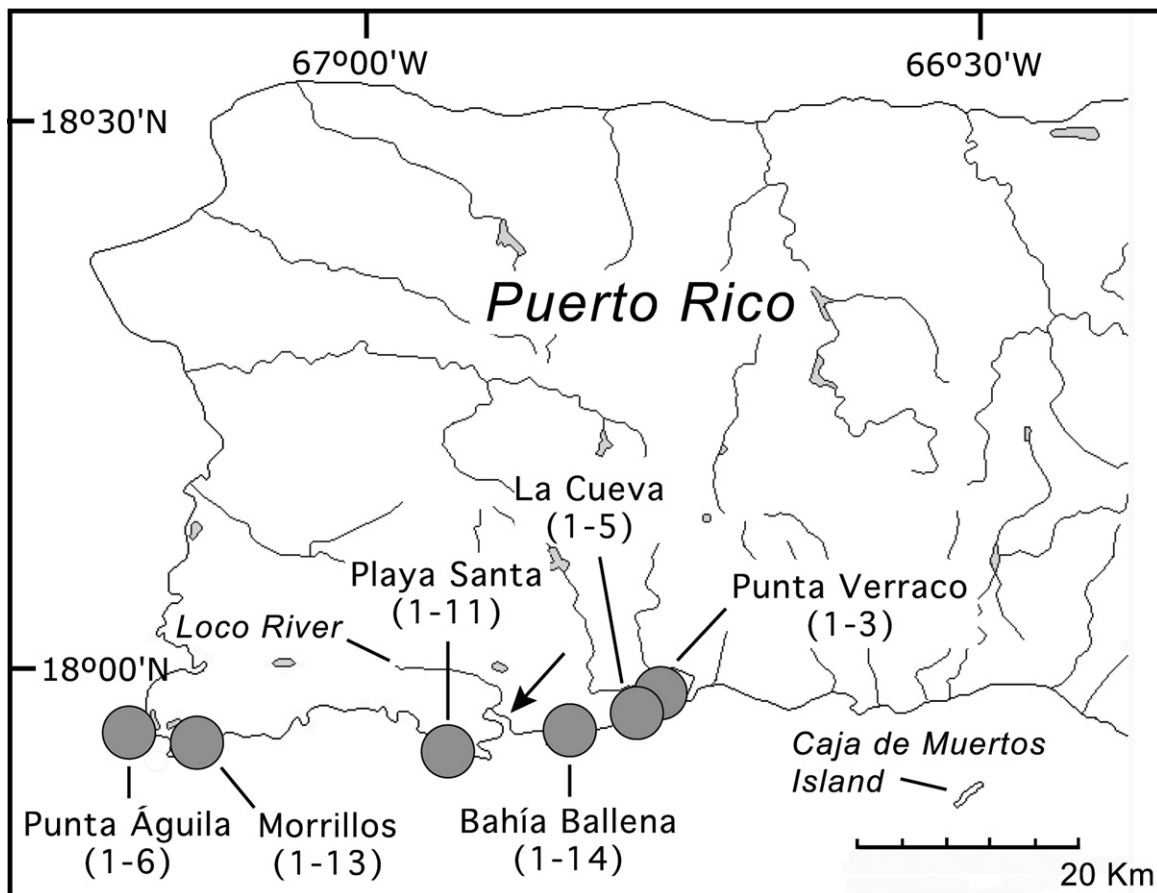


Fig. 1. Map of western Puerto Rico. Circles indicate the approximate locations of the specimens of *Anolis cooki* included in this study (see Table 1 for specific locality information). The arrow indicates the location of Guánica Bay.

are restricted to isolated coastal patches of xeric vegetation surrounded by rocky outcrops or sandy areas (Marcellini et al., 1985; Jenssen, 1990; Ortiz, 1991; Helmer et al., 2002). Because of its limited and disjunct distribution, *A. cooki* was designated in 1991 as a threatened species by the Department of Natural and Environmental Resources of Puerto Rico (Moreno, 1991).

We hypothesized that the fragmented distribution of *A. cooki* has led to noticeable reduction, or even cessation, of gene flow among populations, which in turn has resulted in geographic structuring of genetic variation in this threatened anole. We tested this hypothesis by using mitochondrial DNA (mtDNA) markers to characterize genetic variation within *A. cooki* and to assess the geographic partitioning of this genetic diversity. Additionally, we used our findings to determine whether distinct population segments of this species are on separate evolutionary trajectories (i.e., whether they are “evolutionary significant units”), and therefore worthy of special management consideration in conservation efforts (Fraser and Bernatchez, 2001; Moritz, 2002).

2. Materials and methods

2.1. Taxon sampling, DNA isolation, and sequencing

We secured samples from the known extant populations of *A. cooki* (except from the deme from Caja de Muertos Island; see Section 4.2), and unsuccessfully looked for new localities for the species. We obtained tissue samples from 52 individuals from six populations (Table 1 and Fig. 1) in mainland Puerto Rico. We used *Anolis monensis* and *Anolis cristatellus* as outgroup taxa based on previous phylogenetic studies based on karyotypic, electrophoretic, morphological, and DNA sequence data (Gorman et al., 1968, 1983; Brandley and de Queiroz, 2004; Nicholson et al., 2005; Rodríguez-Robles et al., 2007). *A. monensis*, the sister species of *A. cooki* (references above), is a taxon endemic to Mona and Monito Islands, located ca. 67 and 72 km off the southwestern coast of Puerto Rico, respectively, whereas *A. cristatellus* occurs throughout Puerto Rico and on many islands east of Puerto Rico, including Vieques, Culebra, and the US and British Virgin Islands.

We extracted total genomic DNA from frozen tissue samples (liver, muscle, and tail fragments) with the DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA). Using total cellular DNA as a template, we amplified a fragment of the mitochondrial gene cytochrome *b* (“Cyt *b*”), a segment of the nicotinamide adenine dinucleotide dehydrogenase (NADH) subunit 2 (“ND2”), and two tRNA genes (tRNA^{Trp}, tRNA^{Ala}) using the polymerase chain reaction (PCR). We used the primers MVZ_49 (ATAARAA CAATGACAATYATACGAAG) and MVZ_14 (GGTCT TCATCTYHGGYTTACAAGAC) to amplify ca. 1100 base pairs (bp) of Cyt *b*, and the primers LVT_Metf. 6_AnCr (AAGCTATTGGGCCCATACC) and LVT_5617_AnCr (AAAGTGYTTGAGTTGCATTCA) to amplify

ca. 1150 bp of ND2 and the two adjacent tRNAs. We carried out PCR reactions in 12.5 µl volumes consisting of 1 µl of template DNA, 0.5 µl of each primer (10 µM), 6.25 µl of Takara *Ex Taq*TM Polymerase Premix (Takara Mirus Bio Inc., Madison, WI), and 4.25 µl ddH₂O. DNA was denatured initially at 95 °C for 2.5 min, and then 30 cycles of amplification were performed under the following conditions: denaturation at 95 °C for 1 min, annealing at 57 °C (for ND2) or 51 °C (for Cyt *b*) for 1 min, and extension at 72 °C for 1 min, followed by a final 10 min elongation at 72 °C. Two microliters of all PCR products were electrophoresed on a 0.8% agarose gel stained with ethidium bromide to verify product band size.

We cleaned the double-stranded PCR products with ExoSap-IT[®] (USB Corporation, Cleveland, OH). We sequenced the Cyt *b* fragment with MVZ_49 and MVZ_14. We sequenced the ND2 fragment using the primers LVT_Metf.6_AnCr and LVT_L5002_AnPu (AACCAACACARACTCGAAAAAT). We used the Big Dye Terminator Ready Reaction Kit 1.1 or 3.1 (Applied Biosystems, Foster City, CA) for cycle sequencing, and ran the sequences on an ABI 3130 automated sequencer.

2.2. Phylogenetic and population analyses

Because different partitions of a phylogenetic dataset can produce trees with dissimilar topologies, statistical testing is used to evaluate whether the data portions contain congruent signal. The incongruence length difference (ILD) test (Farris et al., 1994) is commonly employed to assess whether disparities among topologies inferred from different data partitions are likely to have been observed by chance. The ILD test is more susceptible to type I errors (i.e., false inferences of incongruence when the null hypothesis that the partitions combine to produce an accurate estimate of phylogeny is true) than to type II errors (false inferences of congruence when incongruence is in fact present; Hipp et al., 2004). Therefore, the ILD test is a reasonable starting point for identifying potentially incongruent data partitions (Planet, 2006).

Before performing the ILD test we collapsed sequences to unique haplotypes (unique sequences) using the program COLLAPSE (version 1.2; available at <http://darwin.uvigo.es>). An ILD test performed with the program PAUP* (version 4.10b; Swofford, 2003) indicated that the sequences from the Cyt *b* (1083 bp) and ND2 (1041 bp) genes contained congruent phylogenetic signal (1000 replicates, $P = 1.0$). Accordingly, we combined the two datasets for all analyses, and conducted all subsequent phylogenetic tests using maximum likelihood (ML) and Bayesian inference methods.

We used the software MRMODELTEST (version 2.2; Nylander, 2004) to select the best-fit models of nucleotide substitution for the ML analyses. Hierarchical likelihood ratio tests identified HKY + G + I and GTR + G as the most appropriate models for the first plus second codon positions, and for the third codon position, respectively,

Table 1
Taxon, sample number, voucher number, GenBank accession numbers, locality, and coordinates of the specimens used in this study

Taxon	Sample number	Voucher number	GenBank Accession Nos. for cytochrome <i>b</i> and ND2, respectively; locality	Coordinates (latitude, longitude)
Outgroup				
<i>Anolis cristatellus</i>	—	MVZ 242846	EF553539, EF184065; Puerto Rico: Municipality of Lajas, km 3.3 on Rd. 304	17.98, –67.05
<i>Anolis monensis</i>	—	MVZ 235440	EF553612, EF184138; Mona Island: vicinity of Playa Sardinera	18.09, –67.94
	—	MVZ 235454	EF553622, EF184148; Monito Island	18.16, –67.95
Ingroup				
<i>Anolis cooki</i>	Punta Águila 1	MVZ 257364	EU095684, EU095723; Puerto Rico: Municipality of Cabo Rojo, Punta Águila	17.95, –67.21
	Punta Águila 2	MVZ 257365	EU095685, EU095724	
	Punta Águila 3	MVZ 257366	EU095686, EU095725	
	Punta Águila 4	MVZ 257367	EU095687, EU095726	
	Punta Águila 5	MVZ 257368	EU095688, EU095727	
	Punta Águila 6	MVZ 257369	EU095689, EU095728	
	Morrillos 1	MVZ 235170	EU119666, EF184066; Puerto Rico: Municipality of Cabo Rojo, Bosque Estatal de Boquerón, Morrillos de Cabo Rojo	17.94, –67.20
	Morrillos 2	MVZ 235172	EU119667, EF184067	
	Morrillos 3	MVZ 252194	EU119675, EF184068	
	Morrillos 4	MVZ 252195	EU119676, EF184069	
	Morrillos 5	MVZ 252196	EU119677, EF184070	
	Morrillos 6	MVZ 252197	EU119678, EF184071	
	Morrillos 7	MVZ 252198	EU095667, EU095706	
	Morrillos 8	MVZ 252199	EU095668, EU095707	
	Morrillos 9	MVZ 252200	EU095669, EU095708	
	Morrillos 10	MVZ 252201	EU095670, EU095709	
	Morrillos 11	MVZ 252202	EU095671, EU095710	
	Morrillos 12	MVZ 252203	EU095672, EU095711	
	Morrillos 13	MVZ 252204	EU095673, EU095712	
	Playa Santa 1	MVZ 252211	EU095665, EU095704; Puerto Rico: Municipality of Guánica, Balneario Playa Santa	17.94, –66.96
	Playa Santa 2	MVZ 252212	EU095666, EU095705	
	Playa Santa 3	MVZ 257334	EU095675, EU095714	
	Playa Santa 4	MVZ 257335	EU095676, EU095715	
	Playa Santa 5	MVZ 257336	EU095677, EU095716	
	Playa Santa 6	MVZ 257337	EU095678, EU095717	
	Playa Santa 7	MVZ 257338	EU095679, EU095718	
	Playa Santa 8	MVZ 257339	EU095680, EU095719	
	Playa Santa 9	MVZ 257340	EU095681, EU095720	
	Playa Santa 10	MVZ 257341	EU095682, EU095721	
	Playa Santa 11	MVZ 257342	EU095683, EU095722	
	Bahía Ballena 1	MVZ 250896	EU119668, EF184072; Puerto Rico: Municipality of Guánica, Bahía Ballena	17.96, –66.86
	Bahía Ballena 2	MVZ 250897	EU119669, EF184073	
	Bahía Ballena 3	MVZ 250898	EU119670, EF184074	
	Bahía Ballena 4	MVZ 250899	EU119671, EF184075	
	Bahía Ballena 5	MVZ 250900	EU119672, EF184076	
	Bahía Ballena 6	MVZ 250901	EU119673, EF184077	
	Bahía Ballena 7	MVZ 250902	EU119674, EF184078	
	Bahía Ballena 8	MVZ 252206	EU095660, EU095699	
	Bahía Ballena 9	MVZ 252207	EU095661, EU095700	
	Bahía Ballena 10	MVZ 252208	EU095662, EU095701	
	Bahía Ballena 11	MVZ 252209	EU095663, EU095702	
	Bahía Ballena 12	MVZ 252210	EU095664, EU095703	
	Bahía Ballena 13	MVZ 251147	EU095651, EU095690	
	Bahía Ballena 14	MVZ 251148	EU095652, EU095691	
	La Cueva 1	MVZ 252213	EU095653, EU095692; Puerto Rico: Municipality of Guayanilla, La Cueva	17.97, –66.79
	La Cueva 2	MVZ 252214	EU095654, EU095693	
	La Cueva 3	MVZ 252215	EU095655, EU095694	

Table 1 (continued)

Taxon	Sample number	Voucher number	GenBank Accession Nos. for cytochrome <i>b</i> and ND2, respectively; locality	Coordinates (latitude, longitude)
	La Cueva 4	MVZ 252216	EU095656, EU095695	
	La Cueva 5	MVZ 252219	EU095674, EU095713	
	Punta Verraco 1	MVZ 252205	EU095659, EU095698; Puerto Rico: Municipality of Guayanilla, Punta Verraco	17.98, –66.78
	Punta Verraco 2	MVZ 252217	EU095657, EU095696	
	Punta Verraco 3	MVZ 252218	EU095658, EU095697	

Museum abbreviation: MVZ, Museum of Vertebrate Zoology, University of California, Berkeley.

of the combined Cyt *b* and ND2 datasets. (Analyses that partitioned the data by gene only or by gene-specific codon position resulted in trees of significantly lower likelihood scores.) We conducted ML analyses using TREEFINDER (Jobb et al., 2004). TREEFINDER uses a fast sampling algorithm to estimate all model parameters and construct a phylogeny. The accuracy of the program with regard to correctly inferring tree topologies and estimating branch lengths is similar to that of other likelihood programs such as FASTDNAML (Olsen et al., 1994) and PAUP* (Jobb et al., 2004). We assessed nodal support for the ML tree by performing a bootstrap analysis, as implemented in TREEFINDER (HKY + G + I and GTR + G models of nucleotide substitution for the first plus second codon positions, and for the third codon position, respectively, 1000 replicates, consensus level, 50).

We also estimated tree topology and clade support using Bayesian inference methods, as implemented in MRBAYES (version 3.1.1; Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Using the HKY + G + I and GTR + G models selected by MRMODELTEST, we partitioned the data by first plus second and third codon positions, and initiated the analyses from a random starting tree with uniform (uninformative) priors (Brandley et al., 2006). We produced posterior probability distributions by allowing four Monte Carlo Markov Chains (using default heating values) to proceed for 10,000,000 generations each, with samples taken every 100 generations, a procedure that yielded 100,000 trees. We assessed parameter stabilization by examining plots of log-likelihood scores versus number of generations (Leaché and Reeder, 2002). We discarded the first 2,500,000 generations (25,000 trees), as “burn-in” samples (i.e., trees obtained before parameter stabilization occurred), and combined the remaining samples to estimate tree topology, posterior probability values, and branch lengths. We ran the Bayesian analyses three times to ensure that they were not trapped on local optima.

Tree-building methods tend to resolve intraspecific gene genealogies poorly when the different mitochondrial types are separated by few mutations and ancestral haplotypes are still present in the populations (Crandall and Templeton, 1996). Accordingly, we used NETWORK 4.200 (<http://www.fluxus-technology.com>) to construct a median-joining network (Bandelt et al., 1999) to visualize better the relationships among the haplotypes of *A. cooki*. We estimated

mean, pairwise, uncorrected sequence divergences between populations with MEGA (version 3.1; Kumar et al., 2004).

For population genetic analyses, we calculated haplotype (*h*) and nucleotide diversity (π), and conducted tests of selective neutrality using ARLEQUIN (version 3.1; Excoffier et al., 2005). We also assessed genetic differentiation among the six sampling localities using Sewall Wright’s fixation index F_{ST} (10,000 permutations). Using the program SAMOVA (Spatial Analysis of Molecular Variance) 1.0 (Dupanloup et al., 2002; <http://web.unife.it/progetti/genetica/Isabelle/samova.html>), we characterized patterns of genetic divergence among populations of *A. cooki* to identify partitions of sampling areas that are geographically homogeneous and maximally differentiated from each other. We performed these analyses based on 500 simulated annealing steps, and compared maximum indicators of differentiation (F_{CT} values) when the program was instructed to identify $K = 2$ through $K = n - 1$ partitions of the sampling localities, where K is the number of genetically defined units, and n is the number of sampling areas. We further explored the partitioning of variance among and within the maximally differentiated groups with AMOVA (as performed in ARLEQUIN).

3. Results

3.1. Phylogenetic relationships and divergence estimates

Maximum likelihood and Bayesian methods inferred a tree with some nodes that are not strongly supported, suggesting that there is shallow (i.e., recent) genetic differentiation among some populations of *A. cooki* (Fig. 2). Despite this general pattern, the haplotypes of the three westernmost demes (Punta Águila, Morrillos, Playa Santa) of this lizard grouped together, to the exclusion of samples from the three easternmost populations (Bahía Ballena, La Cueva, Punta Verraco). The “western clade” of *A. cooki* comprises three strongly supported subclades, one formed by all the haplotypes from Morrillos and one from Punta Águila, one composed exclusively by three of the five Playa Santa mitochondrial types, and one formed by the other two haplotypes from Playa Santa and one from Punta Águila. Despite the fact that the latter two subclades contain all the haplotypes from Playa Santa, these two groupings are not most closely related to each other. Regarding the three

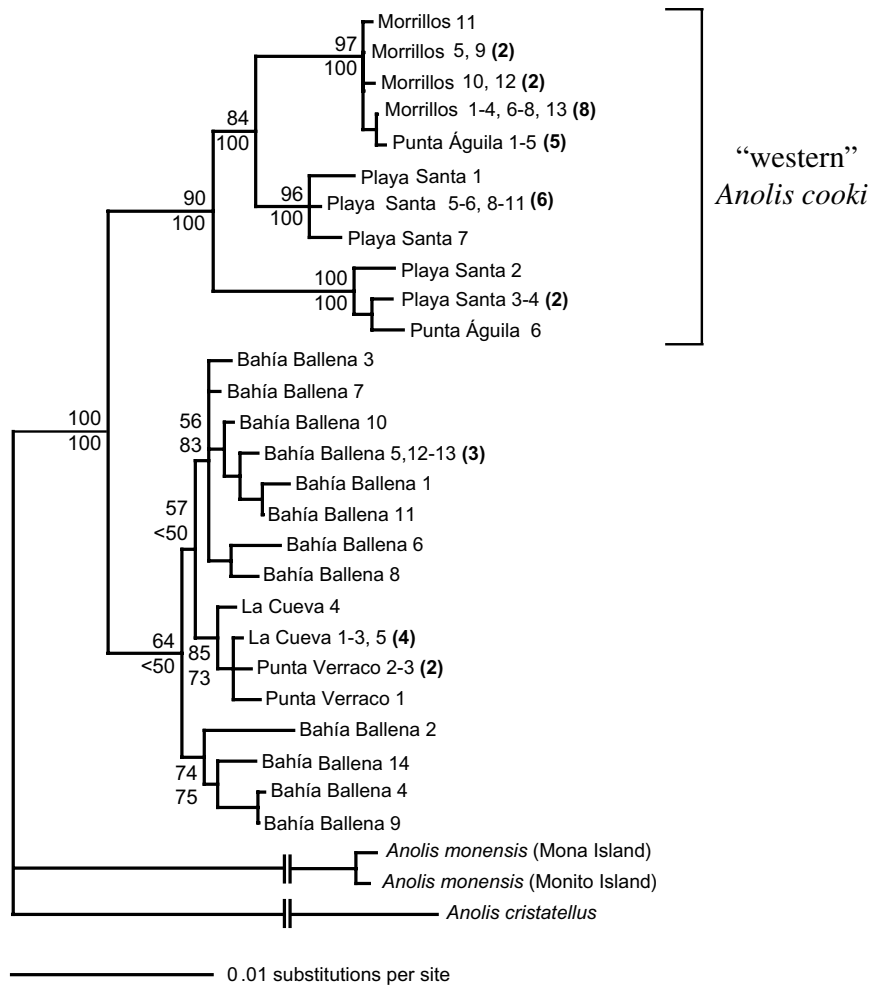


Fig. 2. Maximum likelihood tree for 27 unique mtDNA haplotypes of *Anolis cooki* from southwestern Puerto Rico. *Anolis monensis* and *A. cristatellus* were used as outgroup taxa. Nodal support was assessed with nonparametric bootstrap values (for ML analyses; numbers above) and with Bayesian posterior probabilities (numbers below). See Section 2 for details of phylogenetic analyses.

easternmost populations of *A. cooki*, the mitochondrial types from Bahía Ballena tended to form two groups, whereas those from La Cueva and Punta Verraco nested together.

The median-joining network of *A. cooki* depicts three main clusters (Fig. 3). Two of these clusters constitute the western clade inferred by ML and Bayesian analyses. One of these two clusters is formed by the four haplotypes from Morrillos and one from Punta Águila; these five mitochondrial types are only separated by 1–3 mutational steps. The second cluster is formed by the five haplotypes from Playa Santa (which differ by 5–35 mutational steps) and the second mitochondrial type from Punta Águila. The third cluster of *A. cooki* included all the haplotypes from Bahía Ballena, La Cueva, and Punta Verraco, and exhibited a higher haplotype diversity than the two westernmost clusters combined, particularly in Bahía Ballena. The specimens from the latter population formed two groups, whereas those from La Cueva and Punta Verraco nested together. The mitochondrial types from this third cluster are separated by 1–20 mutational steps.

We could not calibrate a precise molecular clock for *A. cooki* because of lack of fossil specimens. Nevertheless, the average rate of evolution of the fragment of the ND2 gene used in this study is 0.65% (range: 0.61–0.7%) per lineage per million years (Macey et al., 1998), a rate that has been used to estimate divergences in previous investigations of Caribbean *Anolis* (e.g., Creer et al., 2001; Jackman et al., 2002; Glor et al., 2003; Rodríguez-Robles et al., 2007). Applying this rate to the mean, pairwise, uncorrected ND2 sequence divergences obtained in this study yielded an age of ca. 690,000 years (range: 640,000–740,000 years; 0.9% divergence) for the split between the three westernmost populations of *A. cooki* and the three easternmost demes, ca. 310,000 years (range: 290,000–330,000 years; 0.4% divergence) for the separation between the two Cabo Rojo (i.e., Punta Águila and Morrillos) populations and the Playa Santa deme, and ca. 150,000 years (range: 140,000–160,000 years; 0.2% divergence) for the divergence of the Bahía Ballena and the two Guayanilla populations (La Cueva and Punta Verraco). These age estimates are for the split between the mtDNA gene lineages, not for

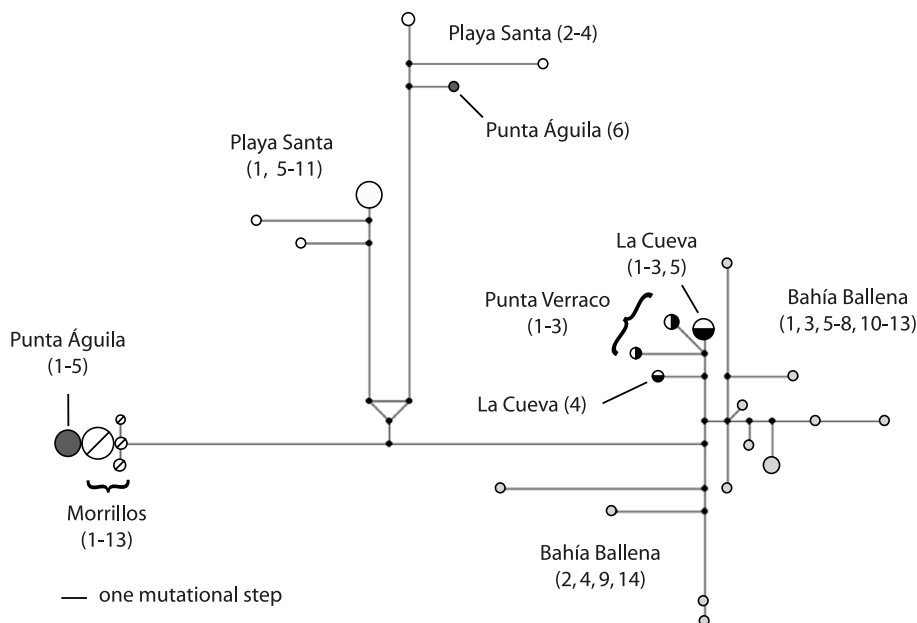


Fig. 3. Median-joining network representing the relationships among haplotypes of *Anolis cooki* from the Punta Águila (dark grey circles, $N = 6$), Morrillos (divided empty circles, $N = 13$), Playa Santa (empty circles, $N = 11$), Bahía Ballena (light grey circles, $N = 14$), La Cueva (half-filled circles, horizontal pattern, $N = 5$), and Punta Verraco (half-filled circles, vertical pattern, $N = 3$) populations. The smallest, black circles indicate missing (i.e., extant unsampled or extinct ancestral) haplotypes. Circle size is proportional to haplotype frequencies, with the smallest circle representing one sample and the largest circle representing eight samples; branch length is proportional to number of mutations separating the haplotypes.

the divergence of the ancestral populations, an event that almost always occurs sometime after the gene split (Edwards and Beerli, 2000). Consequently, these figures almost certainly represent overestimates of the timing of the population divergences.

3.2. Population genetics

Haplotype diversity in the populations of *A. cooki* included in this study ranged from 0.33 (Punta Águila) to 0.97 (Bahía Ballena; Table 2). There were no shared haplotypes between any of the populations (Fig. 3), that is, each of the six demes only has private mitochondrial types. Nucleotide diversity was relatively high in the Playa Santa population, intermediate in the Bahía Ballena deme, and low in the remainder four populations. Pairwise F_{ST} values revealed significant genetic differentiation among all populations of *A. cooki* (Table 3). Except for the deme from Punta Águila, Tajima's D and Fu's F_S statistics did not differ significantly from the expectation under neutrality, suggesting that evolu-

tion in the five populations has been relatively independent of selection, heterogeneity of mutation rates, or major population perturbations during the coalescent history of the Cyt b and ND2 genes (Templeton, 2006; Hartl and Clark, 2007). Tajima's D was significantly negative for the Punta Águila deme, suggesting that this population possibly experienced a relatively recent expansion.

The SAMOVA analyses revealed the existence of distinct groups of genetically defined sampling areas. In analyses where $K = 2$, partitions were identified that suggested "western" (i.e., Punta Águila, Morrillos, Playa Santa) and "eastern" (i.e., Bahía Ballena, La Cueva, Punta Verraco) clusters ($F_{CT} = 0.553$, $P < 0.001$). Analyses where $K = 3$ identified an additional partition that subdivided the western cluster into Punta Águila/Morrillos and Playa Santa groups ($F_{CT} = 0.675$, $P < 0.001$). In analyses where $K = 4$, the haplotypes from Playa Santa were divided into two groups ($F_{CT} = 0.719$, $P < 0.001$). Analyses where $K = 5$ revealed a strong trend suggesting a division between the population from Bahía Ballena and those from Guaya-

Table 2
Measures of haplotype and nucleotide diversity and tests of selective neutrality for populations of *Anolis cooki* (Excoffier et al., 2005)

Population	No. of samples (no. of haplotypes)	Haplotype diversity (\pm SD)	Nucleotide diversity (\pm SD)	Tajima's D	Fu's F_S
Punta Águila	6 (2)	0.33 (\pm 0.22)	0.00471 (\pm 0.00290)	-1.513, $P = 0.001$	8.007, $P = 0.997$
Morrillos	13 (4)	0.62 (\pm 0.14)	0.00045 (\pm 0.00037)	-0.059, $P = 0.46$	-0.628, $P = 0.21$
Playa Santa	11 (5)	0.71 (\pm 0.14)	0.00640 (\pm 0.00352)	0.226, $P = 0.61$	5.490, $P = 0.98$
Bahía Ballena	14 (12)	0.97 (\pm 0.04)	0.00477 (\pm 0.00260)	-1.169, $P = 0.12$	-2.596, $P = 0.09$
La Cueva	5 (2)	0.40 (\pm 0.24)	0.00075 (\pm 0.00062)	-1.094, $P = 0.11$	2.202, $P = 0.85$
Punta Verraco	3 (2)	0.67 (\pm 0.31)	0.00157 (\pm 0.00136)	0.0, $P = 0.80$	2.357, $P = 0.79$

Table 3
Pairwise F_{ST} values for populations of *Anolis cooki*

	Punta Águila	Morrillos	Playa Santa	Bahía Ballena	La Cueva	Punta Verraco
Punta Águila	—	0.235, $P = 0.001$	0.454, $P < 0.0001$	0.710, $P < 0.0001$	0.816, $P = 0.003$	0.773, $P = 0.012$
Morrillos	—	—	0.682, $P < 0.0001$	0.827, $P < 0.0001$	0.965, $P < 0.0001$	0.961, $P < 0.0001$
Playa Santa	—	—	—	0.647, $P < 0.0001$	0.699, $P = 0.001$	0.662, $P = 0.004$
Bahía Ballena	—	—	—	—	0.321, $P < 0.0001$	0.299, $P = 0.002$
La Cueva	—	—	—	—	—	0.392, $P = 0.032$

nilla (La Cueva and Punta Verraco; $F_{CT} = 0.720$; $P = 0.056$). The AMOVA analyses indicated that significant amounts of molecular variation occur among populations within groups for $K = 2$ (22.4%, $P < 0.001$), $K = 3$ (7.8%, $P < 0.001$), and $K = 4$ (1.6%, $P < 0.001$), but not for $K = 5$ (-0.01% , $P = 1.0$). The AMOVA analyses also indicated the presence of significant genetic variation within populations for $K = 2$ (22.3%, $P < 0.001$), $K = 3$ (24.7%, $P < 0.001$), $K = 4$ (26.6%, $P < 0.001$), and $K = 5$ (28.0%, $P < 0.001$).

4. Discussion

4.1. Phylogenetic relationships and population genetics

Anolis cooki is endemic to the southwestern coast of Puerto Rico. One hypothesis accounting for the origin of this anole is that it evolved allopatrically after being isolated from its ancestors on an insular region corresponding to the present southwestern area of Puerto Rico (Gorman et al., 1968). At that time, this region may have been separated from the main island mass by a higher than present sea level. The low llanos (i.e., large, grassy, almost treeless plains) to the north of the current distribution of *A. cooki* completely surround all extant populations of the species, and in this scenario could have been a water canal (Thomas, 1966; Thomas and Schwartz, 1966). Six other species of squamate reptiles (*Anolis poncensis*, the ground lizard *Ameiva wetmorei*, the worm lizard *Amphisbaena xera*, the geckos *Phyllodactylus wirshingi* and *Sphaerodactylus roosevelti*, and the blindsnake *Typhlops granti*) are also restricted to the xeric southern coastal region of Puerto Rico (Thomas, 1999), a pattern consistent with the proposition that this region may have been isolated from the rest of the island (cf. Glor et al., 2004). Molecular estimates suggest that the separation between the ancestors of *A. cooki* and its sister species *A. monensis* occurred ca. 10.2 (range: 9.5–10.9) million years ago (Rodríguez-Robles et al., 2007).

Despite the existence of shallow genetic differentiation among some populations of *A. cooki*, individuals from the same geographic area typically nested together. The most basal division within *A. cooki* is between haplotypes from the three westernmost (Punta Águila, Morrillos, Playa Santa) and the three easternmost (Bahía Ballena, La Cueva, Punta Verraco) populations of the species. This separation coincides with the geographic location of Guánica Bay (indicated by the arrow in Fig. 1) and the Loco

River drainage system. Guánica Bay is a deeply indented, narrow harbor (ca. 0.4 km wide) bordered by steep, high, and rugged hills, and together with the Loco River basin may represent a physiographic barrier for *A. cooki*. Within the “western clade” of *A. cooki*, individuals from the two localities from Cabo Rojo (Punta Águila and Morrillos) nested together (with one exception, see below), to the exclusion of the specimens from Playa Santa. This phylogenetic pattern is not surprising, given the proximity of the Punta Águila and Morrillos localities (ca. 2 km, airline distance), and their geographic separation from Playa Santa (ca. 23 km, airline distance), a break that most likely represents an actual gap in the current distribution of *A. cooki* (Williams, 1972; Jenssen, 1990; Schwartz and Henderson, 1991; Rivero, 1998). However, the Playa Santa samples formed two distinct subclades, one of which is most closely related to haplotypes from Morrillos and Punta Águila. Furthermore, one of the two mitochondrial types from Punta Águila unambiguously nested within one of the Playa Santa groups (Figs. 2 and 3). (We corroborated the locality and the haplotype of this specimen, and the pattern uncovered is unlikely to be the result of a mislabeled or contaminated sample.) Collectively, these findings suggest that the historical distribution of *A. cooki* west of Guánica Bay may have been more continuous than it is nowadays, and that there were higher levels of gene flow across this segment of the species’ range, compared to the present. Eventually the populations between Cabo Rojo and Playa Santa became extinct, but lineage sorting has not yet completely erased the evidence of this historical demographic connection. Relationships among the three easternmost populations of *A. cooki* are not well defined, but there was a tendency for the specimens from Bahía Ballena to form two groups, and for the individuals from the two Guayanilla localities (La Cueva and Punta Verraco, which are ca. 2 km apart, airline distance) to nest together.

Haplotype diversity was generally high in all populations of *A. cooki* (Table 2). This result was unexpected for the demes from Playa Santa and Bahía Ballena, because of the relatively small areas that they inhabit. We collected the individuals from Playa Santa in two ca. 5 m² localities along a ca. 0.5 km transect along the coast, whereas all the specimens from Bahía Ballena were captured in the same ca. 550 × 70 m area. The mitochondrial types from these two demes formed two “subgroups” each (Figs. 2 and 3). (In the case of Playa Santa, both groupings included haplotypes found in the two ca. 5 m² areas.) We interpret

these findings as suggesting that the Bahía Ballena population may have been recently formed by the admixture of two demes that were previously isolated for a period of time. Such event would have resulted in a population containing haplotypes that originated in different localities, and that consequently may not be as closely related to each other as mitochondrial types that arose in the same deme (cf. Kolbe et al., 2007). However, the mechanism responsible for the existence of two well-differentiated haplotype subgroups in Playa Santa remains unknown.

The known extant populations of *A. cooki* seem to be genealogically isolated from one another at the present time. Each deme only has private haplotypes; in other words, there are no shared mitochondrial types between populations. Because the number of private haplotypes can be used as an indirect measure of gene flow (Slatkin, 1985), this finding suggests that there is no migration among demes, and that each is an independent demographic unit, despite the relatively short distances that separate some of them. In other words, the populations of *A. cooki* do not constitute a metapopulation (cf. Hanski, 1999), and if one deme becomes extinct, it is unlikely that the area will be naturally recolonized by individuals from other populations.

4.2. Conservation implications

Genetics can inform conservation planning by identifying components of species that are on separate evolutionary trajectories, and that as such deserve special management considerations (Mockford et al., 2007). In species that are composed of genetically distinctive units, loss of any of these habitat components not only leads to local extinction, but also to a significant decrease of genetic diversity in the species. Because of their demographic isolation and genetic distinctiveness, each of the six populations of *A. cooki* included in this study is a lineage “demonstrating highly restricted gene flow from other such lineages within the higher organizational level (or lineage) of the species” (Fraser and Bernatchez, 2001, p. 247), and therefore represents an evolutionary significant unit that merits special conservation attention. Ensuring the long-term survival of these lineages will accomplish the most important goal of conservation biology, namely the preservation of potentially adaptive genetic variation within species (Moritz, 2002).

The conservation status of the populations of *A. cooki* varies greatly. Those from Cabo Rojo (Punta Águila and Morrillos) and Bahía Ballena are larger, genetically diverse, and seemingly stable. These demes are precisely those that inhabit protected areas (the Cabo Rojo populations lay in the Boquerón State Forest and in an U.S. Fish and Wildlife Refuge, whereas the Bahía Ballena population lies in the Guánica Forest Reserve), which underscores the effectiveness of habitat conservation in preserving biodiversity (Donald et al., 2007). We strongly suggest that the areas where these two *A. cooki* populations occur continue to be protected (cf. Ortiz, 1991; Genet, 2002). The deme from

Playa Santa showed a high level of genetic diversity. However, these individuals occur in an area that has been intensively developed for residential and touristic purposes (e.g., a condominium and several houses have been built 25–50 m of the shore). This proximity to human settlements makes the population vulnerable to continued habitat degradation and to introduced predators (e.g., Domestic Cats, *Felis catus*) known to prey on *Anolis* lizards (García et al., 2001). As previously stated, this deme seems to be restricted to two small patches of vegetation along the shoreline, and without adequate protection and management its long-term survival is uncertain. (We caught and released, unharmed, several individuals from this locality, including males, females, and juveniles.)

The Cabo Rojo population of *A. cooki* was suggested to be the most unstable (Marcellini et al., 1985), because of the absence of limestone ridges for refugia and nearby populations as potential sources for recolonization. We have repeatedly visited this locality since 1998, and have not noticed any apparent decrease in the density of individuals in the area. *Anolis* lizards are highly visual animals (Fleishman, 1992), and *A. cooki* has become specialized to the light conditions characteristic of localities of sparse, xeric vegetation in which it is normally found (Leal and Fleishman, 2002). An important habitat requirement for this anole therefore seems to be the availability of exposed, less heavily vegetated areas, not necessarily of rocky surfaces to perch on.

The requirement of open, xeric areas makes *A. cooki* more susceptible to be negatively impacted by alteration of its habitat due to anthropogenic activities. Because human population centers in the tropics tend to be found in regions having a relatively dry climate, most of the dry forests have been heavily disturbed or totally eliminated (Murphy and Lugo, 1986; Janzen, 1988). This is true in Puerto Rico, where approximately 5000 ha of dry forestlands still remain intact, about 4% of the original dry forest that is believed to have been found on the island. The remainder of the forest has been modified or converted mainly because of agriculture, urbanization, and industrial development (Murphy et al., 1995). One of the changes associated with human activities in the coastal region of southern Puerto Rico is a change in vegetation profile. The native, sparse xeric vegetation is being replaced by introduced species that produce more shade (Molina Colón and Lugo, 2006), which results in a reduction in the suitable habitat for *A. cooki*, and a concomitant increase in the occurrence of *A. cristatellus*, a close relative of *A. cooki* that occupies partially open perches surrounded by vegetation (Leal and Fleishman, 2002).

The status of *A. cooki* on Caja de Muertos Island is uncertain. Our records indicate that the species was most recently collected on the island in 1989 (M. Leal, unpubl. data). Two of us (J.A.R.-R. and M.L.) visited Caja de Muertos on 28 September 2006 and on 11 March 2007. We did not find any *A. cooki* during ca. 24.5 and 6 person/hours of search time around the island during the day (1000–1630 h) and at

night (1900–2030 h), respectively. We noticed that nowadays the understory vegetation of Caja de Muertos is denser and taller, rats (*Rattus* sp.) are more abundant (surprisingly, some of these rodents were active during the day), and the density of the other two anoles native to the island, *A. cristatellus* and *A. pulchellus* (Grant and Roosevelt, 1932; Schwartz and Henderson, 1991), seems to be lower. Irrespective of what effects, if any, these habitat changes may have on the population density of *A. cooki*, a thorough survey of Caja de Muertos is needed to determine the present status of the species on the island.

Relocation of animals is a popular management tool for the conservation of threatened species. Reintroductions involve the release of individuals into previously occupied areas from which they have become extinct (Fischer and Lindenmayer, 2000; Seddon et al., 2007). In supplementation actions, animals are added to an existing population of conspecifics that inhabits an area where habitat deterioration (and/or hunting) has caused a decreased in population size (Storfer, 1999; Ficetola and De Bernardi, 2005). Supplementation could reduce vulnerability of recipient populations to environmental, demographic, and genetic stochasticity, and allow faster population growth (Lubow, 1996), but for translocation efforts to be successful the causes responsible for the population decline need to be identified and mitigated. Furthermore, the source of individuals in supplementation projects should be populations genetically similar to the beneficiary population (to minimize the risks of outbreeding depression; Frankham et al., 2002). Our findings indicate that each sampled population of *A. cooki* is an independent demographic unit, which makes conservation decisions taken to rescue declining populations difficult, for a translocation action would lead to a reduction in the genetic differences between populations.

In conclusion, our survey of *A. cooki* demonstrated a relatively high level of geographic structuring of the genetic variation among populations of this lizard, and provided information that conservation officials in Puerto Rico can use when planning their efforts to protect this threatened species. Phylogeographic studies of closely related, co-distributed taxa allow us to determine whether physiographic events have affected the biota of a given area in similar ways, or whether each species shows an idiosyncratic pattern (e.g., Avise, 2000; Patton et al., 2000; Stuart-Fox et al., 2001; Barber et al., 2006; Soltis et al., 2006; Richards et al., 2007). *A. poncensis* (“Lagartijo Jardinero del Sur,” Dryland Grass Anole) is also endemic to the southwestern coast of Puerto Rico, and has a similar distribution to that of *A. cooki*. It will be interesting to conduct a comparative phylogeographic study of *A. poncensis* and *A. cooki* to determine whether there are similarities in the genetic architecture of the two species.

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