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AMPHIBIAN DNA SHOWS MARKED GENETIC STRUCTURE AND TRACKS PLEISTOCENE CLIMATE CHANGE IN NORTHEASTERN BRAZIL

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The glacial refugia paradigm has been broadly applied to patterns of species dynamics and population diversification. However, recent geological studies have demonstrated striking Pleistocene climate changes in currently semiarid northeastern Brazil at time intervals much more frequent than the climatic oscillations associated with glacial and interglacial periods. These geomorphic data documented recurrent pulses of wet regimes in the past 210,000 years that correlate with climate anomalies affecting multiple continents. While analyzing DNA sequences of two mitochondrial genes (cytochrome *b* and NADH-dehydrogenase subunit 2) and one nuclear marker (cellular-myelocytomatosis proto-oncogene) in the forest-associated frogs *Proceratophrys boiei* and *Ischnocnema* gr. *ramagii*, we found evidence of biological responses consistent with these pluvial maxima events. Sampled areas included old, naturally isolated forest enclaves within the semiarid Caatinga, as well as recent man-made fragments of humid coastal Atlantic forest. Results show that mtDNA lineages in enclave populations are monophyletic or nearly so, whereas nonenclave populations are polyphyletic and more diverse. The studied taxa show evidence of demographic expansions at times that match phases of pluvial maxima inferred from geological data. Divergence times between several populations fall within comparatively drier intervals suggested by geomorphology. Mitochondrial and nuclear data show local populations to be genetically structured, with some high levels of differentiation that suggest the need of further taxonomic work.

KEY WORDS: **Amphibia, Atlantic forest, *Ischnocnema*, phylogeography, Pleistocene, *Proceratophrys*.**

Pleistocene climate changes have strongly influenced the spatial distribution and the genetic makeup of natural populations in areas of high latitudes. Data from plants and animals in the northern hemisphere are consistent with the hypothesis that movement of ice sheets significantly altered the geographical ranges of taxa during the glacial cycles (e.g., Milot et al. 2000; Hickerson and Ross 2001; Alexandrino et al. 2002; Fedorov and Stenseth 2002; Griswold and Baker 2002; Hoffman and Blouin 2004; Kotlik et al. 2004). Although these demographic responses left genetic footprints of population bottleneck and expansion in numerous species currently distributed throughout North America and Europe, we know much less about the extent to which populations at lower latitudes were affected by climate change during the last 2.4 million years (Hugall et al. 2002; Lessa et al. 2003; Grazziotin et al. 2006; Weir 2006; Cabanne et al. 2007).

Pleistocene glacial periods in the neotropics were proposed to have corresponded to times of increased aridity, with evolutionary repercussions (Haffer 1969; Vanzolini 1981; Ab'Saber 1982; Wüster et al. 2005). Although fossil pollen data (Colinvaux et al. 2000; Rull 2005; Urrego et al. 2005; Bush and Oliveira 2006) and

molecular studies (Lessa et al. 2003) indicate that populations in equatorial forests were not fragmented in the late Pleistocene, phylogeographic analyses in the Atlantic rainforest suggest demographic responses to Pleistocene climate-induced change in forest cover in southeastern and southern Brazil (Grazziotin et al. 2006; Cabanne et al. 2007).

Geomorphic features in the presently semiarid Caatinga biome of northeastern Brazil indicate major regional palaeoenvironmental modifications during the Pleistocene, with recurrent pulses of moister climatic regimes happening in interior northeastern Brazil for more than the past two glacial cycles (Auler et al. 2004). Based on newly dated speleothem growth phases and travertine records, a consistent pattern of 12 pluvial phases of varying lengths has been inferred in the last 210 thousand years (kyrs). Geological dating places those events at about 14,670–16,100; 38,510–39,860; 47,700–48,660; 59,130–61,270; 63,320–66,250; 67,440–68,100; 71,910–74,600; 86,110–88,090; 109,100–110,300; 129,500–137,900; 175,900–183,800; 202,300–208,600 years before present (Auler et al. 2004; Wang et al. 2004). These variations in the amount of rainfall are thought to have been driven mainly by solar radiation, which is in turn influenced by the Earth's 23 kyr precessional cycle, a component of the Milankovitch cycles (Cruz et al. 2005). Biogeographic and fossil data support the view that these wet periods corresponded to times of forest expansion in the currently dry Caatinga (Auler et al. 2004; Wang et al. 2004).

Travertine data from interior northeastern Brazil indicate moist phases also around 400,000 and 900,000 years ago, whereas ocean cores off the northeastern coast indicate wet periods at about 13,700–18,500; 29,000; 39,000; and 47,000 years before present (Auler et al. 2004). The broad spatial distribution of sites with well-documented episodes of pluvial maxima suggests that Pleistocene climatic oscillations were common throughout this region.

Were there comparable biologic responses to these oscillations? A recurrent series of humid and comparatively drier phases in northeastern Brazil could lead to population isolation and divergence in humid forest-associated taxa during drier times, and either range expansion or recovery from bottlenecks during periods of pluvial maxima. We used population genetic data from multiple populations of forest-associated frogs in naturally isolated forests within the Caatinga and in man-made remnants of coastal Atlantic rainforest in northeastern Brazil to look for concordant signs of biological response to such geologically inferred environmental changes. We sequenced portions of the mitochondrial genes cytochrome *b* (cyt *b*) and NADH-dehydrogenase subunit 2 (ND2), as well as a nuclear marker, the cellular-myelocytomatosis proto-oncogene (*c*-myc, including the entire intron 2 and flanking exon regions), in the forest-dwelling frogs *Proceratophrys boiei* (six localities) and *Ischnocnema* gr. *ramagii* (10 localities). Given their complex life cycles, permeable skin, and exposed eggs, frogs are among the most sensitive vertebrates to climate change and constitute important and appropriate model organisms for studies of this nature (Pounds and Crump 1994; Pounds et al. 1999; Carnaval 2002).

In documenting patterns of genetic diversity in forests of northeastern Brazil, this study sheds light upon the history of a poorly known neotropical region. This is the first phylogeographic study conducted in forest enclaves within the Caatinga, and one of the few genetic studies of amphibian populations in the megadiverse and highly endangered Atlantic forest. Given northeastern Brazil's high levels of deforestation, it has been proposed that local forest remnants be connected via forest corridors to allow for the persistence of viable populations of seed dispersers such as small mammals and birds (Rodal et al. 1998; da Silva and Tabarelli 2000). Pilot corridor programs are already in place as a result of a consortium between Conservation International, local NGOs, and sugarcane plantations (Uchoa Neto and Tabarelli 2002). If such projects are to be implemented, we need to be mindful of the current levels of genetic structure and understand the history of isolation among populations in northeastern Brazil, so that informed decisions can be made with respect to future landscape changes. This study goes beyond a historical investigation to also discuss the conservation implications of current levels and patterns of genetic diversity in a tropical hotspot.

STUDY AREA

Nearly two decades ago, and a decade after publication of Haffer's (1969) hypothesis of vicariant Pleistocene refuges as promoters of species diversity in the tropics, Andrade-Lima (1982) and Vanzolini (1981) pointed out the existence of present-day montane forest refuges in northeastern Brazil—the so called brejos de altitude—as sites that could provide insight into diversification mechanisms and ecosystem response to isolation and fragmentation. These "brejos" that we will refer to as forest enclaves, correspond to exceptionally humid habitat islands located above 500 m of altitude on the slopes and tops of plateaus and highlands amidst Brazil's semiarid Caatinga. Localized orographic rains and fog condensation enable persistence of these humid enclaves, which show significantly milder temperatures and increased levels of rainfall compared to the surrounding Caatinga (Vanzolini 1981; Andrade-Lima 1982; Rodal et al. 1998; Braga et al. 2002).

From a floristic standpoint, the enclaves of northeastern Brazil are classified as a disjunction of the Atlantic rainforest (Câmara 1992; Rodal et al. 1998; Sales et al. 1998). Their origin has been attributed to forest expansions during humid periods of the Pleistocene and Holocene. Following the contraction of lowland forests in response to increased aridity, these montane forests survived in areas with favorable microclimates (Andrade-Lima 1982; Prado and Gibbs 1993). Evidence of

formerly forested areas that are presently occupied by the dry Caatinga is provided by pollen records, genetic, geological, and fossil data (Cartelle and Hartwig 1996; Hartwig and Cartelle 1996; de Vivo 1997; De Oliveira et al. 1999; Auler and Smart 2001; Carnaval 2002). In the present study, we sampled frogs from 10 sites: five forest enclaves within the Caatinga, two forest remnants located in the transition zone between the Caatinga and the Atlantic forest domain, as well as three human-made fragments in formally continuous Atlantic forest near the coast.

Methods

FIELD AND LABORATORY WORK

We sampled 104 individuals of *P. boiei* and 130 of *Ischnocnema* gr. *ramagii*, ranging from 7 to 22 specimens per species for each site (online Supplementary Table S1). Multiple field trips recorded no *P. boiei* in some of the areas. Sites corresponding to Atlantic forest enclaves within the Caatinga are Baturité, Brejo da Madre de Deus, Maranguape, Pau Ferro, and Ubajara. Água Azul and Brejo dos Cavalos are located in the transition zone between the Caatinga and the coastal Atlantic forest domain. The three recent, man-made Atlantic forest fragments near the coast are Frei Caneca, Guaribas, and Tapacurá (Fig. 1).

Tissue collection and DNA extraction followed Carnaval (2002). Voucher specimens were deposited in the herpetological collection Laboratório de Anfíbios e Répteis, Departamento de Zoologia, Universidade Federal do Rio de Janeiro, Brazil. For all specimens, we sequenced a portion of two mtDNA genes: cyt *b* (666 bp in *Proceratophrys*; 516 bp in *Ischnocnema*) and ND2 (708 bp in *Proceratophrys*; 689 bp in *Ischnocnema*). Forty-two *Proceratophrys* and 53 *Ischnocnema* were sequenced for the nuclear *c*-myc gene (874 bp in *Proceratophrys*; 1,192 bp in *Ischnocnema*).

DNA amplification via polymerase chain reaction (PCR) followed Carnaval (2002). For *Proceratophrys*, we used primers CB1–5′ and CB3–3′ of Palumbi (1996) to amplify a portion of the cyt *b* gene. For *Ischnocnema*, we used primers UBLONGF (5′-TCACAGGACTATTTCTAGCAATACACTACAC-3′) and UBLONGR (5-AGTTATCTGGGTCTCCCAAYATGTTWGG-3′); UBF1 (5′-TAGCAATACACTACACMGC-3′) and CB3–3′ (Palumbi 1996); or UBF1 and UBR1 (5′-TGAAGTTATCY GGGTCTCC-3′), depending on the population.

For the ND2 fragment in *Proceratophrys*, we used primer L4437 (Macey et al. 1997) and HND2PB (5′-GGAT TTAGTTCATGAGGTTG-3′). In *Ischnocnema*, we used either ND2ELEUF1 (5′-CACCCACGMGCCATTGAAGC-3′) and ND2ALAR (5′-TAAAGTGTCTGAGTTGCATTCA-3′) or ND2F1 (5′-CCTATCAATTTTCCTATCAAGTCTAGC-3′) and ND2TRPR (5′-GCTTARGGCTTTGAAGGC-3′).

To amplify a portion of *c*-myc in *Proceratophrys*, we used forward primers cmyc1U (Crawford 2003), cmyc4U (Crawford and Smith 2005), or LCMYCPB (5′-CAGGACTGTATGTGGA-3′), in conjunction with the reverse primer HCMYCPB (5′-GTTGCTGATCTGTTTGA-3′). In *Ischnocnema*, we used forward primers cmyc1U, cmyc4U, or cmyc3Up (Crawford and Smith 2005), in conjunction with primers HCMYCPB or cmyc3L (Crawford 2003).

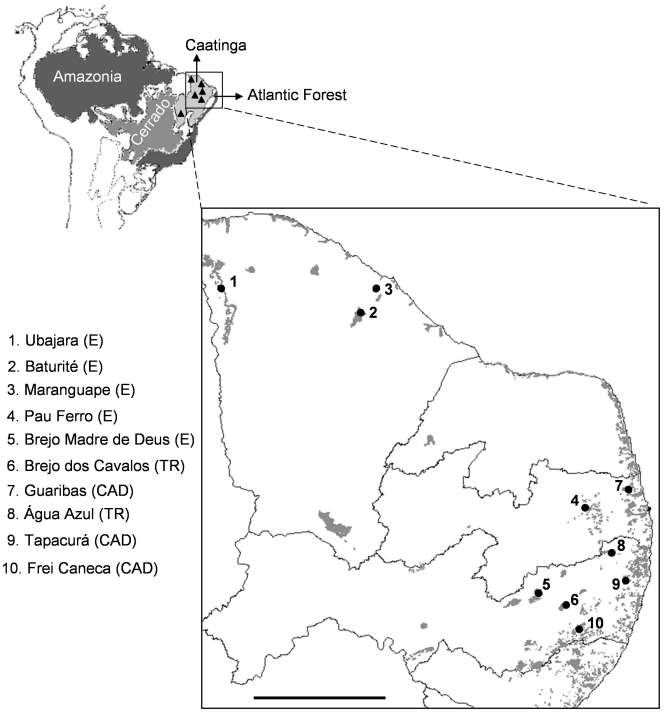

Figure 1. Localities sampled. Above: Brazil's morphoclimatic domains. Triangles represent forest enclaves ("brejos") within the arid Caatinga. Below: Detailed map of sampling localities. In locality key; E, enclave within the Caatinga; TR, fragment within transition zone; CAD, fragment within the coastal Atlantic domain. Forest remnants are pictured in gray. Scale = 200 km.

Following amplification, we purified all PCR products with GELase (Epicentre Technologies) and proceeded with cycle sequencing reactions as described by Carnaval (2002).We precipitated all final products in ethanol and resuspended in 10μl of Hi-Di Formamide (Applied Biosystems, Foster City, CA) as per manufacturer's protocols. Sequencing reactions were run on an ABI PRISM 3100 Genetic Analyzer. Both strands were sequenced.

We aligned and edited all sequences in *Sequencher* version 4.1.2 (Gene Codes Corporation). Cytochrome *b* and ND2 sequences were aligned to the complete mtDNA genome of *Xenopus laevis* available in GenBank (http://www.ncbi.nih.gov/Genbank, accession number NC 001573) and translated to amino acids to confirm the reading frame. No stop codons were encountered. In *P. boiei*, the first nucleotide position of the cyt *b* fragment corresponded to position 16,348 of the complete mitochondrial DNA of *X. laevis*. In *Ischnocnema*, it matched position 16,444. For ND2, the first nucleotide corresponded to positions 5,958

(*Proceratophrys*) and 6,279 (*Ischnocnema*) of *X. laevis* mtDNA sequence.

Because *c*-myc sequencing revealed several indel-heterozygous specimens, we used the TOPO TA Cloning Kit protocol (Invitrogen Corporation, Carlsbad, CA) to clone PCR fragments and clarify allelic sequences, following the manufacturer's protocol. Bacterial colonies were amplified using universal M13 primers.

The total alignment of nuclear sequences in *Proceratophrys* included a 303 bp portion of exon 2, 354 bp of intron 2, and 217 bp of exon 3 of the *c*-myc gene. In *Ischnocnema*, aligned nuclear sequences comprised a 1,192 bp piece corresponding to a 487 bp portion of exon 2, 443 bp of intron 2, and 262 bp of exon 3 of the *c*-myc gene. The first nucleotide position of the fragment obtained for *Proceratophrys* corresponded to position 389 of exon 2 in the complete *c*-myc sequence of *X. laevis* available in GenBank (accession number X53717). The first nucleotide position in *Ischnocnema* corresponded to position 208. All exon sequences coded for amino acids, with no stop codons. Sequences generated for this project were deposited in GenBank, under accession numbers EU017577-EU018044 and EU025551-EU025679.

PHASING OF NUCLEAR HAPLOTYPES

We used the program *PHASE* version 2.0.2 (Stephens et al. 2001; Stephens and Donnelly 2003, available at www.stat.washington.edu/stephens/software.html) to infer (phase) the haplotypes of individuals that were polymorphic for more than one segregating site. To run *PHASE*, we removed all 25 indels observed in intron 2 of *Proceratophrys*. These indels were also excluded from all subsequent sequence analyses. Moreover, two amino acids not shared by all individuals (nucleotide positions 694–696 and 703–705) were removed from exon 3. In *Ischnocnema*, the removal of indels resulted in a dataset of 1,108 bp sequences.

We ran *PHASE* under default conditions, including 200 iterations (of which 100 were used as burn-in), and a thinning interval of 1. To improve reliability, we ran the algorithm multiple times and with different seeds for the random number generator, obtaining slightly different haplotype reconstructions. We then checked all results for consistent goodness-of-fit measurements of the estimated haplotypes to an approximate coalescent model with recombination. Because we had previously cloned PCR products in a few heterozygote individuals—hence uncovering the true sequences of both haplotypes for those specimens—we were able to identify which *PHASE* run provided the most accurate haplotype reconstruction. The results of that run were selected as most reliable and used in subsequent analyses.

To investigate the possible role of recombination in shaping genetic patterns in the nuclear marker, we used the phased haplotypes in the program *DnaSP* version 4.00 (Rozas et al. 2003) to estimate the recombination parameter C as per Hudson (1987). We also estimated the minimum number of recombination events RM through the use of the four-gamete test (Hudson and Kaplan 1985).

ANALYSES OF GENETIC STRUCTURE

We used mitochondrial and nuclear sequences to generate networks and portray relationships among haplotypes with *TCS* version 1.13 (Clement et al. 2000). Population substructure was quantified in *Arlequin* version 2.001 (Schneider et al. 2000) with the fixation index F_{ST} computed from sequence data for all pairs of sampled localities. Statistical significance of F_{ST} values was tested using 10,000 permutations.

We also used *Arlequin* to estimate pairwise genetic distances, including Nei's (1987) average number of nucleotide differences (D) and corrected average number of nucleotide differences (D_a). *Modeltest* (Posada and Crandall 1998) showed that the Tamura–Nei molecular evolution model (Tamura and Nei 1993) best fit the mtDNA data, so we used Tamura–Nei distances as estimators of the number of differences for mtDNA sequences in *Arlequin*. *Modeltest* was not applied to the nuclear data because patterns of polymorphism in *c*-myc were suggestive of recombination events. For this marker, we used uncorrected pairwise differences as a distance method in *Arlequin*.

Evidence of historical changes in population size was explored with Ramos-Onsins and Rozas's (2002) R_2 test in *DnaSP* (Rozas et al. 2003). Because coalescent simulations using different recombination parameters have shown this method to be less sensitive to intragenic recombination compared to other tests, the R_2 statistic was applied both to the mitochondrial and nuclear datasets. After calculating R_2 for each population, we generated an empirical distribution of the test statistic under the assumption of no population growth through 10,000 simulations based on the coalescent process for a neutral infinite-sites model and assuming a large constant population size (Hudson 1990). The empirical distribution of R_2 was used to define a 95% confidence interval to which the observed R_2 value was compared. We report the probability P that the expected value under no population growth is smaller than the observed value. Small values of P ($\leq$ 0.05) indicate significant departures from a hypothesis of constant population size over time.

We used sequences from populations with significant departures from the null hypothesis of constant size in mismatch analyses to estimate the following parameters: θ_0 (theta value, or mutation parameter, prior to population growth or decline, where $\theta = 2N_e u$ for a haploid marker such as mtDNA, N_e = effective population size, u = mutation rate per sequence per generation); θ_i (theta value after population growth or decline); and τ (the date of growth or decline; $\tau = 2ut$, where t = time in generations). We obtained estimates of θ_0, θ_i, and τ through Schneider and Excoffier's (1999) nonlinear least-squares approach implemented

in *Arlequin*, under the assumption of no positive selection or genetic hitchhiking in the markers used (Maynard Smith and Haigh 1974). Given that recombination will rearrange nucleotide variation among haplotypes and lead to biases in parameter estimation (Schneider et al. 2000), and because the nuclear data gathered from both taxa showed patterns consistent with recombination, we decided to date population expansion events based on the mitochondrial data alone. Schneider and Excoffier's (1999) method enabled the generation of approximate confidence intervals for each parameter through a bootstrapping procedure. By using a modified version of Hudson's (1990) coalescent algorithm, we generated 10,000 random samples according to the estimated demography and reestimated θ_0, θ_i, and τ for each simulated dataset. For each parameter, the approximate lower and upper limits of a 95% confidence interval were estimated by the 2.5% and 97.5% percentile values of those 10,000 reestimated values.

We used a one-tailed exact binomial test to determine whether the number of populations with estimated expansion years falling within or close to a pluvial phase was significantly higher than the null expectation of a random distribution based on the proportion of years of pluvial maxima. Because the geomorphological data indicate that the distribution of pluvial periods was not uniform over the 210,000 years of the Pleistocene contemplated by the studies of Auler et al. (2004) and Wang et al. (2004)—but rather much more closely distributed during the first half of this period—we conducted an exact binomial test for each of two partially overlapping time periods: one for 10–160 kyrs before present, and another for 60–210 kyrs before present.

We used both moment-based and likelihood methods to obtain estimates of divergence times between population pairs based on mtDNA data. We started by using Nei's (1987) corrected average number of nucleotide differences (D_a) as a moment-based estimator. When ancestral and daughter populations are of the same size, the expected value of D_a equals $2ut$, where t represents population divergence time and u is the mutation rate at the sampled locus (Arbogast et al. 2002). Based on the D_a values, we selected the group of population pairs whose divergence times approximated that of the target period of our study (the most recent 210 kyrs of the Pleistocene). A refined divergence time for those selected pairs, including credible intervals, was then obtained with likelihood estimators based on Nielsen and Wakeley's (2001) MCMC implementation available in the program *IM* (http://lifesci.rutgers.edu/~heylab/HeylabSoftware.htm). Because *IM* assumes no within-locus recombination, we restricted the use of the software to mtDNA datasets alone.

We used *IM* to estimate divergence times while allowing for migration between population pairs as well as for population size changes through time. The program enabled us to generate integrated likelihood surfaces for population parameters, which were subsequently used to estimate time-since-population-splitting (the value of the bin with the higher residence time, after smoothing), as well as its 95% credible interval. Runs were performed under the Hasegawa–Kishino–Yano finite-sites model of evolution (Hasegawa et al. 1985). We used five Markov Chains with 2,000,000 cycles of length, discarded the first 100,000 cycles as burn-in, and recorded every 10th step of the run. A linear heating mode was applied to the chains, and the heating parameter was set to 0.05. Five chain swaps were attempted per step. Output files were checked for autocorrelation among parameters and for the shape of the likelihood distribution. In the few instances when the autocorrelation among parameter values was high (>0.03), we discarded the results and reran the analysis using twice the number of cycles and twice the thinning interval.

MUTATION RATE AND GENERATION TIME ESTIMATES

Knowledge of the mutation rate u is required to estimate effective population sizes from the mutation parameter θ, and times of divergence and expansion from D_a and τ. No independent fossil or geological records are available to calibrate a local molecular clock for populations of *Proceratophrys* and *Ischnocnema* in northeastern Brazil, so outside calibrations were used. Estimates of amphibian protein-coding mtDNA mutation rates have been shown to vary slightly depending on markers and group of study (Tan and Wake 1995; Macey et al. 1998; Crawford 2003). For anurans, Crawford (2003) estimated ND2 to evolve at a rate of 0.957% per lineage per million years based on a recalibration of Macey et al.'s (1998) Eurasian *Bufo* dataset. In the present study, we used this divergence rate as it is in agreement with estimates for several other vertebrate species (Moritz et al. 1987). An implicit assumption that our study species have comparable DNA divergence rates to those estimated by Crawford (2003) for the *Bufo* data is therefore made. Generation time was assumed as one year, based on studies of other Neotropical frogs (Donnelly 1999).

Results

HAPLOTYPE PATTERNS IN *Proceratophrys*

The *Proceratophrys* mtDNA dataset contained 43 unique haplotypes. The lack of shared sequences between localities and the clustering of sympatric haplotypes within a statistical parsimony network provided evidence of strong geographic structure (Fig. 2). Templeton et al.'s (1992) network algorithm was unable to connect all sequences into a single haplotype cluster under the assumption of no multiple hits at any given segregating site. Instead, haplotypes formed four major geographic groups: a central group of sequences from Água Azul; a northwestern group of haplotypes from Maranguape; and two southern groups—one with a subset of haplotypes from Frei Caneca, and another with remaining haplotypes from Frei Caneca, Brejo da Madre de Deus, Brejo dos Cavalos, and Tapacurá.

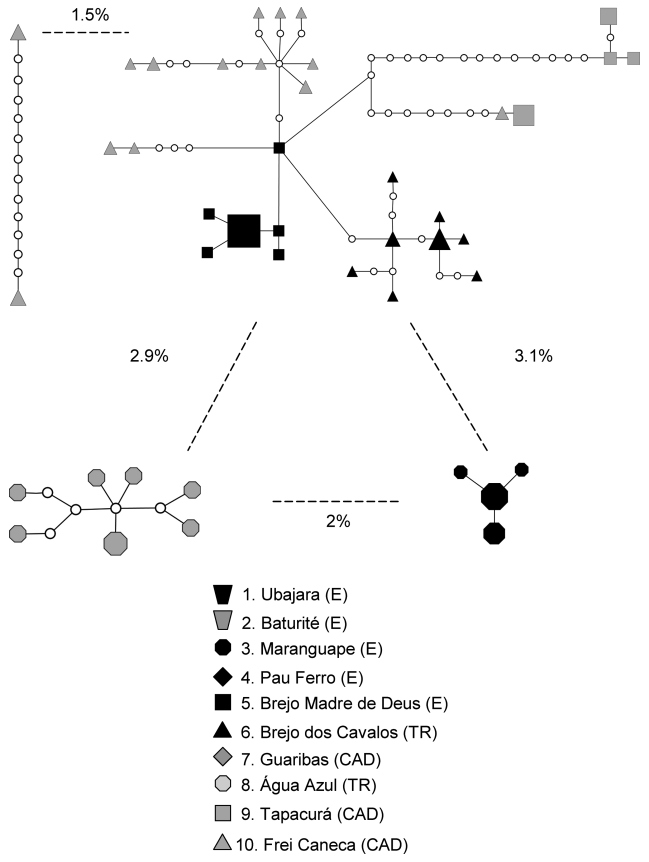

Figure 2. ***Proceratophrys boiei*** **mtDNA haplotype network. Symbols denote haplotypes; hatch marks depict single mutations; empty circles represent unsampled haplotypes. High levels of divergence among haplotype groups precluded connection into a single network under the assumption of no recurrent mutations required by statistical parsimony (Clement et al. 2000). Percent values represent average nucleotide divergence among groups. In locality key: E, enclave within the Caatinga; TR, fragment within transition zone; CAD, fragment within the coastal Atlantic domain.**

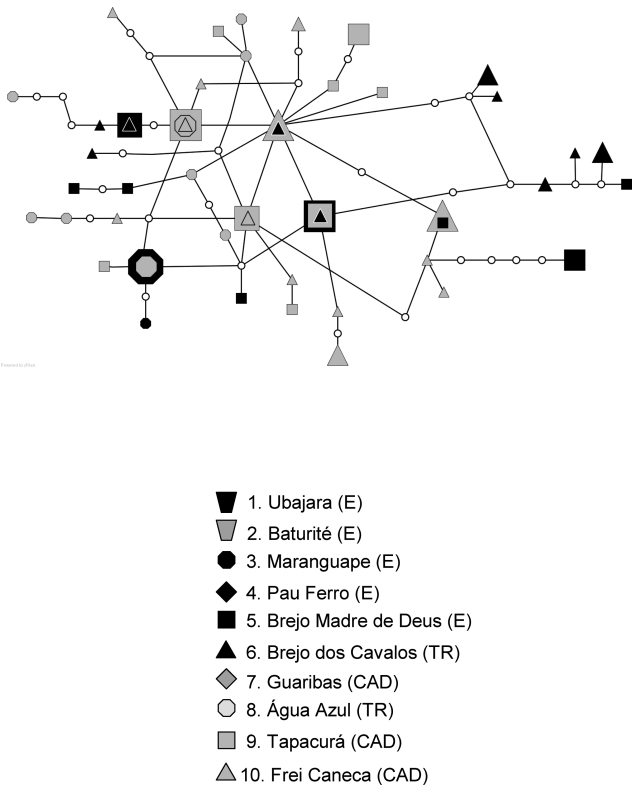

Figure 3. ***Proceratophrys boiei*** **nuclear haplotype network. Symbols denote haplotypes; hatch marks depict single mutations; empty circles represent unsampled haplotypes. In locality key: E, enclave within the Caatinga; TR, fragment within transition zone; CAD, fragment within the coastal Atlantic domain.**

The *Proceratophrys* nuclear dataset contained 42 unique haplotypes, seven of which were present in two or more populations (Fig. 3). Haplotypes were shared between central and northwestern localities (Água Azul and Maranguape), and among southernmost sites (Brejo da Madre de Deus, Brejo dos Cavalos, Frei Caneca, and Tapacurá). One haplotype was shared among Água Azul, Frei Caneca, and Tapacurá populations. Private alleles were detected at all localities. Sympatric haplotypes failed to cluster together in many instances. All haplotypes were connected into a single network with eight loops. At least eight recombination events could be detected in the nuclear dataset; and the recombination parameter C per gene was estimated as 41.2 ($S^2{}_k$: 6.464).

In *P. boiei*, every locality proved to be genetically unique. Mitochondrial F_{ST} estimates for all pairs of populations were significantly large at the $P = 0.05$ level, ranging from 0.30 to 0.98 (online Supplementary Table S2). Nuclear F_{ST} estimates among population pairs were smaller than those based on mtDNA but still significantly large at the 0.05 P-level, ranging from 0.05 to 0.52 (online Supplementary Table S2).

Estimates of mtDNA pairwise population differences corrected for intrapopulation diversity (D_a) revealed populations Frei Caneca, Tapacurá, Brejo dos Cavalos, and Brejo da Madre de Deus to be reasonably similar, with mtDNA distances from 3.02 to 10.18 (online Supplementary Table S2). Corrected mtDNA distances between Água Azul and the remaining populations were larger, ranging from 26.18 to 38.94. Between Maranguape and other localities, these values ranged between 26.18 and 42.85. Nuclear estimates of pairwise population differences corrected for intrapopulation diversity (D_a) were smaller than the mtDNA values (online Supplementary Table S2): population pairs with fewer differences were Brejo dos Cavalos–Maranguape (0.21) and Água Azul–Maranguape (0.34). The largest distances were detected between Brejo da Madre de Deus and Frei Caneca (2.33) and Brejo da Madre de Deus and Tapacurá (2.10).

HAPLOTYPE PATTERNS IN *Ischnocnema*

We found 70 mtDNA haplotypes in 130 samples of *Ischnocnema* from 10 sites. As in *Proceratophrys*, the lack of shared sequences between sites and the clustering of sympatric haplotypes within a statistical parsimony network indicated strong geographic structure (Fig. 4). Likewise, Templeton et al.'s (1992) algorithm was unable to connect all sequences into a single network. Haplotypes formed five largely geographic groups: a central group, including sequences from Água Azul and Tapacurá united through several mutational steps to haplotypes from southern sites such as Brejo da Madre de Deus, Brejo dos Cavalos, and Frei Caneca; two northeastern groups—one encompassing haplotypes from Guaribas and Pau Ferro, and another with haplotypes from Guaribas only—and two northwestern groups—one composed of haplotypes from Ubajara, and another from Baturité and Maranguape. Although the general pattern was similar to that observed in *P. boiei*, the levels of divergence among haplotype groups in *Ischnocnema* were considerably higher. Representatives of the northwestern localities (Baturité, Ubajara and Maranguape) differed from those in the southern and central areas by more than 26% uncorrected sequence divergence, indicating that multiple species may be involved. AC (pers. obs.) noted differences in call structure and microhabitat use between the two distinct haplotype groups that occur sympatrically at Guaribas. Because of these concordant dissimilarities across datasets, and given that haplotypes in this area differed up to more than 16%, we refer to these groups as Guaribas lineage A and Guaribas lineage B throughout the text.

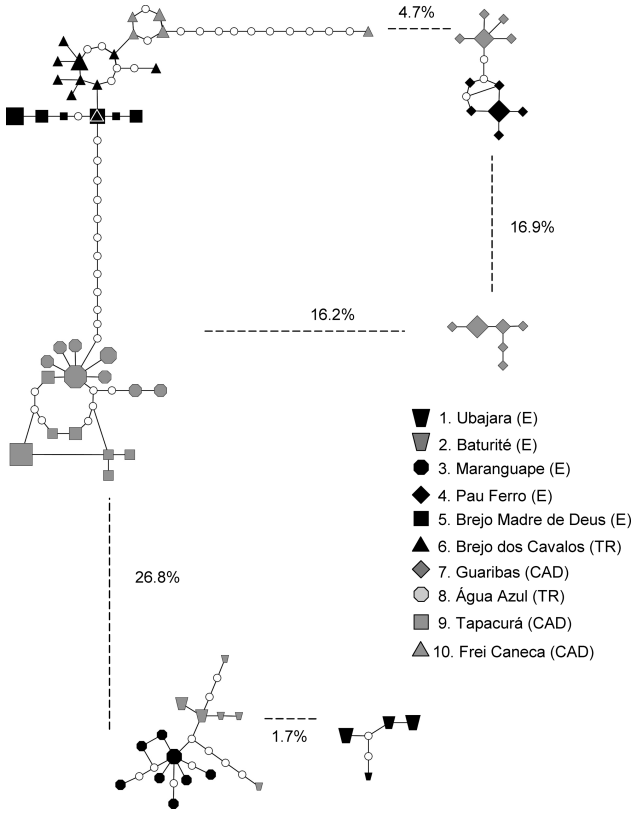

Figure 4. ***Ischnocnema* gr. *ramagii* mtDNA haplotype network. Symbols denote haplotypes; hatch marks depict single mutations; empty circles represent unsampled haplotypes. High levels of divergence among haplotype groups precluded connection into a single network under the assumption of no recurrent mutations required by statistical parsimony (Clement et al. 2000). Percent values represent average nucleotide divergence among groups. In locality key: E, enclave within the Caatinga; TR, fragment within transition zone; CAD, fragment within the coastal Atlantic domain.**

The *Ischnocnema* nuclear dataset totaled 70 haplotypes, five of which were present at two or more localities (Fig. 5). Haplotypes were shared between two northwestern sites (Baturité and Maranguape), between the northeastern locality of Água Azul and Pau Ferro, and between the southernmost sites Brejo da Madre de Deus, Brejo dos Cavalos, Água Azul, and Tapacurá. Private alleles were detected at all localities.

Nuclear haplotypes were connected into three networks, again consistent with the patterns and levels of differentiation detected in the mtDNA dataset, and suggesting the existence of at least three distinct evolutionary lineages. One included samples from the central and southernmost localities (Brejo dos Cavalos, Brejo da Madre de Deus, Água Azul, Tapacurá), Pau Ferro, as well as a subset of the haplotypes from Guaribas (individuals of Guaribas lineage A based on mtDNA). Guaribas lineage B was recovered as a separate network. The third network connected haplotypes from the northwestern localities of Maranguape, Baturité and Ubajara. Loops were recovered in all networks. We were able to detect at least seven recombination events in the first network (central + southern + northeastern sites), and the recombination parameter C per gene was estimated as 87.3 (S_k^2: 5.938). In the northwestern group (Maranguape + Baturité + Ubajara), at least nine recombination events could be detected; C per gene was 28, and S_k^2: 13.266.

As in *P. boiei,* mitochondrial F_{ST} values in *Ischnocnema* were significantly large at the $P = 0.05$ level, ranging from 0.40 to 1.00 (online Supplementary Table S3). Estimates of pairwise population differences corrected for intrapopulation diversity (D_a) ranged from 1.76 (between Água Azul and Tapacurá) up to 609.45 (Ubajara vs. Pau Ferro; online Supplementary Table S3). Nuclear F_{ST} values were significantly large for the majority of population pairs (all but Tapacurá–Água Azul, Tapacurá–Brejo dos Cavalos, Brejo dos Cavalos–Brejo da Madre de Deus, Brejo dos Cavalos–Frei Caneca, and Baturité–Maranguape), and ranged from zero to 0.99 (online Supplementary Table S4). D_a values spanned from 0.05 (Água Azul–Tapacurá) to 79.00 (Ubajara–Frei Caneca; online Supplementary Table S4).

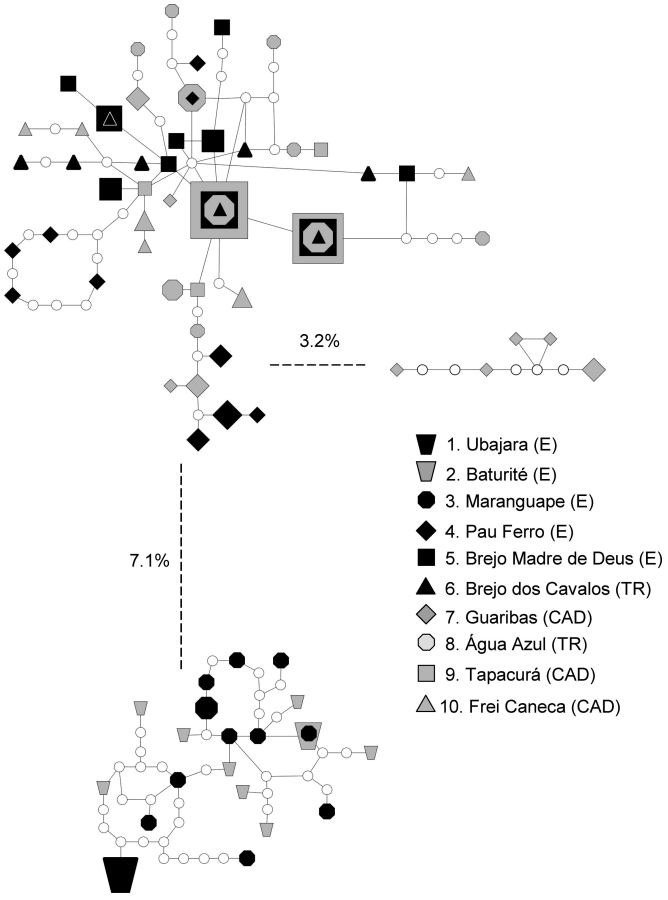

Figure 5. ***Ischnocnema*** **gr.** ***ramagii*** **nuclear haplotype network. Symbols denote haplotypes; hatch marks depict single mutations; empty circles represent unsampled haplotypes. High levels of divergence among haplotype groups precluded connection into a single network under the assumption of no recurrent mutations required by statistical parsimony (Clement et al. 2000). Percent values represent average nucleotide divergence among groups. In locality key: E, enclave within the Caatinga; TR, fragment within transition zone; CAD, fragment within the coastal Atlantic domain.**

SIGNATURES OF POPULATION EXPANSION ACROSS TAXA

When applied to the *P. boiei* mtDNA data, Ramos-Onsins and Rozas' (2002) R_2 test detected traces of historical changes in population size in three localities: Água Azul, Brejo dos Cavalos, and Brejo da Madre de Deus (Table 1). For these areas, methods of Schneider and Excoffier (1999) yielded estimated mean dates of population expansion in generation time (τ) that were slightly larger in Brejo dos Cavalos (4.266, 95% CI: 1.046–8.604) than in Água Azul (3.632, 95% CI: 0.991–5.419), followed by Brejo da Madre de Deus (1.233, 95% CI: 0.000–4.037; Table 2). These point estimates correspond to expansion events dating back to approximately 162, 138, and 47 kyrs before present, respectively. Confidence intervals for expansion times in both Água Azul and Brejo da Madre de Deus fall entirely within the last 210,000 years, whereas expansion at Brejo dos Cavalos extends back to 327 kyrs ago.

In *Ischnocnema*, the mtDNA data showed signatures of historical population expansions in Água Azul, Brejo dos Cavalos, Guaribas, Maranguape, and Pau Ferro (Table 1). Point estimates of the scaled expansion time (τ) ranged from 0.807 to 2.576, corresponding to estimated population expansion events dating to 77 (Água Azul), 101 (Brejo dos Cavalos), 59 (Guaribas, lineage A), 65 (Guaribas, lineage B), 112 (Maranguape), and 35 kyrs ago (Pau Ferro; Table 2), approximately. Confidence intervals for all populations exhibiting demographic changes fall within the last 210,000 years.

With respect to the nuclear data, bell-shaped mismatch distributions were observed in all *Proceratophrys* populations except Maranguape (not shown). However, Ramos-Onsins and Rozas' (2002) R_2 test was unable to detect signatures of demographic expansions in any of the populations. A significant result was only obtained after lumping nuclear haplotypes from all sites into a combined nuclear dataset (Table 1). When applied to the *Ischnocnema* nuclear data, Ramos-Onsins and Rozas' (2002) R_2 test did not detect traces of demographic changes in any of the individual populations or after pooling the entire dataset. However, as in *Proceratophrys*, a significant result was obtained for the eastern group of populations, including Água Azul, Brejo dos Cavalos, Brejo da Madre de Deus, Frei Caneca, Guaribas lineage A, Tapacurá, and Pau Ferro.

After pooling the data from all sites and species, the point estimates of population expansion times based on mtDNA data corresponded well with the periods of Pleistocene pluvial phases inferred from the speleothem and travertine data (Auler et al. 2004; Wang et al. 2004). Particularly close matches were observed for populations in Água Azul, Guaribas, and in forest enclaves located within the dry Caatinga (Brejo da Madre de Deus and Maranguape; Fig. 6). Out of nine localities, only two showed estimates of population expansion times that differed from a geomorphology-based pluvial period by approximately 10 kyrs—and these corresponded to estimates of *P. boiei* and *Ischnocnema* collected at Brejo dos Cavalos. All other populations had an inferred expansion time differing at most by 3,520 years from the upper or lower bound of the time interval of a pluvial phase. An exact binomial test conducted for the last 150 kyrs of the Pleistocene (160 kyrs to 10 kyrs before present) revealed that the number of populations whose estimated expansion years fell within 3,520 years or less of a pluvial phase was significantly higher than the null expectation of a random distribution based on the proportion of years of pluvial maxima ($P = 0.05$). When applied to the period between 60 kyrs and 210 kyrs ago, the test result was not significant ($P = 0.37$).

Table 1. Observed R_2 statistics per taxon and locality (or group of localities) based on mtDNA and nuclear data. Asterisks denote significant departures from a null hypothesis of constant population size through time (*P-value $<$ or $=$ 0.05). In *Ischnocnema*, "Eastern group" comprises the following localities: Água Azul, Brejo dos Cavalos, Brejo da Madre de Deus, Frei Caneca, Guaribas lineage A, Tapacurá, and Pau Ferro. "Western group" includes Baturité, Maranguape, and Ubajara.

Locality	*Proceratophrys boiei*		*Ischnocnema* gr. *ramagii*	
	mtDNA R_2	ncDNA R_2	mtDNA R_2	ncDNA R_2
Água Azul	0.0887*	0.1532	0.0979*	0.1154
Baturité	NA	NA	0.1650	0.1742
Brejo dos Cavalos	0.0829*	0.1283	0.0985*	0.1613
Brejo da Madre de Deus	0.0805*	0.1743	0.2317	0.1475
Frei Caneca	0.1373	0.1343	0.2803	0.1600
Guaribas (Lineage A)	NA	NA	0.1237*	0.2046
Guaribas (Lineage B)	NA	NA	0.1247*	0.1857
Maranguape	0.1382	0.2575	0.0957*	0.1384
Pau Ferro	NA	NA	0.0909*	0.1579
Tapacurá	0.2506	0.1277	0.1542	0.1544
Ubajara	NA	NA	0.1965	no polymorphism
All (pooled)	0.0989	0.0542*	0.1647	0.1253
Eastern group	NA	NA	0.1403	0.0584*
Western group	NA	NA	0.1279	0.1284

DIVERGENCE TIME ESTIMATES

Assuming 0.957% lineage divergence per million years, the following population pairs of *P. boiei* showed D_a values consistent with population divergence within the last 210 kyrs (i.e., $D_a <$ or $= 2 \times (0.00957 \times 1{,}374) \times 0.21 = 5.2$): Frei Caneca–Brejo dos Cavalos; Frei Caneca–Madre de Deus; Brejo dos Cavalos–Madre de Deus (online Supplementary Table S2). Data from all other population pairs indicated divergences dating back to more than 210 kyrs ago (i.e., outside the bounds of the geological data). Although MCMC estimates provided by *IM* are valid only between populations that are each other's closest relatives, we proceeded to obtain parameters of splitting times for these three population pairs given that an a priori inspection of the haplotype network did not allow us to unambiguously determine the closest population pair in the

Table 2. Estimated values of θ_0, θ_i, scaled time of expansion τ, and estimated time of expansion per taxon and locality based on mtDNA data. Values in parentheses represent 95% confidence intervals.

Species	Locality	θ_0	θ_i	τ	Time of expansion
Proceratophrys boiei	Água Azul	0.000 (0.000–2.902)	4,682.500 (26.719–10,267.5)	3.632 (0.991–5.419)	138,108 (37,683–206,058)
Proceratophrys boiei	Brejo dos Cavalos	0.005 (0.000–1.468)	3.599 (0.727–2,800.474)	4.266 (1.046–8.604)	162,215 (39,774–327,169)
Proceratophrys boiei	Brejo da Madre de Deus	0.003 (0.000–0.998)	1.167 (0.000–4,121.167)	1.233 (0.000–4.037)	46,885 (0–153,508)
Ischnocnema gr. *ramagii*	Água Azul	0.218 (0.000–1.611)	3,740.037 (3.147–7,198.438)	1.784 (0.302–3.024)	77,351 (13,094–131,115)
Ischnocnema gr. *ramagii*	Brejo dos Cavalos	0.348 (0.647–3.787)	5,184.071 (23.010–8,565.000)	2.33 (0.647–3.787)	101,025 (28,053–164,197)
Ischnocnema gr. *ramagii*	Guaribas (Lineage A)	0.268 (0.000–2.427)	4,197.162 (8.716–8,595.938)	1.362 (0.000–2.734)	59,054 (0–118,541)
Ischnocnema gr. *ramagii*	Guaribas (Lineage B)	0.293 (0.000–2.981)	4,551.741 (23.979–6731.25)	1.498 (0.000–2.981)	64,950 (0–129,251)
Ischnocnema gr. *ramagii*	Pau Ferro	0.807 (0.000–2.007)	1,800.950 (0.522–5,481.719)	0.807 (0.000–2.007)	34,990 (0–87,020)
Ischnocnema gr. *ramagii*	Maranguape	0.360 (0.000–2.492)	6,543.187 (29.258–8,370.000)	2.576 (0.443–4.505)	111,691 (19,208–195,329)

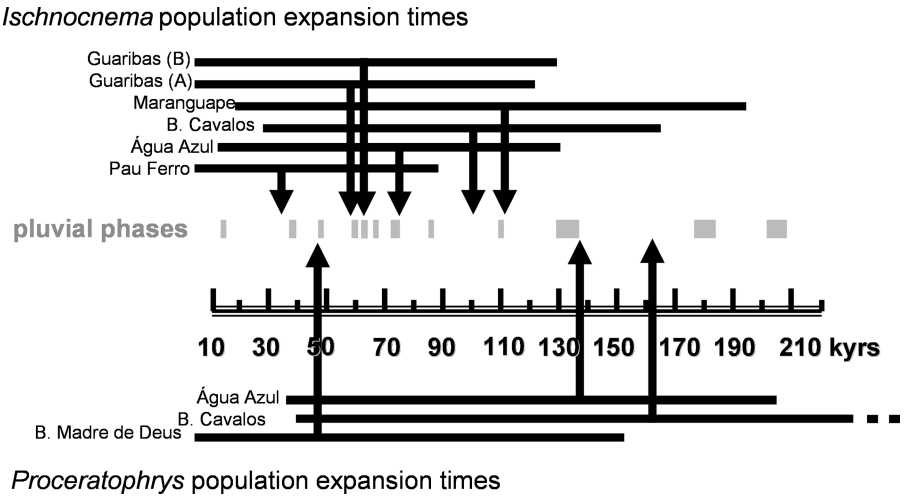

Figure 6. Comparison of Pleistocene pulses of pluvial maxima (Wang et al. 2004), provided as gray boxes over a timeline in kyrs, with point estimates of population expansion times from mtDNA data for *Ischnocnema* gr. *ramagii* (above) and *Proceratophrys boiei* (below), indicated by black arrows. Horizontal bars depict 95% confidence intervals.

group. Minimum dates of the divergence between Brejo dos Cavalos and Brejo da Madre de Deus and that between Frei Caneca and the other two were estimated to be 70,537 (95% CI: 19,203–351, 923), 132,898 (65,593–299,448), and 88,789 (45,820–363,711), respectively (Table 3). These results suggest that the population pair with the most recent divergence time is Brejo dos Cavalos–Brejo da Madre de Deus.

In *Ischnocnema*, levels of genetic divergence between most population pairs were so high that they cannot be considered to date to the Pleistocene (Fig. 3, online Supplementary Table S3). Assuming 0.957% lineage divergence per million years, the following population pairs showed D_a values consistent with population divergence within the last 210 kyrs (i.e., $D_a <$ or $=$ to $2 \times (0.00957 \times 1{,}205) \times 0.21 = 4.8$): Frei Caneca–Brejo dos Cavalos, Frei Caneca–Madre de Deus, Brejo dos Cavalos–Madre de Deus, Água Azul—Tapacurá, Baturité–Maranguape; Pau Ferro–Guaribas lineage A.

As in *P. boiei*, populations of *Ischnocnema* in the enclaves of Brejo da Madre de Deus and Brejo dos Cavalos and in Frei Caneca showed estimated point divergence times within the Pleistocene (Table 3). Despite the relative small D_a value, the divergence time between Brejo da Madre de Deus and Frei Caneca was not estimated given that the haplotype network and D_a values indicated that these two populations were not each other closest relatives, violating the MCMC model assumption. The time of divergence between Brejo dos Cavalos and Brejo da Madre de Deus was estimated in 52,680 years ago (95% CI: 36,204–417,322), and that between Brejo dos Cavalos and Frei Caneca was 77,394 years before present (95% CI: 24,497–412,987). As in *P. boiei*, the *Ischnocnema* data support populations in Brejo dos Cavalos and Brejo da Madre de Deus as each others' closest relatives. Populations in Água Azul and Tapacurá showed somewhat similar divergence time estimates (67,422, 95% CI: 31,001–419,924), whereas Pau Ferro and Guaribas lineage A appeared slightly younger (48,778,

Table 3. Maximum-likelihood estimates of divergence time and 95% credible intervals in years based on MCMC implementation of mtDNA data per taxon and population pair.

Species	Pairwise comparison	Inferred time of divergence in years	95% CI
Proceratophrys boiei	Frei Caneca–Brejo dos Cavalos	132,898	65,593–299,448
Proceratophrys boiei	Frei Caneca–Brejo da Madre de Deus	88,789	45,820–363,711
Proceratophrys boiei	Brejo dos Cavalos–Brejo da Madre de Deus	70,537	19,203–351,923
Ischnocnema gr. *ramagii*	Água Azul –Tapacurá	67,422	31,001–419,924
Ischnocnema gr. *ramagii*	Frei Caneca–Brejo dos Cavalos	77,394	24,497–412,987
Ischnocnema gr. *ramagii*	Brejo dos Cavalos–Brejo da Madre de Deus	52,680	36,204–417,322
Ischnocnema gr. *ramagii*	Pau Ferro–Guaribas (Lineage A)	48,778	17,126–405,616
Ischnocnema gr. *ramagii*	Baturité–Maranguape	92,570	41,841–353,586

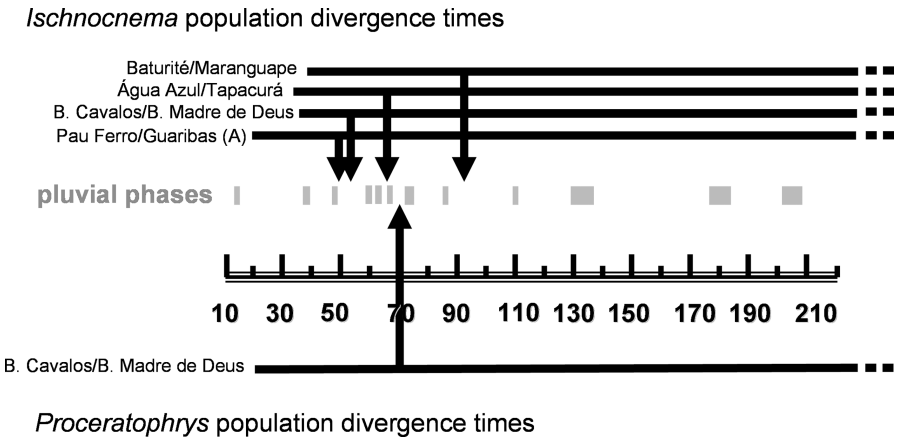

Figure 7. Comparison of Pleistocene pulses of pluvial maxima (Wang et al. 2004), provided as gray boxes over a timeline in kyrs, with maximum-likelihood estimates of population divergence times from mtDNA data for *Ischnocnema* gr. *ramagii* (above) and *Proceratophrys boiei* (below), indicated by black arrows. Horizontal bars depict 95% credible intervals. Estimated divergence times are shown only for populations inferred as closest relatives.

95% CI: 17,126 – 405,616). Baturité and Maranguape were estimated to have diverged around 92,570 years ago (95% CI: 41,841 – 353,586).

In contrast to the pattern of estimated dates of demographic expansions—which corresponded well with periods of pluvial maxima as inferred by the geomorphological data—all valid estimated divergence times in both *P. boiei* and *Ischnocnema* fell within the interpluvial phases suggested by the travertine and speleothem data (Fig. 7). Unfortunately, a power analysis including the period between the earliest and the latest of these estimated divergence times indicates that one would need at least 10 independent estimates of pairwise times of population splitting to tell significance from random results. Because we only have five of such estimates, no test was performed (Fig. 7).

Discussion

HAPLOTYPE PATTERNS IN FOREST POPULATIONS OF NORTHEAST BRAZIL: IMPLICATIONS FOR TAXONOMY, BIOGEOGRAPHY, AND CONSERVATION

Mitochondrial and nuclear DNA data indicate strong geographical structure within *P. boiei*, with each sampled locality being genetically differentiated. The significantly large mitochondrial F_{ST} values highlight the spatial structure of the haplotype network and the lack of shared sequences among localities (Fig. 2, online Supplementary Table S2). Despite the lower degree of geographical structure depicted in the nuclear haplotype network, *P. boiei* populations also show several nuclear private alleles and significantly large nuclear F_{ST} statistics between all pairs of localities (Fig. 3, online Supplementary Table S2). The loops recovered in the nuclear network are consistent with the high inferred number of recombination events.

Ischnocnema shows similar trends: even at the lowest levels of divergence, all pairs of populations exhibit significantly large F_{ST} values based on mtDNA (online Supplementary Table S3). However, estimates of mtDNA *Da* vary widely among populations and suggest that some may belong to distinct species. Also, several population pairs show significantly large nuclear F_{ST} values (online Supplementary Table S4).

Proceratophrys and *Ischnocnema* show comparable haplotype network configurations and largely congruent haplotype patterns (Figs. 2–5). Nonetheless, divergences among haplotype groups in *Ischnocnema* are considerably greater. Both the *P. boiei* mitochondrial network (Fig. 2) and the *Ischnocnema* nuclear network (Fig. 5) show similar haplotype groups comprised by sequences from central and southernmost localities (Brejo da Madre de Deus, Brejo dos Cavalos, Tapacurá, etc). *Proceratophrys* and *Ischnocnema* also have distinctive northern haplotype groups with sequences from Maranguape (which cluster with those from Baturité and Ubajara in *Ischnocnema*). For both taxa, nonenclave sites (such as Guaribas or Frei Caneca) possess sequences so genetically distinct that they form unconnected, unique groups in the haplotype network.

Analyses of mtDNA sequences reveal different evolutionary histories among enclaves. MtDNA haplotypes in the southernmost enclaves of Brejo da Madre de Deus and Brejo dos Cavalos are more closely related to those in nonenclave areas in the Atlantic domain than to other enclaves in both *Proceratophrys* and *Ischnocnema* (Figs. 2, 4). This has been observed in genetic studies of other forest-associated species in northeastern Brazil (Carnaval 2002). These results contradict the notion that all Caatinga enclaves comprise a single biogeographical entity (da Silva and Castelletti 2003), in agreement with parsimony analyses of endemicity of Brazilian woody plants (Santos et al.

2007), as well as studies of reptile distribution patterns in northeastern Brazil (Borges-Nojosa and Caramaschi 2003). They also oppose Santos et al.'s (2007) observation that southern nonenclave areas are more closely related to Amazonian localities and to northern enclaves (such as Baturité) than to southernmost enclaves such as Brejo dos Cavalos and Brejo da Madre de Deus.

The spatial distribution of the forest fragments sampled by this study proved less than ideal for investigations of patterns of isolation by distance (Fig. 1): localities are either relatively close to each other (e.g., Brejo da Madre de Deus and Frei Caneca) or separated by great distances (e.g., Frei Caneca and Ubajara). Despite the existence of a few forest remnants at intermediate distances within the Caatinga north of Água Azul, we were unable to sample populations of the study species in the intervening areas. Given the data at hand, comparisons between areas show a general tendency toward higher levels of mtDNA divergence with increased geographic distance. However, the presence of a pronounced genetic break between Água Azul and nearby southernmost populations in *P. boiei* (Frei Caneca, Tapacurá, Brejo da Madre de Deus, and Brejo dos Cavalos) indicates that isolation by distance cannot be the sole factor responsible for genetic divergence (Figs. 1, 2) and argues for a role for past fragmentation in shaping the distribution of Brazil's biological diversity, as suggested for other organisms (Bigarella et al. 1975; Andrade-Lima 1982; Grazziotin et al. 2006; Santos et al. 2007).

Differentiation between populations of *P. boiei* calls for a reevaluation of the taxonomic status of what is currently recognized as *P. boiei*. This ground-dwelling species is currently thought to be distributed along nearly the entire eastern coast of Brazil, from the southern state of Santa Catarina, to the northern states of Pernambuco and Ceará (Cochran 1955; Izecksohn et al. 1979; Heyer et al. 1990; Santos and Carnaval 2002). Populations sampled in this study roughly represent the northernmost 15% of its putative distribution, yet we found 3% mtDNA divergence between sites separated by 50 km (Água Azul and Tapacurá; Fig. 1). Specimens collected in the southeastern states of São Paulo and Rio de Janeiro differed from samples from northeastern Brazil by an average of 194 bp (i.e., about 14% in sequence composition for mtDNA; data not shown). This suggests that what we are currently recognizing as one taxon may represent a complex of species.

Ischnocnema presents a similar yet more complex pattern because of the high levels of molecular differentiation observed in the mitochondrial and nuclear data, as well as the differences in habitat use and call structure detected between some sympatric haplotype groups (such as observed at Guaribas). The amount of mtDNA divergence observed among some of the lineages identified by this study equals levels of differentiation detected among congeneric species and even confamilial genera of other vertebrate taxa (Johns and Avise 1998). *Ischnocnema*, like other closely related genera, has been recognized as a difficult group from a taxonomic perspective, with a high number of morphologically similar species and high intraspecific polymorphism (Lynch and Duellman 1997); thus multiple suites of characters, including call data, and detailed morphological and molecular analyses are needed to unravel taxonomic limits in the genus (Heyer and Carvalho 2000). *Ischnocnema ramagii* was described from Igarassu, Pernambuco, based on a single specimen (Boulenger 1888). The external morphology and color pattern of the individuals collected for this work resemble that of the original description. However, because we were able to identify differences in call, microhabitat use, and genetic profile among several populations, we purposely avoided making taxonomic inferences, and instead tentatively assigned the various populations to the *ramagii* group. Further comparative work is needed to accurately revise the taxonomy of these frogs.

In both *Ischnocnema* and *Proceratophrys*, mtDNA lineages in enclave populations are either monophyletic or nearly so (e.g., Brejo dos Cavalos, Brejo da Madre de Deus, Maranguape, Ubajara, Baturité; Figs. 2, 4). In contrast, nonenclave areas are polyphyletic and/or possess highly divergent mtDNA haplotypes or lineages (Frei Caneca, Guaribas). This argues for secondary contact or migration among formerly isolated populations along the Atlantic forest domain (Wayne et al. 1990), indicating a dynamic evolutionary history in these coastal forests that deserves further study.

Because they represent areas of high humidity and mild climate in the middle of a semiarid region, the naturally isolated "brejos" have a long history of human exploitation. Agriculture and timber harvesting are the two most important factors driving local habitat loss (Rodal et al. 1998; Sales et al. 1998). Biologists have argued for the conservation value of these enclaves by arguing that they constitute islands of biodiversity within the Caatinga, and are therefore worthy of protection (Uchoa Neto and Tabarelli 2002). Our results bring new insights to this discourse. Although individual enclaves allow the persistence of forest-associated communities in Brazil's semiarid region, they should not be described as reservoirs of genetic diversity. On the contrary, populations in "brejos" show significantly less variation than those in human-made fragments in the Atlantic forest domain. With regards to levels of genetic diversity and the evolutionary potential of local taxa, enclave conservation should be a priority, not because individual "brejos" harbor great amounts of variation, but rather because they each hold genetically unique populations (Figs. 2–5, online Supplementary Tables S2–S4). Their preservation is therefore key for the preservation of genetic diversity at the *regional* level. If we want to preserve populations with high levels of *local* diversity, efforts should focus on populations in the remaining fragments in the coastal Atlantic domain, as the latter are similarly under heavy anthropogenic pressure and still harbor more genetic variation than enclaves in the Caatinga. This is observed

at the haplotype level for *Proceratophrys* and at the lineage level for *Ischnocnema*. Our study demonstrates that naturally isolated enclaves in the Caatinga and recent, human-made fragments along the coast contribute, at different scales, to the genetic diversity of northeastern Brazil, and that both are worthy of preservation.

REVISITING THE PLEISTOCENE IN THE TROPICS

A pattern commonly detected in populations affected by Pleistocene climate change in the northern hemisphere is that of isolation during glacial times and demographic expansions (or recovery of variation after a bottleneck) during subsequent humid, interglacial periods (Milot et al. 2000; Hickerson and Ross 2001; Alexandrino et al. 2002; Fedorov and Stenseth 2002; Griswold and Baker 2002; Hoffman and Blouin 2004; Kotlik et al. 2004). In the lowland tropics, however, the influence of glacial and interglacial cycles on the demography and evolution of local taxa has been questioned (Klicka and Zink 1997; Schneider et al. 1998; Lougheed et al. 1999; Colinvaux et al. 2000; Bush and Oliveira 2006). Based on travertine and speleothem data, Auler et al. (2004) and Wang et al. (2004) have shown that the last 210 kyrs in northeastern Brazil were marked by several alternating pluvial phases that do not match the long-lasting glacial–interglacial periods. Our molecular data show traces of demographic expansions in multiple local populations and across taxa (Tables 1, 2; Fig. 6), as well as several events of population divergence in independent local amphibian lineages (Table 3, Fig. 7) that are strikingly consistent with Auler et al.'s (2004) geomorphological findings.

For both *Proceratoprhys* and *Ischnocnema*, the number of populations whose genetically inferred expansion dates fall within 3,520 years or less of a pluvial phase inferred by the geomorphological data is statistically significant. There is also concordance in point estimates of expansion times between co-occurring populations of two *Ischnocnema* lineages found in Guaribas (59 and 65 kyrs ago; Fig. 6). Point estimates of population expansion times exceeded 3,520 years of a wet period (inferred from the geological data) once in each taxon, and this was observed for the same locality (Brejo dos Cavalos; Table 2, Fig. 6)—suggesting the influence of factors other than regional climatic patterns at this site.

Several instances were observed in which the two species exhibited different responses at a given locality (Fig. 6), possibly due to bottlenecks subsequent to expansion events that have erased signatures of population growth. This may have occurred on mountaintops or slopes of comparatively smaller size such as Brejo da Madre de Deus, Maranguape, and Pau Ferro. Fragments within the transition zone between the Atlantic domain and the Caatinga (i.e., Água Azul and Brejo dos Cavalos) were the only sites showing traces of expansion in both study species. However, the estimated dates of expansion differed between taxa, and *Proceratophrys* expansion dates exceeded those of *Ischnocnema* by approximately 61 kyrs in both localities. Additional data are needed to test whether this time lag is coincidental or whether it reflects an effect of ecological interactions, natural history, reproductive success, or whether it was caused by the imposition of a common mutation rate between species that might have, in reality, evolved at different paces.

It has been suggested that forest enclaves within the Caatinga originated from Atlantic rainforest expansions during Pleistocene's humid cycles. In response to subsequent arid periods and the expansion of the Caatinga, those forests became isolated and restricted to humid uplands (Andrade-Lima 1982; Prado and Gibbs 1993). Divergence time estimates based on likelihood analyses using a rate of 0.957% sequence divergence per lineage per million years place the time of divergence between populations in enclaves Brejo da Madre de Deus and Brejo dos Cavalos (in both *Proceratophrys* and *Ischnocnema*), as well as between Água Azul and Tapacurá, Pau Ferro and Guaribas, and Baturité and Maranguape (for *Ischnocnema* populations) within the last 100,000 years, bracketing the last glaciation (Table 3, Fig. 7). Estimated divergence times between populations in Brejo dos Cavalos and Brejo da Madre de Deus differed only by ca. 18 kyrs between *Proceratophrys* and *Ischnocnema*. Moreover, all likelihood-based divergence estimates obtained for the two taxa fell outside pluvial periods inferred from geomorphological data (Auler et al. 2004). These observations suggest the view of multiple opportunities for population splitting in forest-associated taxa during comparatively dry periods of the late Pleistocene.

Inferred isolation times between northernmost and southernmost enclaves (Maranguape, Ubajara, or Baturité from Brejo dos Cavalos, Brejo da Madre de Deus, or Pau Ferro) predate the last 200 kyrs (Table 3). Levels of differentiation between these populations date to approximately one million years before present in *Proceratophrys*, or still older in *Ischnocnema*. Ab'Saber (1977) proposed that a few forests of northeastern Brazil (including Baturité) had been able to withstand the expansion of dry climates during the Quaternary. In contrast, he suggested that forests on the slopes of the Borborema Plateau such as Brejo dos Cavalos and Brejo da Madre de Deus were likely reduced in size by the expansion of semiarid climates and temporarily displaced by the Caatinga. Our data agree with Ab'Saber's (1977) observations, supporting the view that isolation of the northernmost enclaves such as Maranguape, Ubajara, and Baturité may have occurred as a consequence of the early expansions of the Caatinga, but that these populations were able to persist throughout the Pleistocene instead of disappearing and being subsequently recolonized from coastal or southern populations. The results also suggest that forest expansions during wetter periods of the Pleistocene were insufficient to reconnect populations of the eastern coast with those of the northernmost enclaves. In contrast, the close genetic similarities between populations in the southernmost enclaves of Brejo dos Cavalos and Brejo da Madre de Deus with nonenclave populations

such as Frei Caneca support the idea that these enclaves were apparently displaced by the Caatinga and recolonized by coastal forms through forest expansions during humid phases of the Pleistocene (Carnaval 2002).

In summary, the phylogeographic data demonstrate concordant patterns of population divergence that fall outside periods of pluvial maxima inferred from the travertine and speleothem data, as well as traces of demographic growth that correspond with those Pleistocene pluvial periods. The fact that similar patterns are observed across codistributed frog taxa suggests that these processes have occurred in response to local landscape and/or climate change. Even if we question the precision of the point estimates of population growth and divergence times (due to valid concerns such as stochasticity, limited sampling, reliance on mtDNA dating alone, or assumptions of the methods employed), the confidence/credible intervals obtained from the molecular data from both study species provide evidence for multiple population expansion events and population splits within the late Pleistocene. These results agree with Grazziotin et al. (2006) and Cabanne et al. (2007), who show demographic responses to Pleistocene climate change in the southern portion of the Atlantic forest. Together with Auler et al's (2004) and Wang et al.'s (2004) geomorphological studies, our data support a view of a dynamic Pleistocene period in northeastern Brazil, specifically in the northern portion of the Atlantic forest, with tangible population responses during the last 210 kyrs that were previously unrecognized. Because the oscillations in Pleistocene rainfall shown by Auler et al. (2004) and Wang et al. (2004) are believed to be tied to the Earth's 23 kyrs precessional cycle (Cruz et al. 2005), it is possible that similar processes have occurred in other tropical regions around the world.

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Supplementary Material

The following supplementary material is available for this article:

Table S1. Localities and number of individuals sampled per taxon (*Proceratophrys boiei* and *Ischnocnema* gr. *ramagii*) and marker (mitochondrial and nuclear DNA).

Table S2. *Proceratophrys boiei* mitochondrial DNA (left) and nuclear DNA (right) comparisons across populations. Above diagonal: Fst values (all significantly large at $p = 0.05$ level); diagonal: average number of pair-wise differences within populations; below diagonal: corrected average number of nucleotide differences between populations (Da).

Table S3. *Ischnocnema* gr. *ramagii* mitochondrial DNA comparisons across populations. Above diagonal: Fst values (all significantly large at $p = 0.05$ level); diagonal: average number of pair-wise differences within populations; below diagonal: corrected average number of nucleotide differences between populations (Da).

Table S4. *Ischnocnema* gr. *ramagii* nuclear DNA comparisons across populations. Above diagonal: Fst values (* denotes values significantly large at $p = 0.05$ level); diagonal: average number of pair-wise differences within populations; below diagonal: corrected average number of nucleotide differences between populations (Da).

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