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Phylogeography and species limits in the *Gymnodactylus darwinii* complex (Gekkonidae, Squamata): genetic structure coincides with river systems in the Brazilian Atlantic Forest

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Phylogenetic analyses based on mtDNA cytochrome *b* were performed in 42 lizards of the *Gymnodactylus darwinii* complex from three regions within Brazil's Atlantic Forest. Mainland regions and continental shelf islands in the south-eastern range and mainland areas from the north-east were sampled. The criteria of maximum parsimony (MP), maximum likelihood (ML) and Bayesian methods were explored, with the robustness for nodes assessed by bootstrapping (MP and ML) and posterior probabilities (Bayesian searches). By all methods, three distinctive phylogroups were recovered: a south-eastern clade (SE) and two clades from northern regions (NE_1 and NE_2). The pattern of genetic structure of the major clades coincided with the presence of river systems in the Atlantic Forest, and based on corrected genetic distances between those clades, divergence times were tentatively estimated using mtDNA rates calibrated for squamate reptiles. The putative role of Atlantic Forest rivers in generating differentiation is discussed. We present a hypothesis of species limits for *G. darwinii*, based on concordant lines of evidence including cytogenetic and mtDNA analyses. Two chromosome races (cytotype A, 2n = 38; and cytotype B, 2n = 40) had distributions concordant with clades SE and $NE_1 + NE_2$, respectively. These races are interpreted to be full species on the basis of a number of empirical criteria. *Biological Journal of the Linnean Society*, 2005, **85**, 13–26.

ADDITIONAL KEYWORDS: Ag-NORs – biogeography – Brazil – divergence times – karyotype – mtDNA – phylogroups – riverine barriers – species boundaries.

INTRODUCTION

The Brazilian lizard genus *Gymnodactylus* was first revised by Vanzolini (1953) who recognized a single species, *G. geckoides*, with three subspecies: *G. g. darwinii* occurring throughout the Atlantic Forest, *G. g. geckoides* in the Caatingas of north-eastern Brazil, and *G. g. amarali* from the central Brazilian Cerrados. Although based on poor geographical coverage of collections, this interpretation was widely adopted (Peters & Donoso-Barros, 1970). Later, the availability of larger samples and the absence of morphological intergrades between *G. g. darwinii* and the open formation races led Vanzolini (1982) to

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elevate *darwinii* to full species status. In this same paper he described a new species, *G. guttulatus*, from the 'campos rupestres' of state of Minas Gerais, a higher elevation rocky open habitat in the 'Serra do Espinhaço', a large mountain range extending from the state of Minas Gerais to the state of Bahia. Patterns of geographical variation within, and phylogenetic relationships among these taxa, remain unresolved.

Geckos of the genus *Gymnodactylus* are small (maximum snout–vent length = 48 mm), mostly nocturnal, terrestrial, sit and wait, cryptically coloured lizards (Vanzolini, 1953, 1982). Additional natural history information is restricted largely to *G. g. geckoides* (Vitt, 1995), which is insectivorous and oviposits one egg. *G. darwini* occurs mostly in lowland forests near sea level, but it is occasionally found at forest edges.

At the time of the first revision, the known geographical distribution of *G. darwinii* was restricted to the Atlantic forests extending from the northern state of Bahia to the northern coastal regions of the south-eastern state of São Paulo (Vanzolini, 1953). Its range has recently been expanded farther north into the state of Rio Grande do Norte (Freire, 1998), and to several continental-shelf islands off the coast of São Paulo.

Freire's (1998) comprehensive study included almost all specimens of *G. darwinii* in Brazilian collections from throughout the known range of the species, and revealed conspicuous morphological differentiation among populations along the coast. However, this variation was clinal in all characters analysed, similar to a pattern previously described in the lizard *Tropidurus torquatus,* which is distributed throughout the same general area (Rodrigues, 1987). This shared pattern of morphological variation suggests that both species may have been influenced by the same historical processes operating along broad environmental gradients, but a precise hypothesis based on independent evidence to account for these patterns of morphological differentiation is lacking. A phylogeographical study based on molecular data (Avise, 2000) could provide sufficient resolution of population structure to formulate a more robust hypothesis for the evolution of this genus.

Phylogeographical studies using Atlantic Forest taxa are relatively scarce, and most available data come from studies of small-bodied mammals (e.g. Mustrangi & Patton, 1997; Lara & Patton, 2000; Costa, 2003). An exception is a recent study conducted on four codistributed species of amphibian from Atlantic Forest remnants in north-eastern Brazil (Carnaval, 2002). Additionally, the establishment of DNA phylogenies is useful for suggesting hypotheses about the boundaries of genetically divergent groups (e.g. cryptic species or evolutionary significant units), but these should also receive supporting evidence from other sets of characters (Avise & Ball, 1990; Moritz, 1994; Wiens & Penkrot, 2002), as recently demonstrated in a phylogeographical study of Atlantic Forest pitvipers of the genus *Bothrops* (Puorto *et al*., 2001).

In addition to molecular data, chromosome data are important in this case for two reasons. First, karyotypes in geckos exhibit a high level of variation both in diploid number, which varies from 2n = 16 (Schmid *et al*., 1994) to 2n = 48 (Ota *et al*., 1992), with most karyotypes characterized by a series of acrocentrics gradually decreasing in size. Surprisingly, only a few karyotypes have been reported for Neotropical species, which represent about 23% of the total number of recognized species. Until now, few data have been published on karyotypes of *Gymnodactylus*; intraspecific variation due to the occurrence of a Robertsonian polymorphism and supernumerary chromosomes has been detected in one well-sampled population of *G. g. amarali* (2n = 38–41) from Serra da Mesa, on the central Brazilian Cerrados (Pellegrino, 1998). This pattern suggests that additional chromosomal variation may be present in these geckos. Second, issues of species boundaries remain unresolved in *Gymnodactylus*, and chromosomes may prove to be useful independent characters to corroborate any well-supported clades recovered from mtDNA analyses.

The purposes of the present study were to: (1) describe patterns of genetic divergence and phylogeographical structure of mtDNA haplotypes across the geographical range of *G. darwinii*; (2) formulate a preliminary biogeographical hypothesis about the Atlantic Forest region, which is characterized by high species diversity and endemism, and is recognized globally for its conservation significance (Mittermeier *et al*., 1999; Myers *et al*., 2000); (3) present a hypothesis of species limits in this group, based on concordant lines of evidence.

MATERIAL AND METHODS

Taxon sampling and outgroups

Forty-two specimens of *G. darwinii* were used to infer a molecular phylogeny, and sampling included specimens from three major geographical areas across most of the range within the Atlantic Forest of eastern Brazil. The south-eastern samples included specimens from the mainland regions of São Paulo and Espírito Santo, and some islands off the coast of São Paulo. Further north, populations from the states of Bahia, Alagoas and Rio Grande do Norte were also sampled (Table 1). The congeneric *G. geckoides*, and two other more distantly related genera (*Hemidactylus* and *Phyllopezus*), were used as outgroups.

Table 1. Samples of *Gymnodactylus darwinii* and outgroup taxa used in this study with respective field numbers and localities

Specimen	Field number	Locality	Coordinates
Island			
South-east Brazil (SE)	LG 872, 871, 883, 880	Ilhote do Sul, Ubatuba, SP (A) ●	23°34′S, 45°05′W
	LG 870, *882	Ilhota das Cabras, Ubatuba, SP (B) ●	23°31′S, 45°02′W
	LG 881	Ilha das Palmas, Ubatuba, SP (C)	23°33′S, 45°02′W
	LG 914, 924	Ilha do Mar Virado, Ubatuba, SP (D) ●	23°34′S, 45°09′W
	LG 930	Ilha Redonda, Ubatuba, SP (E) ●	23°21′S, 44°54′W
	LG 931	Ilha Comprida, Ubatuba, SP (F) ●	23°24′S, 44°51′W
	LG 933, 935	Ilha do Prumirim, Ubatuba, SP (G)	23°23′S, 44°57′W
	LG 937, 942, 944	Ilha da Pesca, Ubatuba, SP (H)	23°23′S, 44°53′W
	LG 943	Ilha das Couves, Ubatuba, SP (I)	23°25′S, 44°51′W
	LG 961	Ilha dos Porcos Pequena, Ubatuba, SP (J)	23°23′S, 44°54′W
	LG 962, 963	Ilha Rapada, Ubatuba, SP (K)	23°26′S, 44°54′W
	LG 802, 803	Ilhote de Fora, Ubatuba, SP (L)	23°33′S, 45°09′W
Mainland			
South-east Brazil (SE)	LG 1371, *1372, *1373, 1375, *1378	Corcovado, Ubatuba, SP (1) ●	23°20′S, 44°45′W
	LG 1600, 1601, 1602	Barra do Una, SP (2) ●	23°45′S, 45°24′W
	MRT 1265, 1266	UHE Rosal, ES (3) ●	19°38′S, 39°49′W
North-east Brazil (NE)	LG 957, 958, 991	Porto Seguro, BA (4) ■	16°26′S, 39°03′W
	LG 1349, 1742, 1740	Una, BA (5) ■	15°17′S, 39°04′W
	*LG 2062	Almada, BA (6) ■	14°47′S, 39°02′W
	MRT A1, A2, A3	Praia do Forte, BA (7)	12°34′S, 38°00′W
	MRT A5, A6	Mata São João, BA (8)	12°32′S, 38°18′W
	LG 1128, 1129	Mata do Catolé, Maceió, AL (9) ■	09°39′S, 35°44′W
	LG 1410	Natal, RN (10) ■	07°47′S, 35°12′W
Outgroups			
Gymnodactylus geckoides	LG 911	Xingó, AL	09°24′S, 37°58′W
	LG 1050	Barra dos Coqueiros, SE	10°54′S, 37°02′W
Phyllopezus pollicaris	LG 1310	Niquelândia, GO	14°27′S, 48°27′W
Hemidactylus mabouia	LG 1137	Ilha dos Búzios, SP	23°48′S, 45°08′W

Political units of Brazil (under 'locality') are: AL, Alagoas; BA, Bahia; ES, Espírito Santo; GO, Goiás; SE, Sergipe; SP, São Paulo; RN, Rio Grande do Norte.
Underlined specimens have mtDNA sequences and karyotypes available.
Specimens marked with * have only karyotype data. Letters and numbers in parentheses and symbols under 'locality' correspond to those plotted in Fig. 1.
Symbols ● and ■ represent occurrence of Cytotype A (2n = 38) or Cytotype B (2n = 40), respectively.

Collection and alignment of sequences

Total genomic DNA was extracted from liver and tail samples according to Fetzner (1999), and a fragment of about 800 bp of the mtDNA cytochrome *b* gene was amplified with the light-strand primers GLUDGL (5′-TGACTTGAARAACCAYCGTTG-3′; Palumbi, 1996) and CB1 (5′-CCATCCAACATCTCAGCATGATGAAA-3′; Kocher *et al.*, 1989), and the heavy-strand primer CB3 (5′-GGCAAATAGGAARTATCATTC-3′; Palumbi, 1996). The PCR cycle protocol consisted of 94 °C (1 min), 48 °C (1 min), 72 °C (1 min) × 40, and the products were directly purified with the GeneClean III Kit (BIO 101, INC., Vista, CA, USA). Both light and heavy strands were sequenced using the Perkin Elmer ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction (PE Applied Biosystems, Foster City, CA, USA), with sequences being run on an ABI PRISM 377 automated DNA sequencer (PE Applied Biosystems, Foster City, CA, USA).

Sequences were edited using the program Sequencher 3.1.1 (Gene Codes Corp., Inc., 1995), and the matrix of 794 bp of aligned sequences revealed no indels. Translation into amino acids confirmed

the alignment, and the few questionable bases were coded as '?'. Sequences were deposited in GenBank (accession numbers AY 630356–630401), and the NEXUS file is available from the first author upon request.

Phylogenetic analyses, profiles of genetic divergence and Mantel tests

Phylogenetic analyses under maximum parsimony (MP) and maximum likelihood (ML) optimality criteria were performed using PAUP* (version 4.0b4b, Swofford, 2001). All characters were equally weighted in the MP analyses, and heuristic searches were implemented with 100 replicates of random taxon addition with tree-bisection-reconnection branch-swapping (TBR). Rooting included three outgroup taxa: *G. geckoides, Phyllopezus pollicaris* and *Hemidactylus mabouia*.

Prior to implementing ML analyses, the general time reversible model with a proportion of invariable sites and a discrete gamma distribution (GTR + I + Γ; Yang, 1994; Gu, Fu & Li, 1995) was selected as the best-fit model of evolution for the cytochrome *b* sequences by ModeltTest (version 3.06, Posada & Crandall, 1998). The parameters of this model were used to implement the ML search with 20 random addition replicates using the TBR algorithm.

Confidence in the recovered nodes from MP and ML analyses was assessed by non-parametric bootstrapping (BS; Felsenstein, 1985), with nodes of BS ≥ 70% being considered strongly supported (Hillis & Bull, 1993). Bootstrap proportions were obtained on searches with 1000 (MP) and 500 (ML) pseudoreplicates.

Bayesian analyses were also performed using MrBayes 2.0 (Huelsenbeck & Ronquist, 2001), based on the GTR + I + Γ model of DNA substitution, with the parameter values estimated as part of the analysis. Two independent runs were conducted. The longest ran for 2×10^6 generations and four incrementally heated Markov chains were sampled at intervals of 100 generations to include 20 000 data points. Stationarity was reached before 150 000 generations, and after discarding the first 150 trees as 'burn-in', a 50% majority rule tree from the remaining 19 850 data points was obtained. The shortest analysis ran for 920 900 generations, with the 'burn-in' at 30 000; the majority rule consensus tree resulted from 8909 data points. Nodes on consensus trees from both runs with posterior probability (PP values) ≥ 0.95 were considered as evidence of significant support for a given clade (Huelsenbeck & Ronquist, 2001). These three methods of tree reconstruction are based on different assumptions about molecular evolutionary processes, and we interpreted similarities in tree topologies and nodal support as evidence that neither was strongly dependent on any particular method or assumed process.

Corrected average pairwise genetic distances, taking into account the intrapopulation mean divergences of two groups being compared, were calculated for the major clades recovered in the phylogenetic analyses using the software Arlequin vers. 2000 (Schneider, Roessli & Excoffier, 2000). These percentages of sequence divergence were then used to estimate times of divergence following mtDNA rates of evolution in squamate reptiles, which varied from 0.47% to 1.32% per million years (Myr). These represent the high and low point estimates compiled from a review of squamate mtDNA sequence divergence (Zamudio & Greene, 1997). Prior to these estimates, sequences were tested for deviations from neutral expectations using the *F*-test (Fu, 1997) as implemented in Arlequin vers. 2000 (Schneider *et al*., 2000).

Mantel tests were implemented following Legendre & Legendre (1998) and Lampert *et al*. (2003). Incorporating Mantel tests into a partial regression design allows partitioning of the variance in a response matrix (in this case, genetic distances) due to the effects of different predictor matrices. In this study, two predictors for genetic distances were used: geographical distances (km) among sampling localities, as a surrogate for an isolation-by-distance (IBD) process, and a model matrix defining whether each pair of haplotypes was within the same geographical region defined by possible allopatric isolation processes created by the major rivers on the Atlantic coast (Fig. 1). Partial coefficients were tested using 5000 random permutations using NTSYS 1.5 (Rohlf, 1989). This approach permits the total genetic divergence to be partitioned into influence from long-term historical processes (e.g. river effect) vs. that caused by ongoing demographic events (e.g. restricted gene flow with IBD). Additionally, since barriers are also structured into geographical space, the two predictors may be correlated in such a way that some amount of interaction (overlap) between these effects is expected.

Chromosome data

Chromosomes from 16 specimens representative of the three major geographically distinct populations of *G. darwinii* (Table 1; Fig. 1) were obtained from intestines following Bogart (1973), and from fibroblast cultures according to Yonenaga-Yassuda *et al*. (1988). A minimum of ten metaphases per specimen was analysed after routine Giemsa staining to establish the diploid number and chromosome morphology. The Ag-NOR staining technique (Howell & Black, 1980) was used to localize nucleolar organizer regions (NORs).

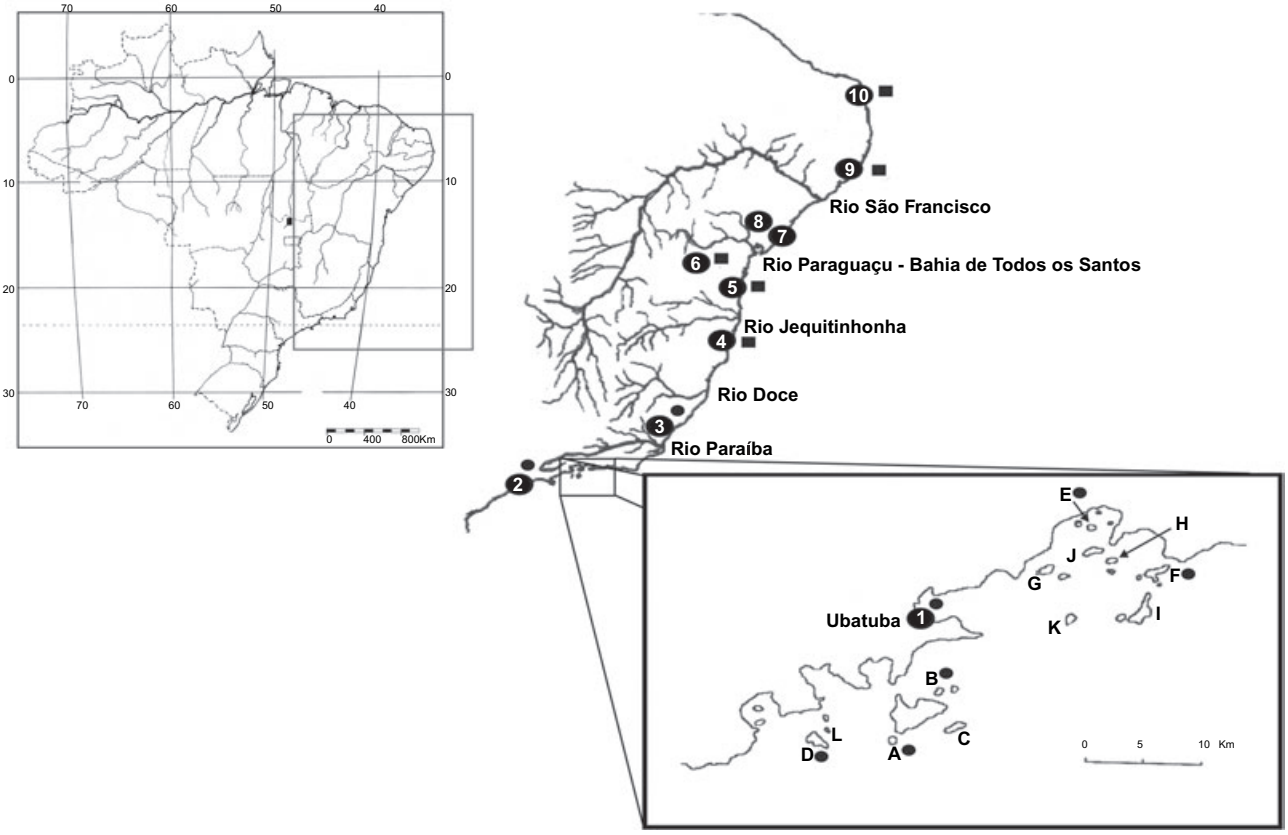

Figure 1. Map of eastern Brazil with the geographical location of populations of *Gymnodactylus darwnii* sampled for this study, relative to the major river systems of the Atlantic coast. See Table 1 for details of localities. Black dots and squares represent the distributions of Cytotype A (2n = 38) and Cytotype B (2n = 40), respectively.

RESULTS

PHYLOGENETIC ANALYSES

Phylogenetic analyses using multiple outgroups simultaneously (not constrained as monophyletic with respect to the ingroup) recovered the *G. darwinii* complex as monophyletic, with varying levels of support depending on the method of tree reconstruction: strong in MP analyses (BS = 99), moderate in Bayesian analyses (PP = 0.86) and weak in ML analyses (BS = 51). The congeneric *G. geckoides* was resolved as the sister taxon to the *darwinii* complex, followed by the two more distantly related geckos, *P. pollicaris* and *H. mabouia*, respectively (Fig. 2).

The MP analyses produced 10 000 most parsimonious reconstructions (limit set for number of trees to be saved) that were 699 steps in length; the strict consensus tree (CI = 0.72, RI = 0.88) is shown in Figure 2A. Three major mtDNA phylogroups were recovered. The first was a well-supported clade comprising populations from south-eastern Brazil (hereafter referred to as SE clade; BS = 100); this clade included: (1) a weakly supported monophyletic island–mainland complex (the SE_1 clade) from the southern mainland area and the adjacent islands of São Paulo (BS < 50); (2) a strongly supported haploclade from Espírito Santo (SE_2 clade; BS = 100). Second and third were two strongly supported northern clades that included all samples from north-eastern Brazil (Table 1; Fig. 2A); these are referred to here as NE_1 and NE_2, and both were recovered with strong support (BS = 100), but were only weakly supported as sister groups (BS = 56). Two phylogroups were recovered within each of the NE clades, and these are identified as southern or northern (NE_1S, NE_1N; NE_2S, NE_2N) clades in this region of Atlantic Forest. All of these were strongly supported: NE_1S and NE_1N clades both with BS = 100, and NE_2S and NE_2N clades with BS values of 98 and 85, respectively (Fig. 2A).

Figure 2B shows one of 106 ML trees (–lnL = 4048.88155) which were similar to the MP topology in that the same three major phylogroups (SE, NE_1 and NE_2 clades) were recovered. However, placement of these groups differed between ML and MP trees. The ML analysis recovered a strongly supported NE_1 clade (BS = 90) as the sister group of the SE clade, but with weak support (BS < 50; Fig. 2B). Furthermore, ML support for monophyly of the NE_2 clade was weak

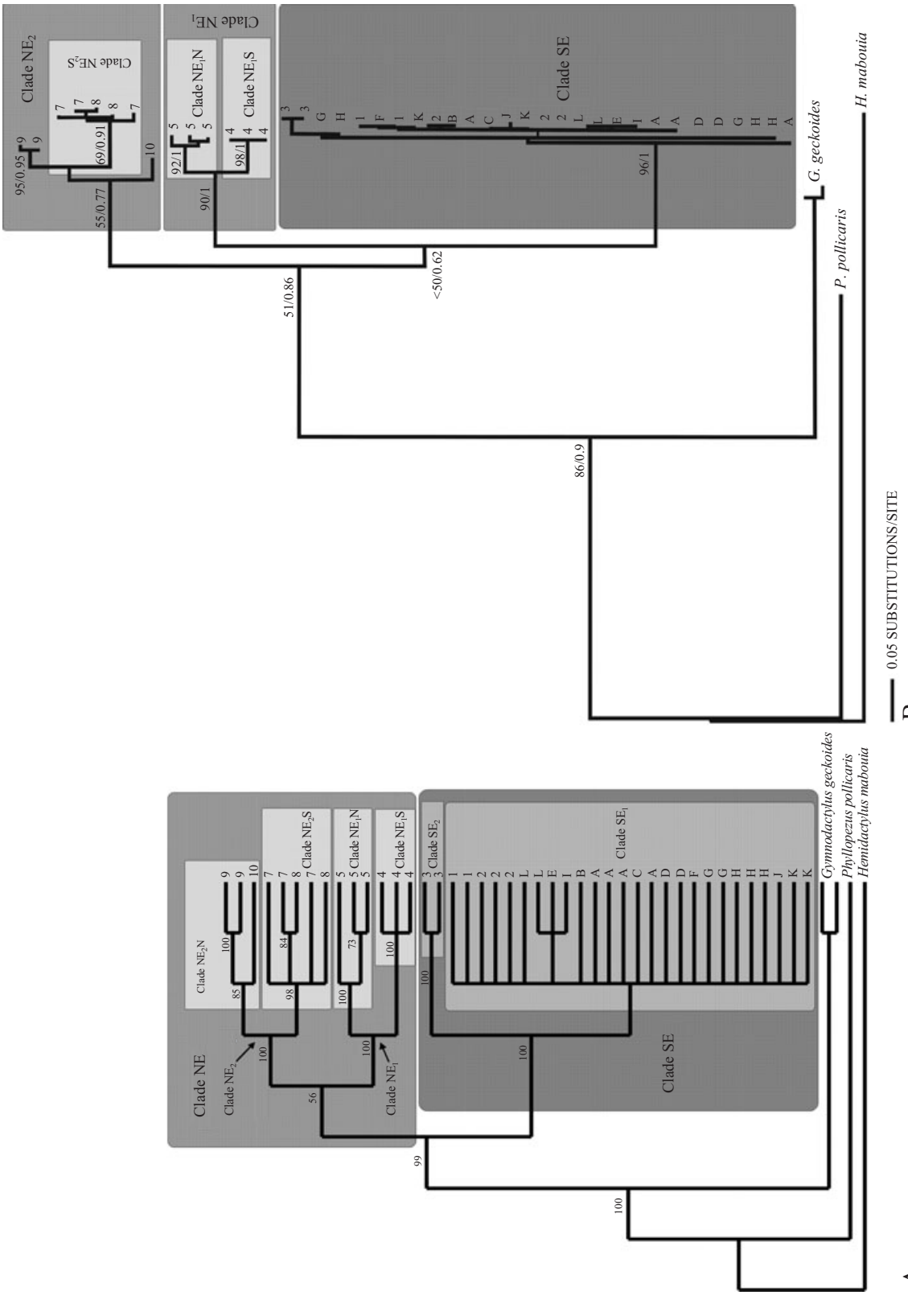

Figure 2. Molecular phylogeny of the *Gymnodactylus darwinii* complex for a region of mtDNA cytochrome *b* recovered by (A) maximum parsimony (L = 699; CI = 0.72; RI = 0.88) and (B) maximum likelihood (–lnL = 4048.88155). Numbers at nodes are bootstrap values (> 50%)/posterior probability (Bayesian searches). Numbers and letters at terminals correspond to sampling localities (see Table 1 and Fig. 1 for details).

Table 2. Upper and lower time estimates for divergences among major clades within *Gymnodactylus darwinii* (Figs 1, 2), and their coincidence with major river systems in the Atlantic Forest of Brazil

Evolutionary divergence	River system	Sequence divergence (% corrected)	mtDNAclock rate (%/myr)	Estimated time (Mya) (upper and lower)
Between SE_1 and SE_2 clades	Paraíba	1.29	0.47	2.7
			1.32	0.9
Between SE and NE clades	Doce	7.88	0.47	16.7
			1.32	5.9
Between SE and NE_1 clades	Doce	10.241	0.47	21.7
			1.32	7.7
Between SE and NE_2 clades	Paraguaçu – Bahia de Todos os Santos Jequitinhonha, Doce	10.983	0.47	23.3
			1.32	8.3
Between NE_1N and NE_1S clades	Jequitinhonha	6.06	0.47	12.8
			1.32	4.5
Between NE_1 and NE_2 clades	Paraguaçu – Bahia de Todos os Santos	10.52	0.47	22.3
			1.32	7.9
Between NE_2N and NE_2S clades	São Francisco	4.31	0.47	9.1
			1.32	3.2

mtDNA clock rates for reptiles followed Zamudio & Greene (1997).

(BS = 55), and within it, the monophyly of the NE_2N clade was not recovered with the specimen from Natal (terminal number 10; Table 1; Fig. 1). The Natal haplotype was resolved as the sister terminal to a group clustering the monophyletic NE_2S (BS = 69) plus two specimens (labelled as number 9; Table 1; Fig. 1) from Maceió.

A majority rule consensus of 19 851 trees estimated using Bayesian methods produced a topology almost identical to those recovered from ML analyses, and the same results were obtained in both runs (although one ran fewer than half of the generations of the other [not shown]). Common features of the Bayesian and ML analyses included: (1) weak support (PP = 0.62) for the sister relationship between the NE_1 clade (PP = 1.0) and the SE clade (PP = 1.0); (2) weak support for monophyly of the NE_2 clade (PP = 0.77); (3) absence of structuring within the NE_2 clade, but the NE_2S clade was recovered with quite high support (PP = 0.91); (4) non-monophyly of the NE_2N clade, due to the unresolved position of the Natal sample (terminal number 10; Table 1; Fig. 1). Differing from the ML analyses, the Bayesian method recovered the split between the SE_1 and SE_2 (PP = 0.92) clades (not illustrated), in agreement with the MP analyses.

Estimation of divergences and Mantel tests

Corrected sequence divergences among the major clades recovered in the phylogenetic analyses within the *Gymnodactylus* complex (Fig. 2) varied from 1.29% (between the SE_1 and SE_2 clades) to 10.9% (between the SE and NE_2 clades; Table 2).

Considering that Fu's *F*-test did not reject the hypothesis of neutrality, we used the percentage sequence divergence to estimate times of divergence among the major clades, following a range from low (0.47%/Myr) to high (1.32%/Myr) rates of squamate evolution. Approximated times of divergence varied from 0.9 to 2.7 Mya (between the SE_1 and SE_2 clades) to 8.3–23.3 Mya (between the SE and NE_2 clades; Table 2).

Mantel tests showed that about 64.7% of the variation in corrected pairwise genetic distances (under the GTR + I + Γ model) within the *G. darwinii* complex could be explained by the combined effects of geographical distances and long-term historical divergence. Separating these effects, about 9% of the total genetic variation could be attributed to long-term historical divergence (river effect), independent of the geographical distances, whereas 4% of this variation was due to geographical distances, reflecting ongoing demographic processes (IBD) independently of long-term historical processes. These partial coefficients, although relatively small in magnitude, were significant at $P < 0.01$ under 5000 random permutations.

Chromosomal analyses

Two different karyotypes were found in the sample of 16 specimens of *G. darwinii* analysed in this study, here referred to as Cytotypes A and B.

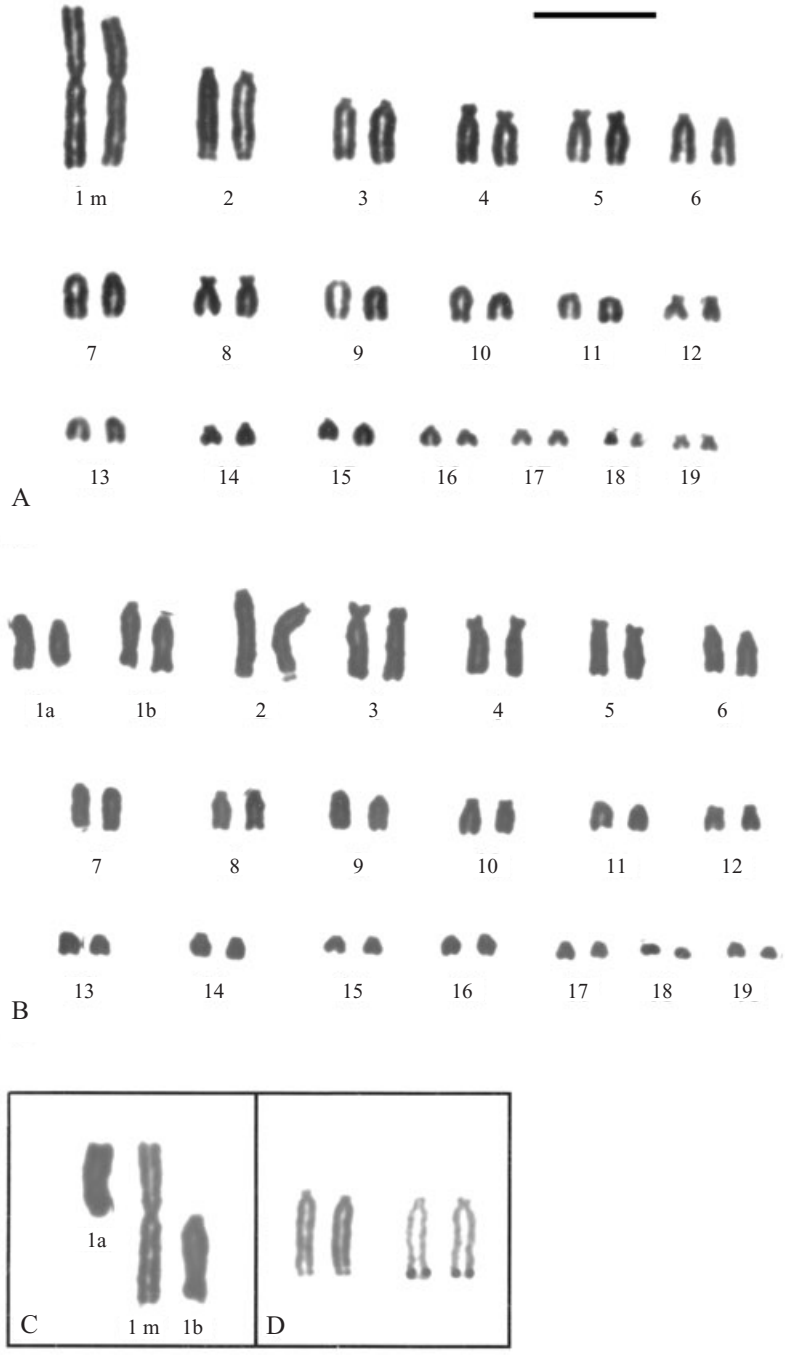

Figure 3. A, Cytotype A (2n = 38) and B, Cytotype B (2n = 40) of the *Gymnodactylus darwinii* complex, after conventional staining. C, the Robertsonian rearrangement of pair 1: acrocentrics 1a and 1b of Cytotype B correspond to the short and long arms, respectively, of metacentric pair 1 (1m) of Cytotype A. D, Pair 2 bearing the secondary constriction at the distal end of the long arm, after conventional (left) and Ag staining (right). Scale bar = 10 μm.

Cytotype A (2n = 38)

Specimens from the southern-island–mainland complex (SE clade; Table 1; Figs 1, 2) were all characterized by a karyotype comprised of 38 chromosomes, with a large metacentric pair 1 (here labelled as 1m), two medium-sized subtelocentric pairs (2 and 3), and two medium-sized submetacentric pairs (4 and 5), all clearly distinct from each other and from all other chromosome pairs (Fig. 3A). Pairs 6–18 were mostly acrocentric chromosomes decreasing gradually in size,

with the exception of pairs 8 and 12, which were biarmed, and pair 19 that was a small metacentric.

Cytotype B (2n = 40)

Specimens collected in localities from the northern range of the species (NE clades; Table 1; Figs 1 and 2) were all characterized by a karyotype comprised of 40 chromosomes (Fig. 3B), with the major difference being the presence of two medium-sized acrocentric pairs (here labelled as 1a and 1b) in place of the single large metacentric pair of Cytotype A. These two acrocentric pairs likely represent the homologous elements resulting from a Robertsonian rearrangement involving the large metacentric pair (1m) of Cytotype A (Fig. 3C). In the remaining chromosomes, Cytotype B also differed from Cytotype A by the presence of a submetacentric pair 3 and an additional biarmed pair (number 10).

Ag-NORs

The NOR was located at the secondary constriction at the distal end of the long arm of pair 2 (Fig. 3D) in specimens from both SE and NE clades.

DISCUSSION

Genetic divergence and phylogeography of *G. darwinii*

Phylogenetic analyses using either parsimony or model-based methods recovered three distinctive mtDNA phylogroups across populations of the *G. darwinii* complex: the SE clade including specimens from southern regions of the range, and clades NE_1 and NE_2 grouping populations from northern regions (Table 1; Fig. 2). Although the strength of support for each of these three clades varied according to the tree-building method used, as did their relationships (Fig. 2), the general pattern that emerged from all analyses is that the deep genetic structure recovered within the *G. darwinii* complex coincides with the major river systems reaching the Atlantic coast of Brazil (Table 1; Figs 1, 2).

The southern island–mainland complex (SE_1 clade) did not show evidence of population structure, as demonstrated by the lack of resolution or very short branch lengths in the MP and ML topologies, respectively (Fig. 2A, B). This pattern is expected in a scenario of recent dispersal, or gene flow coupled with recent isolation, and could represent the effects of rising sea level related to the last marine transgression at about 11 000 BP, when these islands were likely isolated from the mainland and from each other (Vanzolini, 1973). The split between the SE_1 and SE_2 clades recovered in the MP trees (Fig. 2A) was characterized by a lower level of sequence divergence (1.29%) compared with those estimated for the other clades, but nevertheless showed geographical concordance with the Paraíba river system (Table 2; Fig. 1). The deeper phylogeographical break between the SE and NE clades (recovered in the MP trees) had an average haplotype divergence of almost 8%, and was coincident with the Rio Doce system (Table 2; Figs 1, 2). The MP and ML topologies differed with respect to the monophyly of the NE_1 and NE_2 clades, and the structuring within the latter (Fig. 2A, B). The support for monophyly of NE_1 and NE_2 clades as represented in the MP phylogeny was weak (BS = 56), while in the ML hypothesis, the NE_1 clade (BS = 90) appeared as the sister group of the SE clade (10.2% sequence divergence; Table 2), but also with weak support (BS < 50).

The hypothesis recovered by the ML topology implied that a split between populations currently distributed above (clade NE_2) and below (clades NE_1 and SE) the Bahia de Todos os Santos may have occurred first, with a subsequent and almost contemporary second split that isolated populations below (SE clade) and above (NE_1 clade) Rio Doce (Figs 1, 2B; Table 2).

Another deep phylogeographical split was correlated with haplotype clades within the northernmost NE clade. Here, the NE_1 and NE_2 clades were separated by the Bahia de Todos os Santos (Fig. 1) and other smaller rivers which historically may account for the relatively large divergence (10.5%) between these two clades. The NE_1 and NE_2 clades each reflected further phylogeographical structure, in the form of strongly supported clades whose ranges were also coincidental with isolation by the Rio São Francisco (clades NE_2N and NE_2S, Figs 1, 2A) and the Rio Jequitinhonha (clades NE_1N and NE_1S; Figs 1, 2A, B).

Regardless of which (MP or ML) hypothesis is considered, the overall pattern of genetic differentiation of the major clades within *G. darwinii* as revealed by the mtDNA coincides with the presence of major river systems in the Atlantic Forest of eastern Brazil.

Time of divergence among major clades of *G. darwinii*: can rivers be putative candidates for barriers in the Atlantic Forest?

We are aware of the potential problems of using molecular clock estimates, the large error terms (Graur & Martin, 2004), and the uncertainty about which rates are more appropriate for a given group of organisms in the absence of external data for calibration. However, the mtDNA rates of evolution we have applied here are considerably broad (from 0.47 to 1.32%/Myr) and the divergence times we obtained are only a first approximation, but might provide a crude estimate of the relative order of events isolating the major haplotype clades of *G. darwinii* in the Brazilian Atlantic

Forest (Table 2). These estimates may further provide a relative database for developing more explicit hypotheses about the establishment of major river systems in this rainforest.

As an example, estimates of divergence times suggested that the oldest historical events accounting for the split between southern (SE) and northern (NE) clades, and the opening of Rio Doce and Bahia de Todos os Santos, may have occurred within a period between 8 and 23 Mya (Table 2). These divergence estimates are broad, but are compatible with the inferred time for deposition of the Barreiras Formation (Martin, Suguio & Flexor, 1993). All of the Atlantic Forest and associated sandridge habitats along the coast now occupied by *G. darwinii* are on the Barreiras Formation sediments, which extend widely from the state of Pará in the north to the state of Rio de Janeiro in the south-east. The deposition of the Neogene sediments of the Barreiras Formation occurred by flash-flood sedimentation under a semiarid climate when the sea level was lower than it is at present, and this likely took place at the Miocene/Pliocene boundary**.** Current opinion holds that only in the Pliocene, under high humidity conditions, did the rivers settle into their approximately present courses, bisecting the Barreiras deposits (Martin *et al*., 1993; Suguio & Nogueira, 1999).

Although our data suggest that major Atlantic coastal rivers act as potential gene flow barriers and therefore may have played a role in generating diversification among *G. darwinii* populations, we cannot be certain of this without better geographical sampling throughout the area, under a statistical design that rejects alternative explanations. Our study was not formerly designed to test the 'riverine barrier hypothesis' as originally proposed by Wallace (1852) for Amazonia, and detailed studies aimed at explicitly testing this idea have provided ambiguous results. There is some support from studies of primates (Ayers & Clutton-Brock, 1992), while other vertebrate groups (Gascon, Lougheed & Bogart, 1998; Patton & da Silva, 1998; Lougheed *et al*., 1999; Symula, Schulte & Summers, 2003) do not manifest phylogeographical patterns consistent with riverine barrier expectations. Considering that river courses shift constantly (see Symula *et al*., 2003), they are not all likely to serve as effective barriers to gene flow.

For example, patterns of the meandering of Amazon rivers in their midstream and downstream courses create cutoffs that physically transfer blocks of habitats from one riverbank to the other over time (Kalliola, Puhakka & Danjoy, 1993; Gascon *et al*., 2000). The frequency of this mechanism suggests that the period of isolation of habitats may be insufficient to account for differentiation of the isolated populations in the Amazon, and in fact older geological events may have predominated over river barriers in structuring genetic variation within and between species (see Patton, da Silva & Malcolm, 2000). In contrast, the portion of Atlantic Forest river courses in which such a meandering might have occurred in the past was flooded by the last marine transgression (11 000 ya; Vanzolini, 1973; Suguio & Nogueira, 1999) and, indeed, the areas we sampled for *G. darwinii* are now geologically stable and less likely to undergo major shifts in river courses. Given the limited support for riverine barriers in Amazonia, we had no reason to expect these patterns in the Atlantic Forest when we began this study, but the phylogeographical pattern evident in the *G. darwinii* complex is suggestive enough to warrant serious follow-up studies.

An important piece of evidence that may favour the hypothesis of the putative role of Atlantic Forest rivers in generating differentiation among taxa comes from studies conducted at the sand dune region of the middle Rio São Francisco in the state of Bahia (for a review, see Rodrigues, 1996). From that unique region, some endemic lizards, snakes and amphisbaenians have been described as being likely to form vicariant allopatric sister species inhabiting opposite and adjacent sandbanks along the São Francisco river (Rodrigues & Juncá, 2002).

We implemented Mantel tests to evaluate statistically the simultaneous influence of long-term historical processes (in our case, the hypothesized barriers represented by the major Atlantic Forest rivers) and more recent events (e.g. restricted gene flow with IBD) on the observed genetic variation within the *G. darwinii* complex. This test revealed that about 9% of the total genetic variation within the *G. darwinii* complex could be attributed only to the effect of a putative barrier, whereas less than half of the influence on the genetic diversity (4%) would be due to geographical distances (IBD) alone. Therefore, the Mantel tests provide statistical support for the predominant influence of historical isolation in structuring the major clades within the *G. darwinii* complex (although ongoing demographic processes [IBD] cannot be ignored). As expected, the majority of genetic divergence (64.7%) could not be partitioned between historical and IBD events, showing a strong interaction of the effects of geographical distances and the putative barriers (rivers).

The coincidence of rivers and patterns of genetic differentiation in the Atlantic Forest is not restricted to the genus *Gymnodactylus.* A few examples indicate that what appear to be river-limited distributional patterns disclosed by our study also characterize other taxa and are suggestive of a shared vicariant history. For example, among tropidurid lizards, *T. hispidus* and *T. hygomi* do not occur south of the Bahia de Todos os Santos (Rodrigues, 1987), and *Strobilurus torqua-*

tus does not occur south of the Rio Doce. The geckonid *Coleodactylus meridionalis* occurs in the northern part of the Atlantic Forest but does not occur south of the Rio Jequitinhonha. In the lizard family Gymnophthalmidae, *Stenolepis ridleyi* and an undescribed genus until now referred to as *Anotosaura* sp. nov. (Rodrigues *et al*., in press) have similar distributions; neither extends south of the Rio São Francisco (Rodrigues, 1990).

Distributional observations alone do not prove that rivers were the causal mechanisms of divergence in these groups, but rigorous tests of the putative role of Atlantic Forest rivers as barriers to gene flow are now possible in phylogeographical studies. These must be planned with sampling designs based on a series of transects along and parallel to the coast and from both banks throughout the drainages of the rivers hypothesized to be gene flow barriers (e.g. Patton *et al*., 2000). Sampling should include a greater density of geographical localities as well as more individuals per locality to capitalize on the strengths of coalescent approaches (see Morando *et al*., 2004 for a recent example), and to permit statistical tests of alternative hypotheses derived from predictions based on external data (e.g. bioclimatic, paleoecological; see Knowles, 2003).

Taxonomically, future studies should target the *G. darwinii* complex and other endemic taxa from multiple groups, as well as widely distributed taxa that appear not to display strong geographical structure (Stuart-Fox *et al*., 2001). If the palynological record is sufficient, paleodistributions of original Forest under various climatic regimes can be modelled, and then tested using molecular phylogeographical approaches to separate the possible role of refugial isolation in causing most of the genetic divergence, followed by subsequent postrefuge expansion in which rivers form only current barriers to gene flow (see Hugall *et al*., 2002). Lastly, powerful methods now exist for comparing phylogenies and phylogeographies of codistributed taxa to test for evolutionary concordance (vs. associations that might have arisen by chance; Charleston, 1998; Charleston & Page, 2002); these kinds of studies will contribute to the development of a broadly synthetic hypothesis for the evolution of this unique and threatened biota (see examples in Moritz *et al*., 2000, for the World Heritage tropical Forests of Australia).

Species boundaries within the *G. darwinii* complex

We have confirmed the monophyly of the *G. darwinii* complex by mtDNA genealogy (Fig. 2) and also by the location of Ag-NORs on the large chromosome pair 2 (Fig. 3D), which is a synapomorphy for the complex. The related taxa from Caatinga (*G. g. geckoides*) and Cerrado (*G. g. amarali*) are characterized by the presence of NORs on a medium-sized chromosome pair that is morphologically indistinguishable from other autosomes (Pellegrino, 1998). Our genetic data corroborate the full species status of *G. darwinii* previously proposed on the basis of morphological characters by Vanzolini (1982).

The molecular and karyotype data presented here provide qualitative support for some of the abrupt clinal variation observed in morphological characters among Atlantic Forest populations of *G. darwinii* (Freire, 1998). Freire suggested the possibility of subspeciation within *darwinii*, but was aware of the need for analyses of non-morphological characters to confirm such a hypothesis. A similar pattern of morphological variation was also described in lizards of the genus *Tropidurus* sharing the same general distribution along the eastern coast of Brazil (Rodrigues, 1987). Although our sampling was limited for karyotypes (Table 1), the concordance between the two major mtDNA phylogroups (the SE and NE clades) and the two different chromosome races (Cytotype A [2n = 38; SE clade] and Cytotype B [2n = 40; NE clades]), was unambiguous (Fig. 1), and supports a hypothesis of at least two distinct species under the name *G. darwinii*. By any number of empirical methods for delimiting species (reviewed by Sites & Marshall, 2003, 2004), these clades would meet acceptable criteria for species recognition, regardless of whether the MP or ML topology is more accurate for the mtDNA genealogy. A number of species delimitation methods do not require genealogical monophyly for recognition, as long as paraphyly is accompanied by evidence of gene flow or ecological interchangeability (Templeton, 2001; Wiens & Penkrot, 2002). We therefore here recognize each chromosome race as a valid species; further morphological study is in progress to identify external diagnostic characters for each.

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