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Genetic diversity and structure of two species of *Enyalius* (Squamata: Leiosauridae) from neotropical biodiversity hotspots

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Abstract

Genetic diversity and structure of two species of *Enyalius* (Squamata: Leiosauridae) from neotropical biodiversity hotspots. *Enyalius*, a lizard genus endemic to South America, is mostly distributed in the remains of the Atlantic Forest and in the Cerrado. The genus has been the topic of a few studies but none has quantified the genetic diversity and structure within and among populations of *Enyalius*. The genetic diversity and structure of populations of *E. bilineatus* ($N = 20$) and *E. perditus* ($N = 28$) are examined using a 234-bp fragment of the cytochrome b gene and compared with the sequences reported in other published data. Nineteen distinct haplotypes (eleven for *E. perditus* and eight for *E. bilineatus*) were found, eight of which were recorded for the first time. The haplotype diversities are highly similar for both species (0.684 for *E. perditus* and 0.647 for *E. bilineatus*). The genetic distance between the two species is 20.3% and the distance within species is 2.0% and 5.6% for *E. perditus* and *E. bilineatus*, respectively. Our data suggest that populations of *E. bilineatus* are genetically divergent and may reveal cryptic diversity. This is the first study to quantify the genetic diversity of species of *Enyalius* from Neotropical biodiversity hotspots. These data facilitate a better understanding of both

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within and among population variation, and highlight the distribution of genetic lineages of an endemic and poorly studied genus.

Keywords: Atlantic Forest, Cerrado, cytochrome b (*cyt b*), *Enyalius bilineatus*, *Enyalius perditus*.

Resumo

Diversidade e estrutura genética de duas espécies de *Enyalius* (Squamata: Leiosauridae) em hotspots neotropicais de biodiversidade. Os lagartos do gênero *Enyalius* são endêmicos da América do Sul, sendo predominantemente encontrados no Cerrado e em fragmentos da Floresta Atlântica. Este é um gênero pouco estudado, e não foram encontrados dados relacionados à diversidade e à estrutura genética das espécies do gênero. Neste trabalho, investigamos a diversidade genética de populações de *E. bilineatus* ($N = 20$) e *E. perditus* ($N = 28$), usando um fragmento de 234-pb do citocromo b, e comparamos as sequências geradas com outras publicadas. Dezenove haplótipos distintos foram encontrados (11 de *E. perditus* e oito de *E. bilineatus*), sendo oito deles novos registros. Os valores de diversidade haplotípica foram muito similares para as duas espécies (0.684 para *E. perditus* e 0.647 para *E. bilineatus*). A distância genética entre as duas espécies foi de 20.3%, e as distâncias intraespecíficas foram 2.0% para *E. perditus* e 5.6% para *E. bilineatus*. Nossos dados sugerem que as populações de *E. bilineatus* são altamente divergentes e que a espécie deve apresentar diversidade críptica. Este é o primeiro estudo medindo a diversidade genética de espécies do gênero *Enyalius* oriundas de regiões consideradas hotspots da biodiversidade neotropical e apresenta dados relevantes para um melhor entendimento das relações inter e intrapopulacionais, assim como a distribuição das linhagens genéticas desse gênero endêmico.

Palavras-chave: Cerrado, citocromo b (*cit b*), *Enyalius bilineatus*, *Enyalius perditus*, Floresta Atlântica.

Introduction

Ecological, demographic, and genetic studies provide critical data for conservation studies. Data documenting the genetic structure and levels of genetic diversity within and between populations are useful in the formulation of conservation strategies that ensure a species or local populations retain sufficient genetic diversity to respond to environmental change (O'Brien 1994, Wallace *et al.* 2011). Thus, maintenance of genetic diversity is an important factor in fostering adaptative evolution in the medium and long terms in natural populations (Santos and Redondo 2003, Frankham *et al.* 2010).

Enyalius (Leiosauridae) comprises at least 10 endemic South American taxa; systematic

relationships among these taxa are challenging given the variation in their color patterns (Rodrigues *et al.* 2014). The species are widely distributed through the Atlantic Forest; one species is found in the Amazon, and others occur in remote locations of the Caatinga and Cerrado gallery forests (Etheridge 1969, Vanzolini 1973, Jackson 1978, Ávila-Pires 1995). *Enyalius perditus* occurs in remnants of the Atlantic Forest in the states of Rio de Janeiro, São Paulo, Paraná, and Minas Gerais (Jackson 1978, Sousa *et al.* 2012). *Enyalius bilineatus* inhabits regions of the Atlantic Forest in the states of Minas Gerais, Rio de Janeiro and Espírito Santo and areas of Cerrado and Caatinga habitats in Minas Gerais (Etheridge 1969, Jackson 1978, Zamprogno *et al.* 2001, Bertolotto *et al.* 2002, Colli 2003, Sousa *et al.* 2010, Novelli *et al.*

2012). *Enyalius bilineatus* is sympatric with *E. perditus* at high elevations in the Atlantic Forest (Barreto-Lima 2012, Rodrigues *et al.* 2014).

The few studies that examine the distribution (Barreto-Lima 2012) and evolutionary history of these species (Bertolotto 2006, Rodrigues *et al.* 2014) emphasize the need to increase the number of localities sampled to better understand the relationship between the levels of genetic diversities, genetic structure, and phylogeny of the genus. These data will reveal cryptic diversity and inform effective conservation actions. Based on an analysis of chromosomes, and mitochondrial and nuclear markers from *Enyalius* spp., Bertolotto (2006) identified two divergent lineages of *E. bilineatus* from Minas Gerais; the author suggested that *E. bilineatus* represented two different species. In a more inclusive biogeographic study, Rodrigues *et al.* (2014) examined the 10 recognized species of *Enyalius* from 51 Brazilian localities; they reported high diversity and several taxonomic problems. The authors found *E. perditus* to be paraphyletic and *E. bilineatus* to contain cryptic diversity expressed as two coherent, divergent geographic clades. None of the locations sampled in this study was included in previous studies; therefore, the new data presented herein provide important records on genetic structure of these species, as well as useful information that can be applied to future taxonomic decisions.

Neither *Enyalius perditus* nor *E. bilineatus* is cited in the Brazilian list of threatened species (Martins and Molina 2008) or the Red List of the International Union for Conservation of Nature (IUCN 2015). We think there are insufficient data to assess the status of the species properly. Both species are relatively abundant in the areas that we targeted; thus, we were able to collect appropriate numbers of samples (desirable greater than five per location) to perform a sound population genetic study.

We propose to increase the knowledge about the genetic diversity of lizards from the Atlantic Forest and Cerrado regions. Specifically, we aimed to: (1) understand the distributions of the

genetic variation of the two species of *Enyalius* in the fragments studied; (2) reveal evidence of cryptic diversity for *E. bilineatus* based on the *cyt b* fragment, as well as from samples at two additional localities; (3) analyze the parphyly of *E. perditus* on the basis of specimens from three additional locations; and (4) to analyze the relationship among the *E. perditus* and *E. bilineatus cyt b* haplotypes from novel locations and compare our results with other published data.

Materials and Methods

Sampling

Enyalius bilineatus was collected in Cerrado areas of Área de Proteção Ambiental da Serra de São José (APASSJ), which is located in the foothills of the APASSJ in the municipality of Tiradentes ($N = 12$), and in the Reserva Biológica Unilavras-Boqueirão (RBUB) in the municipality of Ingaí ($N = 8$). We collected *E. perditus* in Atlantic Forest areas in Parque Estadual do Ibitipoca (PEI) in the municipality of Lima Duarte ($N = 19$) around Sítio Malícia, and in the Área de Proteção Ambiental Mata do Krambeck (APAMK; $N = 6$) and Parque Municipal da Lajinha (PML; $N = 3$) in Juiz de Fora (Figure 1).

All samples were collected in accordance to permits from the Sistema de Autorização e Informação da Biodiversidade—SISBIO (System for Biodiversity Information and Authorization), Instituto Estadual de Florestas—IEF (State Forestry Institute) and Agência Ambiental de Juiz de Fora (Juiz de Fora Environmental Agency) (AGENDA-JF) (license numbers 17917-1 to collect in Tiradentes, 17917-1 and 24661-1 in Ingaí, 772-1 in Lima Duarte and 17074-1 in Juiz de Fora, permission number 118/09 to collect in APASSJ, 125/09 to collect in PEI and 69995/08 to collect in Conservation Units from Juiz de Fora). The project also was approved by the ethics committee of the animal experimentation, Comitê de Ética na Experimentação Animal da Pró-Reitoria de Pesquisa / UFJF (Protocol number 010/2008-CEA).

We took liver parts from euthanized animals or tail samples (2 cm) from live animals. Tissues were stored at room temperature in 98% ethanol and added to the tissue collection of the UFJF, Minas Gerais. Total DNA was extracted using a phenol: chloroform: isoamyl-alcohol protocol (Sambrook *et al.* 1989) at the Genetics and Biotechnology Laboratory of UFJF.

Cyt b Sequencing

Partial *cyt b* mitochondrial sequences were amplified using primers LGL 765 and H15144 (Bickham *et al.* 1995). PCR reaction mixes of

20 μ L, which included 4 μ L of genomic DNA (~ 40 ng), 0.5 U of *Taq* polymerase (Phonetrutria[®]), 200 μ M of dNTPs, 1X Tris-KCl buffer with 1.5 mM MgCl₂ (Phonetrutria[®]) and 0.5 μ M of each primer. The PCR profile consisted of 5 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 52°C, 1 min at 72°C and a final extension step of 9 min at 72°C. After amplification, the PCR products were checked by electrophoresis in 0.8% agarose gels and stained with Sybr Green. Negative controls in which template DNA was omitted, were used in all amplification runs. Only products with a single and well-defined 400-bp band were used in the sequencing

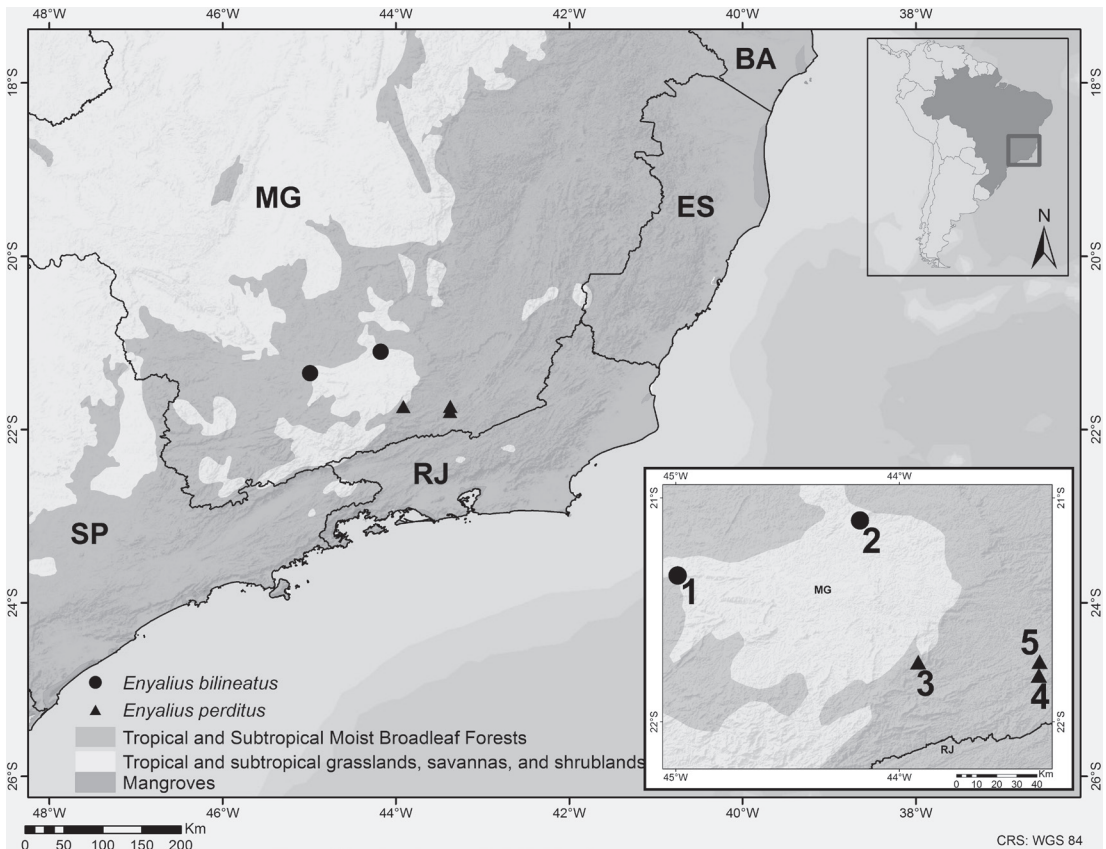


Figure 1. Map showing approximate location of sampling along Minas Gerais state, southeastern Brazil. Localities: 1. RBUB (Ingaí), 2. APASSJ (Tiradentes), 3. PEI (Lima Duarte), 4. PML (Juiz de Fora), 5. APAMK (Juiz de Fora).

reactions. Before sequencing, PCR products were cleaned by precipitation using polyethyleneglicol (20% PEG 8000, 2.5 M NaCl). Forward and reverse sequencing reactions were performed at Embrapa Recursos Genéticos e Biotecnologia (Embrapa Genetic Resources and Biotechnology) using a final volume of 10 μL that contained 2 μL of purified PCR product, 3 μL of ultrapure water, 1 μL of primer (5 μM) and 4 μL of sequencing kit (ET DYE Terminator Kit, GE Healthcare). Sequencing products were initially precipitated with ammonium acetate and ethanol, then dried at room temperature and dissolved with formamide-EDTA before being run in the automatic sequencer MegaBACE 1000 (GE Healthcare).

To generate high quality sequences, we undertook the following measures: (1) sequences longer than 400-bp were amplified; (2) chromatograms were checked carefully for ambiguities; (3) high quality consensus sequences were generated with forward and reverse sequences; and (4) consensus sequences produced for each individual in the present study were aligned and compared among them and with other sequences of species of *Enyalius* obtained from GenBank.

Sequence alignments were performed in MEGA v.6 (Tamura *et al.* 2013) and edited manually if necessary. Sequence divergences among different haplotypes within and among populations from this study and from Rodrigues *et al.* 2014 (GenBank 234-bp *cyt b* matched sequences from *E. perditus*—KM517705.1 to KM517713 and *E. bilineatus*—KM517659.1 to KM517663) were estimated in the same program using an uncorrected *P* distances and Kimura 2-parameter (K2p) substitution model. Haplotypes numbers (H), haplotype (h) and nucleotide diversities (π), the number of polymorphic sites (S), F_{ST} and Φ_{ST} using K2p distance method were calculated with Arlequin version 3.1 (Excoffier and Lischer 2010) only for the populations with $N \geq 3$ (our study). Median Joining (MJ) networks were constructed in the Network 4.6.1.3 (Bandelt *et al.* 1999), to depict the relationships among the 11 haplotypes of *E.*

perditus and the eight of *E. bilineatus* (this study and Rodrigues *et al.* 2014).

Results

Despite amplifying a fragment of 400 bp, only sequences 234 bp long were suitable for consensus sequencing. The 234-bp *cyt b* fragment was analyzed for 20 samples of *Enyalius bilineatus* and 28 of *Enyalius perditus*. Eight novel haplotypes were found, five for *E. perditus* (two for PEI, two for APAMK and one for PML) and three for *E. bilineatus* (two for APASSJ and one for RBUB). We identified seven polymorphic sites in the haplotypes of *E. perditus*; 19 polymorphic sites were discovered in *E. bilineatus* (Table 1 and Figure 2). Figure 2 illustrates the relationship network among the haplotypes and frequencies identified for both species. A minimum of 37 steps was also found between the two groups of haplotypes (data not shown). Figure 2A shows the relationship among the five haplotypes found for individuals from PEI, PML and APAMK that, although not sharing haplotypes, have similar sequences (Table 1 and Figure 2A). Figure 2B shows the divergent haplotypes found in APASSJ and RBUB for *E. bilineatus*. Standard molecular diversity indices for analyzed samples are $h = 0.684$ and $\pi = 0.01015$ for *E. perditus* and $h = 0.647$ and $\pi = 0.04163$ for *E. bilineatus*. The standard diversity indices for each population studied are summarized in Table 2. The Φ_{ST} values for among populations of *E. perditus* range from 0.368–0.700, and 0.988 between the two populations of *E. bilineatus* (Table 3).

When the 14 sequences from 13 locations were included in the analysis (Rodrigues *et al.* 2014), we identified 11 and 8 haplotypes for *E. perditus* and *E. bilineatus*, respectively (Figure 3). Nucleotide divergences among populations for both species, which also included GenBank sequences (matched the 234-bp), are summarized in Tables 4 and 5. Nucleotide divergence between species is 20.30% (17.37% for uncorrected *P* distance) and the mean distances within

Table 1. *Cyt b* polymorphisms and haplotype designations for *Enyalius perditus* (H1 to H5) and *E. bilineatus* (H6 to H8) assayed using 234-bp. The numbers at the top indicate the locations of polymorphic sites within the 234-base sequence.

	Base position																		
<i>E. perditus</i> haplotypes	1	2	3	1	1	1	2												
	2	6	5	7	7	9	1												
				1	6	1	2												
H1	G	A	C	G	A	C	G												
H2	A	A	C	A	G	T	G												
H3	A	A	A	A	G	T	G												
H4	G	G	C	A	A	T	A												
H5	G	G	C	A	A	T	A												
<i>E. bilineatus</i> haplotypes	8	2	8	8	9	1	1	1	1	1	1	1	1	1	1	1	2	2	
		6	3	6	6	0	1	2	3	3	4	5	5	7	8	8	9	0	0
						4	3	2	4	7	3	2	8	3	5	8	7	5	6
H6	T	T	C	T	G	C	T	C	C	A	A	C	T	C	T	T	T	G	A
H7	T	T	C	T	G	C	T	C	C	A	A	C	T	C	T	T	T	G	C
H8	C	C	T	C	A	T	C	T	T	G	C	T	C	T	C	C	C	A	T

Table 2. Number of samples (N), number polymorphic sites (S), absolute haplotype frequencies (H), haplotype (h) and nucleotide (π) diversities for *Enyalius perditus* and *E. bilineatus* populations using 234-bp of the mtDNA gene *cyt b*.

Location	Size of sampled areas (ha)	N	S	H	h	π
PEI (Lima Duarte)	1,488	19	4	2	0.351 ± 0.111	0.00600
APAMK (Juiz de Fora)	292.89	6	4	2	0.600 ± 0.129	0.01026
PML (Juiz de Fora)	88	3	0	1	0	0
Overall <i>E. perditus</i>		28	7	5	0.683 ± 0.081	0.01015
APASSJ (Tiradentes)	4,758	12	1	2	0.409 ± 0.133	0.00175
RBUB (Ingaí)	159.5	8	0	1	0	0
Overall <i>E. bilineatus</i>		20	19	3	0.647 ± 0.057	0.04163

Table 3. Population pairwise F_{ST} based on haplotype frequencies (below diagonal) and Φ_{ST} using K2p distance method (above diagonal) between *Enyalius perditus* and *E. bilineatus* populations (all values were significant with $P < 0.05$).

Location	PEI	APAMK	PML	APASSJ	RBUB
	<i>E. perditus</i>	<i>E. perditus</i>	<i>E. perditus</i>	<i>E. bilineatus</i>	<i>E. bilineatus</i>
PEI (Lima Duarte)	–	0.368	0.700	0.979	0.978
APAMK (Juiz de Fora)	0.571	–	0.601	0.978	0.977
PML (Juiz de Fora)	0.710	0.600	–	0.993	1.000
APASSJ (Tiradentes)	0.628	0.518	0.681	–	0.988
RBUB (Ingai)	0.763	0.741	1.000	0.759	–

species are 2.03% (1.97% for uncorrected P distance) for *E. perditus* and 5.63% (5.20% for uncorrected P distance) for *E. bilineatus* (data not shown).

Figure 3 illustrates the two networks of our data, as well as those of Rodrigues *et al.* (2014), for both species. The first network (Figure 3A) depicts 11 *E. perditus* haplotypes (corresponding 24 polymorphic sites) that are grouped in two divergent clades. The second network shows *E. bilineatus* haplotypes (corresponding 41 polymorphic sites) grouped in, at least, four divergent clades (Figure 3B).

Discussion

The genetic diversity values of the two species of *Enyalius* examined are similar; however, the patterns of genetic structure are distinct. Most species of *Enyalius* have highly restricted habitat preferences (Atlantic Forest), but *E. bilineatus* is more broadly distributed. This species also occurs in open gallery forests of Cerrado and close to coffee plantations (Teixeira *et al.* 2005). *Enyalius bilineatus* also is an older lineage (mean time to the most recent common ancestor around of 6.86 mya) than *E. perditus* (mean time to the most recent common ancestor around of 4.2 mya) (Rodrigues *et al.*

2014); thus, *E. bilineatus* has had a longer time to accumulate genetic variation.

Enyalius perditus

Despite the low sample size ($N = 6$), the population of *E. perditus* from APAMK (the larger intact Atlantic Forest fragment studied here; 292 ha) has the highest haplotype and nucleotide diversity values (Table 2). The mean sequence distance found among populations of *E. perditus* are highly similar (Table 4). We identified an exclusive haplotype from the PML (Juiz de Fora) population; thus, this population likely is isolated from the other populations.

Although more distant (55 Km apart), the mean sequence divergence and the Φ_{ST} between PEI and APAMK (1.2% and 0.37, respectively) are lower than in the two populations of Juiz de Fora (APAMK and PML—1.7% and 0.60) that are only 6.5 Km apart. This absence of shared haplotypes among populations of *E. perditus* may reflect a sampling artifact, given that the sample sizes for PML and APAMK are low ($N = 3$ and $N = 6$, respectively). Thus, it is possible that our sampling may not represent overall population diversity. Based on a comparison of overall diversity for *E. perditus* (Figure 3), two divergent clades are apparent. Our samples are

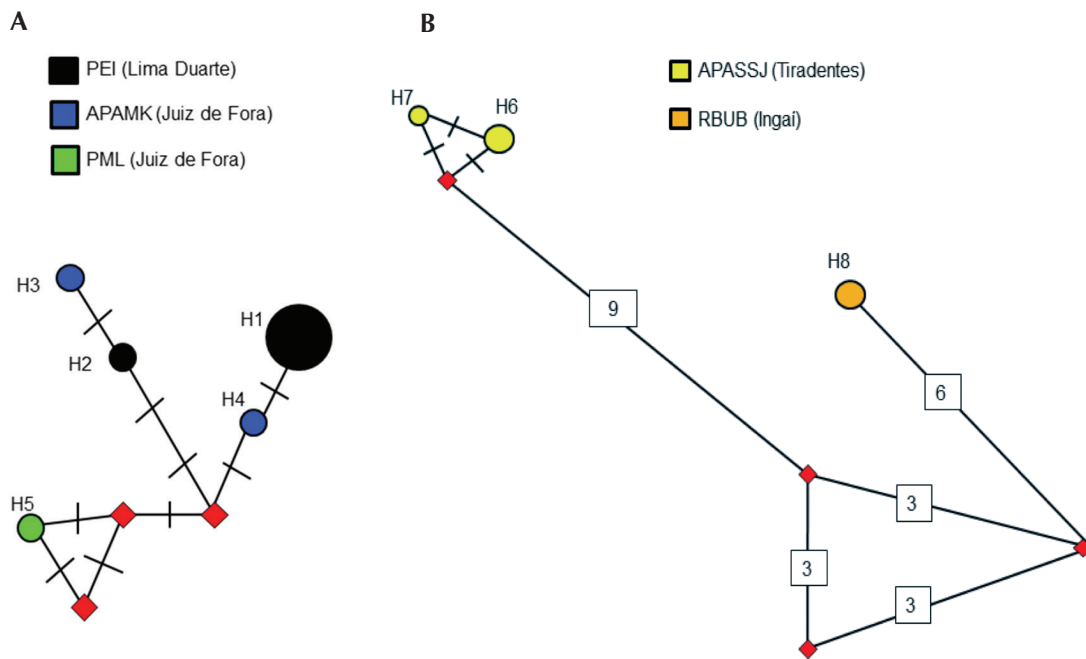


Figure 2. MJ networks describing the relationship among the *Enyalilus* mtDNA haplotypes (234-bp). The relationship among the five haplotypes of *E. perditus* is shown on the left (A), that of the haplotypes of *E. bilineatus* is on the right (B). H1 to H8 represents the Haplotypes 1–8. The median vectors are represented by the red diamonds. The values inside the squares are mutational points. The sizes of the circles size represent the frequencies of each haplotype.

more similar to those sequences reported by Rodrigues *et al.* (2014) for clade “Eper2.” Thus, the inclusion of samples of *E. perditus* from other localities from Minas Gerais did not clarify the putative paraphyly of the species (Rodrigues *et al.* 2014). The “Eper1” clade is associated with the coastal areas of the state of Sao Paulo; thus, additional samples should be collected from these areas, and from transitional areas between coastal and inland areas in the state of Rio de Janeiro.

Enyalilus bilineatus

Enyalilus bilineatus from APASSJ has the highest diversity values (Table 2). The samples from both RBUB and APASSJ were identified as

E. bilineatus; however, the populations did not share any haplotypes, and they differed by 19 mutational steps (Figure 2).

The mean uncorrected sequence distance of 8.8% between the two populations of *Enyalilus bilineatus* is greater than expected for divergence values within a single species. The lack of shared haplotypes, the many mutational steps, the high level of F_{ST} (0.759) and Φ_{ST} (0.988), and the high genetic distance between these two populations of *E. bilineatus* suggests that there is great genetic divergence within the same species. Furthermore, comparison of our sequences with those of Rodrigues *et al.* (2014) yields a sequence divergence value of 14.5% between samples from Brasilia (DF) and Mariana (MG). The two haplotypes found at APASSJ are also divergent

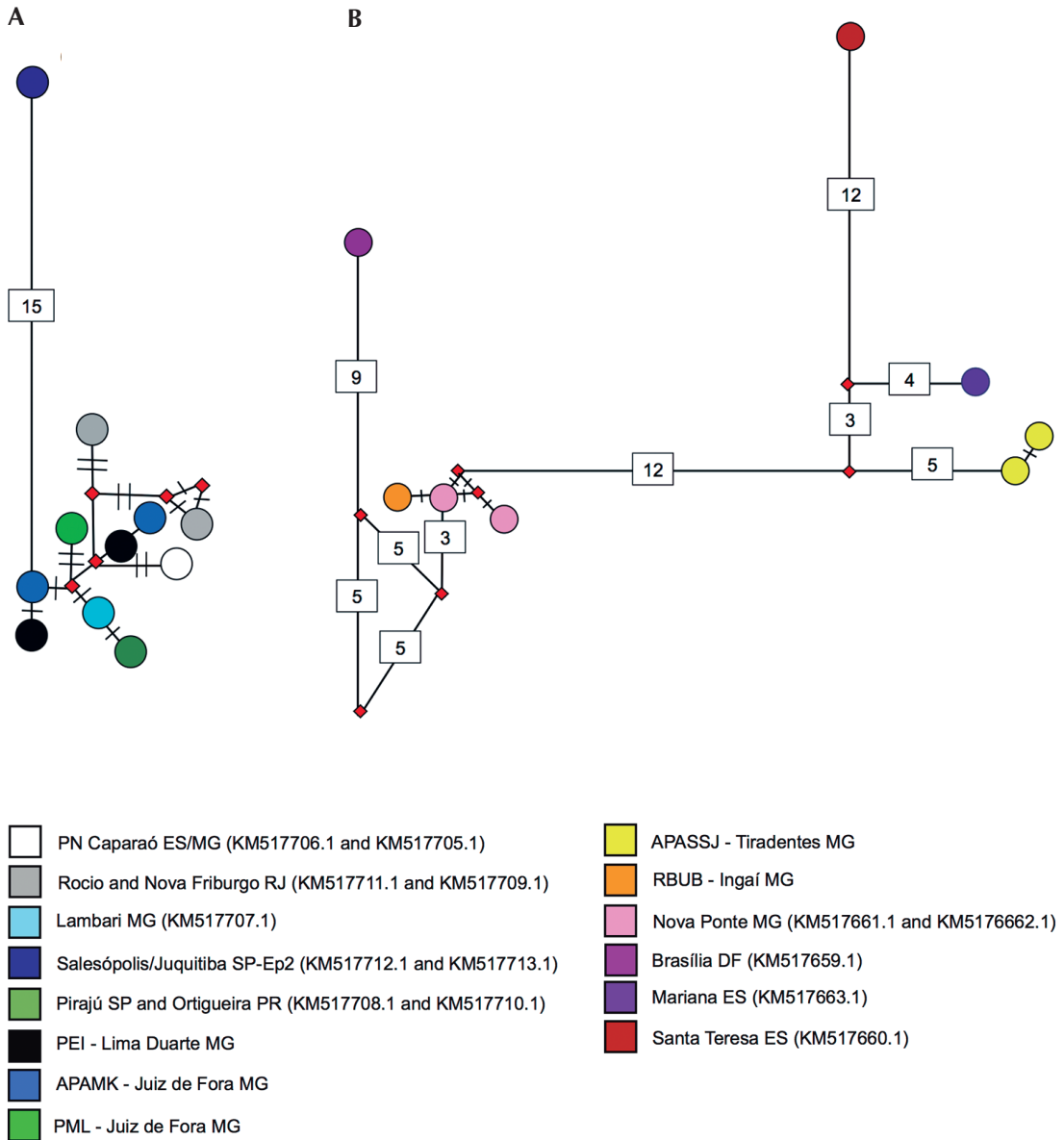


Figure 3. MJ networks describing the relationship among the *Enyalius* mtDNA haplotypes (234-bp). The relationship among the 11 haplotypes of *E. perditus* is shown on the left (**A**), and that of the eight haplotypes of *E. bilineatus* is on the right (**B**; this study and Rodrigues *et al.* [2014]). The median vectors are represented by the red diamonds. The values inside the squares are the mutational points. The sizes of the circles size do not reflect the frequencies of samples from each haplotype.

Table 4. Mean sequence divergence between 12 pairs of populations of *Enyalius perditus* and within populations (diagonal – underlined numbers), from this study and Rodrigues *et al.* (2014). Above diagonal - uncorrected *P*-distance and below diagonal - corrected K2p.

<i>E. perditus</i> populations	1	2	3	4	5	6	7	8	9	10	11	12
1 - PEI - Lima Duarte MG	<u>0.006</u>	0.012	0.017	0.020	0.020	0.013	0.010	0.028	0.010	0.032	0.070	0.070
2 - APAMK - Juiz de Fora MG	0.012	<u>0.010</u>	0.017	0.017	0.017	0.013	0.017	0.026	0.017	0.028	0.073	0.073
3 - PML - Juiz de Fora MG	0.017	0.017	<u>0.000</u>	0.021	0.021	0.013	0.017	0.030	0.017	0.034	0.068	0.068
4 - Caparaó ES	0.020	0.017	0.022	<u>0.000</u>	0.000	0.017	0.021	0.026	0.021	0.030	0.073	0.073
5 - Caparaó MG	0.020	0.017	0.022	0.000	<u>0.000</u>	0.017	0.021	0.026	0.021	0.030	0.073	0.073
6 - Lambari MG	0.013	0.013	0.013	0.017	0.017	<u>0.000</u>	0.004	0.026	0.004	0.030	0.073	0.073
7 - Ortigueira SP	0.010	0.017	0.017	0.022	0.022	0.004	<u>0.000</u>	0.030	0.000	0.034	0.077	0.077
8 - Rocio RJ	0.029	0.026	0.031	0.026	0.026	0.026	0.031	<u>0.000</u>	0.030	0.021	0.085	0.085
9 - Pirajú SP	0.010	0.017	0.017	0.022	0.022	0.004	0.000	0.031	<u>0.000</u>	0.034	0.077	0.077
10 - Nova Friburgo RJ	0.033	0.028	0.035	0.031	0.031	0.031	0.035	0.022	0.035	<u>0.000</u>	0.081	0.081
11 - Juquitiba SP	0.074	0.077	0.072	0.077	0.077	0.077	0.082	0.092	0.082	0.087	<u>0.000</u>	0.000
12 - Salesópolis SP	0.074	0.077	0.072	0.077	0.077	0.077	0.082	0.092	0.082	0.087	0.000	<u>0.000</u>

(5.5% and 12 mutational steps) from the haplotype found for Mariana (MG), despite the populations being separated by only 120 Km (Table 5).

The least genetic divergence found in populations of *Enyalius bilineatus* (0.9%) is between RBUB and Nova Ponte (MG) sequences (Table 5). Although these populations are separated by more than 380 Km, the individuals sampled occupy similar open areas of Cerrado. These individuals differ from the other clade, which comprises samples from eastern areas of high altitude near or along the Atlantic Forest (Figure 3B).

It is apparent that *Enyalius bilineatus* probably is composed of two major lineages that are associated with different habitats rather than geographic distance—one is associated with the

open areas of Cerrado, whereas the other occupies high-altitude environments in and around Atlantic Forest fragments. In addition, we also noted greater divergence among haplotypes within each clade than for *E. perditus* (Figure 3B).

The genetic divergence values identified among populations of *Enyalius bilineatus* (Table 5) are higher than those among individual lizards reported in studies that used the same *cyt b* marker. For example, Thorpe *et al.* (2005) reported 5.2–5.7% sequence divergence within *Anolis extremus* in Barbados. Others studies have documented values of uncorrected genetic distances from 4.2–6.5% for three Fijian iguana (*Brachylophus*) species (Keogh *et al.* 2008) and from 0.0–3.57% for species of *Uma* in North America (Trépanier and Murphy 2001). However, one study of scrub lizards (*Sceloporus*

Table 5. Mean sequence divergence between six pairs of populations of *Enyalius bilineatus* and within populations (diagonal – underlined numbers), from this study and Rodrigues *et al.* (2014). Above diagonal - uncorrected *P*-distance and below diagonal - corrected K2p.

<i>E. bilineatus</i> populations	1	2	3	4	5	6
1 - APASSJ - Tiradentes MG	<u>0.002</u>	0.088	0.111	0.084	0.079	0.052
2 - RBUB - Ingaí MG	0.081	<u>0.000</u>	0.077	0.073	0.009	0.090
3 - Brasilia DF	0.124	0.083	<u>0.009</u>	0.103	0.077	0.128
4 - Santa Teresa ES	0.092	0.079	0.114	<u>0.000</u>	0.071	0.068
5 - Nova Ponte MG	0.085	0.009	0.083	0.076	<u>0.000</u>	0.088
6 - Mariana MG	0.055	0.098	0.145	0.073	0.095	<u>0.000</u>

woodi) in Florida reported a genetic distances of 1–10% (Clark *et al.* 1999); the authors questioned the taxonomic status of this species, given the evolutionary partitions evident in the data.

This study is the first to document the genetic diversity and structure of two species of *Enyalius*. The results suggest that populations of *Enyalius bilineatus* are genetically divergent and contain cryptic diversity, thereby confirming the conclusions of Rodrigues *et al.* (2014). The divergence reflects the habitat preferences of the species (open areas of Cerrado vs. Atlantic Forest fragments). We also found abundant variation among haplotypes within clades, suggesting the existence of more hidden diversity than previously documented.

Complementary studies on the ecology, behavior, and morphology—as proposed by Pellegrino *et al.* (2005) for species limits in the *Gymnodactylus darwini* complex, should be undertaken to determine whether (1) the differences reported herein are associated with other biological aspects of this species, or if (2) they are unique to the molecular marker used in this study. When viewed in an ecological context, our results will contribute to a better understanding of the taxonomic and conservation status of *Enyalius*.

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