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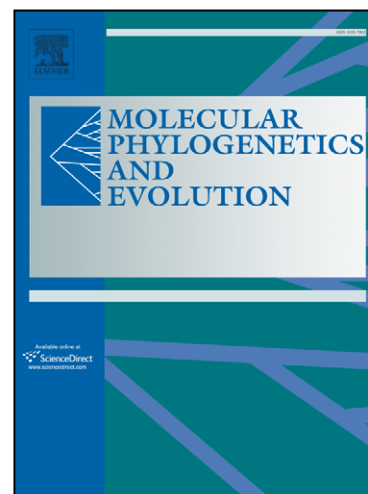

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Phylogeny, species limits, and biogeography of the Brazilian lizards of the genus *Eurolophosaurus* (Squamata: Tropiduridae) as inferred from mitochondrial DNA sequences

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Abstract

Phylogenetic relationships and divergence times for ten populations of the three recognized "species" of Brazilian lizards of genus *Eurolophosaurus* were estimated from 1,229 bp of cyt *b*, COI, 12S and 16S rRNA mitochondrial gene segments. *Eurolophosaurus* is monophyletic and the basal split within the genus separates *E. divaricatus* from a clade comprising *E. amathites* and *E. nanuzae*. Three populations of *E. divaricatus,* which occurs along the western bank of Rio São Francisco, were consistently grouped together. On the east bank of the river, *E. amathites* and *E. nanuzae* from state of Bahia were recovered as the sister group of *E. nanuzae* populations from state of Minas Gerais. The paraphyly of *E. nanuzae* and the high divergence levels among populations of *E. divaricatus* strongly suggest that species limits in *Eurolophosaurus* should be revised. Even considering an extreme evolutionary rate of 2.8% sequence divergence per million years for the four gene segments analyzed together, *E. divaricatus* would have separated from the two other species by at least 5.5 my ago, and *E. amathites* from *E. nanuzae* populations from Bahia and Minas Gerais, respectively by 1.5 my and 3.5 my. The paleolacustrine hypothesis and changes in the course of the river potentially explain faunal divergence in the area, but divergences are much older than previously admitted.

Keywords: cyt *b*; COI; 12S; 16S; mtDNA phylogeny; species limits; biogeography; divergence times; *Eurolophosaurus*; Tropiduridae; Rio São Francisco sand dunes; Brazil.

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1. Introduction

The tropidurid lizard genus *Eurolophosaurus* (Frost et al., 2001) is a monophyletic group of three species formerly called the *Tropidurus nanuzae* group (Rodrigues, 1986). It occurs along the Serra do Espinhaço, a large mountain ridge extending along eastern Brazil in the states of Minas Gerais and Bahia, and in sandy areas close to the banks of the middle Rio São Francisco in Bahia (Fig. 1). *E. nanuzae* is predominantly saxicolous and occurs in several localities throughout the Serra do Espinhaço, from Serra do Cipó in the southern part of Minas Gerais to Rio de Contas and Caetité, in Bahia, always at altitudes near or above 900 m. The other two species are psammophilous and occupy opposite banks of Rio São Francisco below 600 m, close to the northern portion of Serra do Espinhaço in the state of Bahia. *E. amathites* is found at Santo Inácio, Gameleira do Assuruá and Lagoa de Itaparica, on the sands of the right bank of the river, and *E. divaricatus* in Queimadas, Ibiraba, Mocambo do Vento, Manga and Alagoado, on the opposite margin, in two dune fields separated by an area of rocky soils approximately 150 km long. Additional data on distribution, physiography and ecology of the group have been reported by Rodrigues (1986, 1996).

According to morphological and distributional data, Rodrigues (1986) suggested that *E. nanuzae* (at the time in genus *Tropidurus*) was the sister taxon to a clade comprising *E. amathites* and *E. divaricatus*. Based on morphology, Frost (1992) placed *E. nanuzae* outside a clade comprising the rest of the *Tropidurus* radiation (*E. amathites* and *E. divaricatus* were not sampled in this study). This was the reason why, in a later paper on tropidurid phylogeny using both morphological and molecular characters, the genus *Eurolophosaurus* was created (Frost et al., 2001).

The chromosomal studies of Kasahara et al. (1987) detected a nucleolar organizer region (NOR) on the long arm of macrochromosome pair 6 in *E. amathites* and *E. nanuzae*, but on the

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short arm of the same chromosome pair in *E. divaricatus*. As NORs are absent from this chromosome pair in phylogenetically related tropidurids, this character was used to support the monophyly of the group and a pericentric inversion was proposed to explain the condition observed in *E. divaricatus* (Kasahara et al., 1983, 1985, 1986 a, b). Frost et al. (2001) considered location of NOR on chromosome 6 and the XXAA:XAY/A sexual determination type (Kasahara et al., 1987) as synapomorphies of the genus *Eurolophosaurus*. In allozymic analyses (Martins, 1995), however, monophyly of the *nanuzae* group could not be demonstrated with confidence. Finally, in the study of Frost et al. (2001) monophyly of *Eurolophosaurus* was corroborated, and *E. divaricatus* was shown as the sister taxon to a clade comprising *E. amathites* and *E. nanuzae*.

Based on geomorphological and paleoclimatic data on the São Francisco sand-dunes (Ab'Saber, 1969; Tricart, 1974), and assuming the close relationship of *E. amathites* and *E. divaricatus*, Rodrigues (1986) proposed a vicariant model of speciation to explain their origin. He hypothesized an ancestral population bisected by the Rio São Francisco after the last glacial period, about 12,000 years ago. According to the model, in the Pleistocene the river flowed into a paleolake and, at the end of the Würm-Wisconsin glaciation it would have flowed to the Atlantic Ocean. Other psammophilous endemic pairs of closely related species of gymnophthalmid lizards, snakes and amphisbaenians, which occur in the area and show the same vicariant pattern of geographic distribution, lend support to the model (Rodrigues, 1991 a, b, c, d, 1996, 2003). More recently it was also argued that, although the paleolacustrine hypothesis could account for the speciation in the area, forsaken meanders of the river may have acted as barriers (Rodrigues, 1996). Palaeoclimatic and geomorphological data on this region support the model (Souza-Lima et al., 2005). Nevertheless, allozymic data (Martins, 1995)

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suggested that the *E. amathites*/*E. divaricatus* divergence should be much older, of the order of millions of years, and not of 12,000 years as originally proposed.

To obtain phylogenetic relationships and divergence times between and within species of *Eurolophosaurus,* based on DNA sequencing data, we analyzed mtDNA sequences for populations of these three species.

2. Materials and methods

2.1. Taxonomic sampling and DNA extraction

Ten populations of the three species of *Eurolophosaurus* and three outgroup species were sampled and assayed for regions from mitochondrial genes encoding 12S and 16S ribosomal RNAs (rRNA), cytochrome *b* (cyt *b*) and subunit I of cytochrome *c* oxidase (COI) proteins. A total of 25 specimens was used. Technical problems in DNA amplification and sequencing precluded the inclusion of a larger number of individuals (see *2.2*). Table 1 shows the sample sizes that we had as a result. The geographic distribution of populations studied is shown in Fig. 1. Samples of *E. divaricatus* from Queimadas and Ibiraba were considered the same population (Queimadas/Ibiraba) according to Passoni et al. (2000). One specimen of *Uranoscodon superciliosus*, one of *Microlophus quadrivittatus* and one of *Tropidurus hispidus* were used as outgroups, according to Frost et al. (2001). Heart, liver and portions of skeletal muscle were stored at –120 ^{o}C or –196 ^{o}C. DNA enriched in the mitochondrial fraction was obtained by the methods of Hillis and Davis (1986) and Dowling et al. (1990), as modified by Passoni et al. (2000). Approximately 2-4 mm^3 of these tissues from each specimen were homogenized in 800 µl STES (0.01 M NaCl, 0.01 M Tris, 0.1 M EDTA, 0.25 M sucrose, pH=7.5) and then centrifuged for 5 min at 1,200 g at 4 ^{o}C to pellet the nuclei. The supernatant

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was centrifuged at 23,000 g for 20 min at 4 ^{o}C for mitochondrial precipitation. The pellet was suspended in 250 µl STE (0.1 M NaCl, 0.01 M Tris, 0.1 M EDTA, pH=7.5), with 1% sodium dodecyl sulfate (SDS) and 10 U/ml proteinase K. After incubation for 2 h at 55 ^{o}C, the preparation was extracted with one volume of phenol, and then with one volume of 24:1 chloroform: isoamyl alcohol solution. DNA was precipitated from the supernatant by the addition of 1/10 volume buffer (3 M NaCl, 0.25 M Tris, 0.1 M EDTA) and 2.5 volumes 100% ethanol at –20 ^{o}C overnight. The DNA pelleted after 15 min centrifugation at 23,000 g at 4 ^{o}C was washed in 70% ethanol, air dried and dissolved in 20-80 µl TE (0.01 M Tris, 0.001 M EDTA, pH=8.0), and stored at –20 ^{o}C.

2.2. Amplification and sequencing

DNA amplification and sequencing trials presented many technical problems hindering the analysis of the desirable number of individuals for each of *Eurolophosaurus* populations. Therefore, we decided to limit the present analysis to phylogenetic issues. Segments of 12S and 16S rRNA genes, and segments of cyt *b* and COI protein-coding genes of the mitochondrial genome were amplified via polymerase chain reaction (PCR) in 25 or 50 µl reaction volumes containing 20-30 ng DNA, 1x PCR buffer, 4 mM $MgCl_2$, 0.2 mM each dNTP, 1 µM each primer, and 2.5 U AmpliTaq DNA Polymerase (Perkin-Elmer, Roche). Amplification primers and thermocycler conditions are specified on Table 2. Negative controls were made to avoid contamination. Amplification products were checked by electrophoresis on 0.8% agarose gel with 0.4 µg/ml ethidium bromide (the target fragment size was estimated from molecular-weight markers). Products were cleaned with Exonuclease and Shrimp Alkaline Phosphatase (Amersham Pharmacia Biotech) per 1h at 37 ^{o}C and, subsequently, per 10 min at 80 ^{o}C. The

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Concert Rapid PCR Purification System (Gibco BRL-Life Technologies) was also used with some samples. In cases of non-specific amplifications, the Concert Rapid Gel Extraction System (Gibco BRL-Life Technologies) was used. Purified PCR products containing about 100 ng DNA were utilized for sequencing using the Big Dye Terminator Ready-Reaction Kit (Perkin-Elmer Applied Biosystems). A Perkin Elmer-2400 thermocycler was used with the following parameters: 96 ^{o}C (0:10), 50 ^{o}C (0:05), 60 ^{o}C (4:00) X 25 (0:05 at 96 ^{o}C before first cycle). Both strands of each segment were sequenced for each specimen. An ABI PRISM 310 Genetic Analyzer (Perkin-Elmer Applied Biosystems) was used and the sequences were edited using Sequence Navigator 1.0.1 (Perkin-Elmer Applied Biosystems).

2.3. Phylogenetic analyses

Multiple alignments were accomplished using Clustal X 1.64b (Thompson et al., 1997). For *12S* and *16S* segments, gap-opening values of 6, 8, 10 and 12, and a gap-extension value of 0.05 were used to assess the effects on sequence alignment according to Gatesy et al. (1993), Wiens and Reeder (1997) and Reeder and Montanucci (2001). Regions of ambiguous alignments were excluded. 12S rRNA secondary structure models for a scincid lizard (Hickson et al., 1996), for the lizard family Opluridae (Titus and Frost, 1996) and for hyperoliid treefrogs (Richards and Moore, 1996), and the 16S rRNA secondary structure models for humans (Gutell and Fox, 1988) and for mammals (Burk et al., 2002) were considered. Cyt *b* and COI protein-coding gene sequences were translated into amino acid sequences to check for unexpected occurrences of stop codons, which might indicate that pseudogenes had been amplified (Sorenson and Fleischer, 1996; Zhang and Hewitt, 1996). The degree of saturation in each gene segment was investigated by plotting transitions and transversions against Lake 94 distances employing DAMBE program (Xia, 2000; Xia and Xie, 2001). In the case of protein-coding

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segments, these analyses were accomplished considering all codon positions, the first and second codon positions together, and each position separately.

Maximum parsimony (MP) analyses based on all individuals of Table 1 along with molecular (Martins, 1995; Passoni et al., 2000) and morphological data (Rodrigues, 1986, 1996; Skuk, 1994) have evidenced monophyletic population units in *Eurolophosaurus* (see *3.2*). Therefore, the four gene segments of one or two individuals of each monophyletic population unit could be used for main phylogenetic analyses, as follows.

The partition homogeneity test (Farris et al., 1994) using PAUP* (1,000 replicates) indicated that the four gene regions could be analysed together. Phylogenetic signal was detected for the combined data set using the g_1 statistic (Sokal and Rohlf, 1981; Hillis, 1991; Huelsenbeck, 1991; Hillis and Huelsenbeck, 1992), which measured the skewness of the distribution of 10,000 random cladograms. Phylogenetic analyses were conducted on ribosomal sequences and protein-coding sequences separately, and on combined mitochondrial data for all populations, using maximum parsimony (MP), maximum likelihood (ML), and Neighbor Joining (NJ) methods implemented in PAUP* 4.0b10 (Swofford, 2002). Parsimony trees were constructed using a branch-and-bound search, and the confidence tested by 1,000 bootstrap replicates (Felsenstein, 1985). Due to saturation on third codon positions, MP analyses were done considering two different weightings for those characters, 0 and 1. In the case of the ribosomal segments, gap was treated as a fifth character state. For ML heuristic searches and NJ tree estimates, the evolutionary model that best fits each data set was selected employing PAUP* and MODELTEST 3.06 (Posada and Crandall, 1998) programs. The substitution model for all four concatenated segments was found to be the General-Time-Reversible model (GTR+Γ+I; Rodríguez et al., 1990). In ML analyses, tree bisection-reconnection (TBR) branch

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swapping, MULTREES, and random addition of sequences (100 replicates) were employed. Bootstrap values (BVs) were estimated by 100 replicates with 10 replicates of random addition of sequences. In NJ analyses, BVs were calculated by 1,000 replicates. The interpretation of BVs is still a controversial issue. We followed Shaffer et al. (1997): BVs ≥ 90% were considered as highly significant values; 70 ≤ BVs < 90% as marginally significant proportions, and 50% ≤ BVs < 70% as suggesting limited evidence of monophyly. For discussion purposes, we consider only highly significant BVs.

2.4. Divergence time analyses

Absence of fossils and geomorphological records precludes independent testing of divergence times for the *Eurolophosaurus* radiation. Previous molecular divergence time estimates were based only on a reduced sample of allozymic loci (Martins, 1995). Therefore, in the present *Eurolophosaurus* evolutionary history context, it is worthwhile determining time intervals in order to have at least rough date estimates and also to test the vicariant hypothesis of 12,000 years ago. With this purpose, the corrected genetic distances based on the gene sequences of the main phylogenetic analyses were used.

The hypothesis of rate constancy was tested by the likelihood-ratio test (Muse and Weir, 1992) for each gene sequence separately, for ribosomal sequences, for protein-coding sequences, and for all mitochondrial sequences. This test compares the log likelihood (ln L) of a tree using an assumption of clock-like evolution with the log likelihood of a tree calculated without this assumption (P=0,05). PAUP* and MODELTEST were used to select the most appropriate model of evolution for each data set and PAUP* was used to calculate each log likelihood value. Evolutionary rate constancy was detected only for cyt *b*, 16S and the four

concatenated gene segments.

Since we obtained the first considerable amounts of mtDNA sequence data for *Eurolophosaurus* populations, we have an opportunity to obtain secure time limits for the evolutionary history of this genus. The highest rates of molecular evolution found in literature, along with lower rates, were applied for the combined data set. To allow comparison with published data, the Kimura Two-Parameter (K2P) model (Kimura, 1980) was used to correct the cyt *b* genetic distances.

3. Results

3.1. Molecular characterization

About 1,360 mtDNA base pairs (bp) were sequenced for 22 specimens of *Eurolophosaurus* and the three outgroup individuals. Appendix I shows the aligned 12S DNA sequences used in the main phylogenetic analyses, with the identified stem and loop regions, from which 33 ambiguously aligned characters were removed. Seven insertion/deletion events - indels - (characters 123, 124, 128, 129, 189, 194 and 275) could be precisely aligned in 12S sequences which, along with others, did not disrupt the RNA secondary structure. For 16S ribosomal sequences (Appendix II), a region of 98 positions was excluded due to ambiguous alignment. Indels in 16S DNA sequences, six of which could be unambiguously aligned (characters 16, 17, 317, 331, 355 and 365) were all located in unpaired regions. Cyt *b* and COI protein-coding gene sequences contained no indels. The resulting phylogenetic data matrix was composed of 282 characters of 12S rRNA gene, 389 characters of 16S rRNA gene, 209 bp of cyt *b* and 349 bp of COI genes. These sequences, used in the main phylogenetic analyses, have been deposited in GenBank under accession numbers: cyt *b* (**DQ848725** to **DQ848735**); COI (**DQ848747** to **DQ848757**); 12S (**DQ848758** to **DQ848768**); 16S (**DQ848736** to **DQ848746**).

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The remaining sequences, used in other analyses, have been deposited in GenBank under accession numbers: cyt *b* (**DQ848713** to **DQ848724**); COI (**DQ848769** to **DQ848772**); 12S (**DQ848773** to **DQ848776**); 16S (**DQ848777** and **DQ848778**). Several observations indicate that the sequences analyzed here correspond to functional mitochondrial genes (see Zhang and Hewitt, 1996). Protein-coding sequences do not have premature stop codons, and ribosomal sequences apparently code for rRNAs with stable secondary structures. Nucleotide frequencies shown in Table 3 were estimated from all sequences referenced in Table 1. The bias against guanine on light strands of the protein-coding sequences and the bias against guanine and thymine on light strands of the ribosomal sequences support the conclusion that the sequences do not represent nuclear-integrated copies of mitochondrial genes (e.g. Zhang and Hewitt, 1996; Macey et al., 1997, 1999; Honda et al., 2000a, b; Tu et al., 2000; Ast, 2001; Daniels et al., 2002; Schulte II et al., 2002).

3.2. Phylogenetic relationships

Our data diagnose at least six monophyletic population units in *Eurolophosaurus*. Although our cladograms based on all cyt *b* and COI sequences taken separately do not present any phylogenetic resolution among populations, they group with BVs of 97-100% (1,000 replicates; branch-and-bound search) sequences representing each of the following population units: 1) *E. divaricatus* of Alagoado, 2) *E. divaricatus* of Queimadas/Ibiraba/Mocambo do Vento, 3) *E. amathites* of Santo Inácio/Gameleira do Assuruá, 4) *E. nanuzae* of Caetité/Rio de Contas, 5) *E. nanuzae* of Serra do Cipó and 6) *E. nanuzae* of Pedra Menina. These results corroborate inferences from morphological data (Rodrigues, 1986, 1996; Skuk, 1994), an allozymic survey (Martins, 1995) and a mtDNA RFLP study (Passoni et al., 2000). These data collectively include a comprehensive survey of all known *Eurolophosaurus* populations as

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diagnosed by morphological and molecular phylogenetic characters. In other words, there are characters that diagnose each of six well differentiated population units that show reciprocal monophyly for mtDNA haplotypes (Avise, 1994).

The main phylogenetic inferences were conducted with the two rRNA gene sequences (671 characters), with cyt *b* and COI protein-coding sequences (558 bp), and with the concatenated data including all four genes. The partition homogeneity test (Farris et al., 1994) detected no significant heterogeneity among the four DNA segments, and the g_1 statistic (Hillis and Huelsenbeck, 1992) for the total data matrix showed a value of -0.728, therefore indicating the presence of phylogenetic signal. Thus, most of the mtDNA analyses were based on the combined data, which include 1,229 unambiguously aligned sites, with 204 of the 318 variable sites being informative under parsimony. Graphical analyses of transition/transversion substitutions at third codon positions plotted against Lake 94 distances for all cyt *b* and COI mitochondrial sequences indicate some saturation in both segments. No other evidence for saturation was detected at protein-coding gene sequences. At 12S and 16S ribosomal gene sequences no saturation was detected in nucleotide transitions or transversions.

A single most parsimonious tree was obtained with the four concatenated gene segments, taking *Uranoscodon superciliosus*, *Microlophus quadrivittatus* and *Tropidurus hispidus* as outgroups, and two different weightings (1 and 0) for third codon positions. Fig. 2 shows the resulting cladogram with bootstrap supports. In Table 4, the step numbers, CI and RI values are presented for both different weightings. High bootstrap supports were obtained for all ingroup clades, except for the clade of *E. divaricatus* haplotypes from Queimadas/Ibiraba and Mocambo do Vento when third codon positions were eliminated (BV=61%). The General Time-Reversible (GTR+Γ+I) model (Rodríguez et al., 1990) was identified by MODELTEST as the best

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nucleotide-substitution model for mtDNA combined data, considering *Eurolophosaurus* and outgroup taxa. The estimated gamma distribution shape parameter was 0.7270 and the proportion of invariable sites was 0.5297. ML and NJ analyses produced the same phylogenetic relationships, also supported by high bootstrap values (Fig. 2). When ribosomal and protein-coding sequences were analyzed separately the same topology was also obtained, although with different bootstrap supports for some nodes. Monophyly of *Eurolophosaurus* was significantly supported by ribosomal data under MP and ML methods, respectively with 91% and 92% of bootstraps.

Two major clades were recovered in all phylogenetic analyses. One is composed of the populations of *E. divaricatus*, the species found on the left margin of Rio São Francisco in the state of Bahia, with highly significant BVs (see Fig. 2). The other monophyletic group is composed of the two species on the right margin, *E. amathites* and *E. nanuzae*, with 92% to 100% BVs. In this group, two other clades are evidenced. In one of them, *E. amathites* is shown as the sister taxon to *E. nanuzae* populations from Caetité and Rio de Contas, in the state of Bahia. This clade is consistently supported by BVs of 90% to 100%, except for ribosomal sequence analyses (76% to 89%). The other clade is composed of *E. nanuzae* populations from the state of Minas Gerais (Pedra Menina and Serra do Cipó) with BVs of 100%. Therefore, *E. nanuzae* is consistently shown as a paraphyletic species. *E. divaricatus*, on the left margin of the Rio São Francisco, also shows two well differentiated clades, the population of Alagoado, in the northern dune field, and the populations of Queimadas/Ibiraba and Mocambo do Vento, on the south dunes, with high bootstrap supports, except for MP analyses with the four concatenated sequences excluding third codon positions, and for ribosomal analyses (see Fig. 2).

3.3. Divergence times

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The hypothesis of evolutionary rate constancy was tested (Muse and Weir, 1992) for each gene sequence separately, for ribosomal sequences, for protein-coding sequences, and for the concatenated four gene sequences, with the best molecular evolution model selected in each case. Evolutionary rate constancy was statistically accepted (P=0.05) only for cyt *b*, 16S and the four concatenated gene sequences. Table 5 shows pairwise distances among *Eurolophosaurus* populations and outgroup taxa estimated for the concatenated gene sequences with the GTR+Γ+I substitutional model, and also for cyt *b* sequences with the K2P model, to allow comparisons with the literature.

Studies on mtDNA evolution among vertebrates indicate a rate of approximately 2% sequence divergence per million years (Upholt and Dawid, 1977; Brown et al., 1979). Deviations from this "conventional" rate have been reported, and have been correlated with generation length, metabolic rate and body size of the organisms (Martin and Palumbi, 1993; Li, 1997). Avise et al. (1992), based on restriction assays of Testudines, noted up to 2-14-fold slowdown deviations from the universal mtDNA molecular clock of 2%/my. On the other hand, rates from 1.7 to 2.85% were considered for the Iberian lizard *Lacerta schreiberi* (Paulo et al., 2001). Carranza et al. (2000) used a rate of 1.96%/my for concatenated sequences of cyt *b* and 12S rRNA genes in *Tarentola* genus (Gekkonidae), based on similar rate estimated for *Gallotia galloti* of the Canary Islands (Thorpe et al., 1994; González et al., 1996). Those lizards are all similar in body size to *Eurolophosaurus*. Benozzati and Rodrigues (2003), based on RFLP analyses of gymnophthalmid lizards, endemic from the dune fields of the Rio São Francisco, also considered a sequence divergence of 2%/my rate in their evolutionary studies. Based on those references, a sequence divergence of 2%/my and the maximum rate of 2.8%/my (Paulo et al., 2001) were considered for divergence-time estimations in *Eurolophosaurus*. As those rates

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were the highest we found in literature, they were used to determine minimal ages for vicariant events in the group. Table 6 shows time estimations for *Eurolophosaurus* divergence events with both molecular clocks. According to those data, *E. divaricatus* would have diverged from the two other species by 5.4 to 9.5 my, and *E. amathites* from *E. nanuzae* by 1.5 to 5.2 my. *E. amathites* was shown to be closer in time to *E. nanuzae* populations from Bahia (1.5 to 2.1 my) than to those from Minas Gerais (3.5 to 5.2 my). Divergence times of 2.1 to 2.9 my have been estimated for *E. divaricatus* populations living in different dune fields, on the left margin of the river. *E. nanuzae* populations of Bahia would have diverged from those of Minas Gerais by 3.5 to 5.1 my. Divergence between Pedra Menina and Serra do Cipó populations (Minas Gerais) was estimated at 1.2 to 1.7 my. The Tropidurinae ancestor would date from approximately 9.8 to 13.7 my. Separation of *Eurolophosaurus* and *Microlophus quadrivittatus* would date from 8.4 to 11.7 my, and the separation between *Tropidurus hispidus* and *Eurolophosaurus*, from 7 to 9.8 my. The maximum likelihood tree of Fig. 3 illustrates minimal divergence times, estimated with an evolutionary rate of 2.8%/my.

4. Discussion

4.1. Phylogenetic relationships and taxonomic changes

Our study supports monophyly of *Eurolophosaurus* and places *E. divaricatus* as the sister taxon to a clade comprising *E. amathites* and *E. nanuzae*, as previously proposed by Frost et al. (2001). The phylogenetic relationships recovered for ten populations of *Eurolophosaurus* including representatives of the three recognized "species" were strongly supported. The three populations of *E. divaricatus*, the species restricted to the left bank of the river, were consistently grouped together. This grouping was reinforced by the discovery of a precisely aligned gap in Helix 42 of 12S ribosomal RNA secondary structure (position 189 in Appendix

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I), present only in those three populations. Populations of *E. amathites*/*E. nanuzae*, all restricted to the right bank of the river, were also strongly supported as a monophyletic group and recovered as the sister group to *E. divaricatus*. Our analyses demonstrate that *E. amathites* and the populations of *E. nanuzae* from Bahia (Caetité and Rio de Contas) form a monophyletic group, which is recovered as the sister group of that formed by the populations of *E. nanuzae* from Minas Gerais (Pedra Menina and Serra do Cipó). Paraphyly of *E. nanuzae* and high divergences found among populations of *E. divaricatus* strongly suggest that species limits in *Eurolophosaurus* should be revised, because present taxonomy of the group is inconsistent with recovered relationships. Contrary to Rodrigues (1986), *E. amathites* and *E. divaricatus* are not sister species, nor does their separation correspond to the opening of the Rio São Francisco to the sea at 12,000 years ago. Indeed, divergence data support a much older origin and differentiation of this group of species than previously proposed.

Considering mean genetic distances in *Eurolophosaurus* and all outgroup taxa, molecular evolutionary rates for ribosomal sequences were shown to be three to four times slower than those for the protein-coding sequences. Their more conservative nature therefore provided better resolution for deeper nodes of phylogeny. This was probably the reason why the monophyly of *Eurolophosaurus* could be consistently demonstrated only by ribosomal sequence data. On the other hand, more recent nodes could be consistently supported mainly by protein-coding sequences.

Our sequence data indicate considerable evolutionary divergence between the populations of *E. divaricatus* from Alagoado and Queimadas/Ibiraba; these populations also differ morphologically in color pattern and in size and conspicuousness of the dorsal crest of scales (Skuk, 1994). Likewise, we know that the populations of *Eurolophosaurus* from Caetité and Rio de Contas, although similar to *E. nanuzae* in external appearance, have an almost black

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ventral color. These differences can be attributed to long-term isolation of these populations indicated by the molecular data and justify a rediagnosis of this species complex. The populations of *E.* "*nanuzae*" from the states of Minas Gerais and Bahia are actually allopatric, living in isolated areas at high altitudes. The same is true for those of *E. divaricatus*, which occupy two isolated dune fields separated by 150 km of unfavorable habitats (Rodrigues, 1996). Taxonomic changes in *Eurolophosaurus* are, therefore, urgent. The populations of *E. nanuzae* from Bahia should be considered a new undescribed species, and the name *nanuzae* should be restricted to those of Minas Gerais (Extração, Pedra Menina and Serra do Cipó). Similarly, the population of *E. divaricatus* from the isolated sand dunes of Alagoado should be described as a new species.

4.2. Genetic distances and biogeography

Sequences of cyt *b* and 16S rRNA genes, analyzed separately, and the four mitochondrial gene segments, collectively, were shown to be evolving according to the "molecular clock" hypothesis. Considering the evolutionary rates of 2%/my to 2.8%/my for the four combined gene sequences, *E. divaricatus* would have diverged from the *amathites*/*nanuzae* complex approximately 5.4 to 9.5 my ago. These evolutionary times are compatible with those previously estimated for the group from allozymic data (Martins, 1995). Since no intrinsic calibration point is available for *Eurolophosaurus* evolutionary history, the high rates proposed here must be considered tentative. Although ectothermy of lizards favors low metabolic rates, *Eurolophosaurus* organisms are small and live in drastic climatic conditions (average annual temperature between 24 ^{o}C and 26 ^{o}C), suggesting high metabolic rates and high rates of molecular evolution (Martin and Palumbi, 1993; Li, 1997). With lower evolutionary rates, the

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basal *Eurolophosaurus* split would be older than 7.5-9.5 my ago. Thus, the hypothesis of a few thousand years ago for this split, as initially proposed (Rodrigues, 1986), was not corroborated.

Cyt *b* K2P genetic distances in *Eurolophosaurus* (see Table 5) are compatible with intrageneric comparisons reported by Johns and Avise (1998) in a comprehensive survey in vertebrates. They detected lower genetic distances for birds than for fish, amphibians, reptiles and mammals, considering congeneric species and confamilial genera. The average genetic distance of 16% that we found among the three recognized *Eurolophosaurus* species is close to the maximum value identified by Johns and Avise for reptilian congeneric species, and twofold higher than the average genetic distance estimated for bird congeneric species. The 21% mean value obtained among the tropidurid genera *Uranoscodon*, *Microlophus*, *Tropidurus* and *Eurolophosaurus* is within the interval reported by those authors for confamilial genera of reptiles (9 to > 25%). The mean divergence times of 5.7 my (with sequence divergence of 2.8%/ my) and 8 my (with sequence divergence of 2%/ my) estimated for the mtDNA lineages in *Eurolophosaurus* species, based on cyt *b* K2P genetic distances, are also in accordance with the mean time estimated by Johns and Avise for reptiles (6 my, with a sequence divergence of 2%/ my).

Evolutionary history of *Eurolophosaurus* would have featured an original separation between lineages on the east and west banks of Rio São Francisco, followed by the isolation between Bahia and Minas Gerais populations, at the right margin, and between populations of the two dune fields, at the left margin. Considering a range of rates between 1% and 2.8%/my, most vicariant events in this genus would have occurred in the Pliocene and Miocene. Only two splits, *E. amathites*-*E. nanuzae* of Caetité/Rio de Contas and *E. nanuzae* of Pedra Menina-*E. nanuzae* of Serra do Cipó, could be situated in the Pleistocene (Table 6). The Tropidurinae ancestor could date from Miocene or Paleocene, and separation of *Eurolophosaurus* and

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Microlophus quadrivittatus date from Miocene or from the Miocene/Paleocene border. On the other hand, the separation between *Tropidurus hispidus* and *Eurolophosaurus* would be restricted to Miocene even with this broader range of rates.

Although data do not support either the divergence time or the species originally proposed in the first vicariance model, both the paleolacustrine hypothesis and changes in the course of the river remain valid as putative vicariant barriers to explain faunal divergence in the area (Rodrigues, 1986; 1991 a, b, c, d; 1996). Paleoclimate and historical geomorphology of this region are poorly understood. All available studies encompass timescales of thousands and not millions of years ago (see Cartelle and Hartwig, 1996; Barreto et al., 1999; De Oliveira et al., 1999). Otherwise, the herpetofauna of these Quaternary sand dunes is a very rich one and shows a high degree of endemism (Rodrigues, 1996), providing a great opportunity to compare evolutionary patterns in many taxa. Additional phylogenetic studies in progress with other reptilian groups of the continental sand dunes of the middle Rio São Francisco will determine whether the basal east-west split observed in the *Eurolophosaurus* radiation is a general pattern for fauna of the region.

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Legends and footnotes

Table 1. Number of *Eurolophosaurus* populations and outgroup samples successfully sequenced for different gene regions. All localities are in the Brazilian state of Bahia, except for Pedra Menina and Serra do Cipó (Minas Gerais), and Manaus (Amazonas). Punta Blanca is located in Chile.

Table 2. List of PCR and sequencing primers, and a summary of the PCR conditions for all four gene segments. Incubation times are given in parentheses in minutes:seconds.

* At 92°C or 95°C for 5:00 before first cycle, and for 7:00 at 68°C or 72°C after the last cycle. Reference for primers are: [a] Fu et al. (1997); [b] Frost et al. (1998); [c] Kocher et al. (1989); [d] Palumbi (1996).

Table 3. Mean base frequencies, X^2 and P values in homogeneity test for base composition among taxa for each DNA segment.

[a] Number of bases.

Table 4. Tree measures with different character weightings under the maximum parsimony (MP) criterion. CI (consistency index) values considering only informative characters. RI (retention index).

Table 5. Percent genetic distances (GTR+Γ+I) for the combined data (below diagonal) and percent genetic distances (K2P) for cyt *b* data (above diagonal) among ingroup and outgroup taxa.

U= *Uranoscodon superciliosus*; M= *Microlophus quadrivittatus*; Th= *Tropidurus hispidus*; d= *Eurolophosaurus divaricatus*; a= *E. amathites*; n= *E. nanuzae*; Al= Alagoado; Qu= Queimadas/ Ibiraba; MV= Mocambo do Vento; SI= Santo Inácio; Ca= Caetité; RC= Rio de Contas; PM= Pedra Menina; SC= Serra do Cipó.

Table 6. Divergence times (my) based on combined data set (cyt *b*, COI, 12S and 16S) considering two different molecular clocks. Slower molecular clocks must also be considered (see text).

d= *Eurolophosaurus divaricatus*; a= *E. amathites*; n= *E. nanuzae*; Al= Alagoado; Qu= Queimadas/ Ibiraba; MV= Mocambo do Vento; SI= Santo Inácio; Ca= Caetité; RC= Rio de Contas; PM= Pedra Menina; SC= Serra do Cipó.

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Legends

Figure 1. Above, South America inter-tropical region. Below, sand dune region of the middle Rio São Francisco, Brazil. Localities: (1) Caetité; (2) Rio de Contas; (3) Pedra Menina; (4) Extração; (5) Serra do Cipó; (6) Alagoado; (7) Queimadas; (8) Vacaria; (9) Ibiraba; (10) Xique-Xique; (11) Ilha do Gado Bravo; (12) Mocambo do Vento; (13) Lagoa de Itaparica; (14) Barra; (15) Santo Inácio; (16) Gameleira do Assuruá.

Figure 2. Tree estimated for combined gene sequences (cyt *b*, COI, 12S and 16S), with bootstrap values for equal weighting of all characters (above) in MP (bold), ML (italics), and NJ (underlined) analyses, and without 3rd codon positions in MP (below). Localities: Al= Alagoado; Qu= Queimadas/ Ibiraba; MV= Mocambo do Vento; SI= Santo Inácio; Ca= Caetité; RC= Rio de Contas; PM= Pedra Menina; SC= Serra do Cipó.

Figure 3. Maximum likelihood tree of combined gene sequences (cyt *b*, COI, 12S and 16S) with minimal dates for splitting events estimated with a sequence divergence of 2.8%/my. Slower molecular clocks must also be considered (see text). Localities: Al= Alagoado; Qu= Queimadas/ Ibiraba; MV= Mocambo do Vento; SI= Santo Inácio; Ca= Caetité; RC= Rio de Contas; PM= Pedra Menina; SC= Serra do Cipó.

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Species	Population	mtDNA			
		Cyt *b*	COI	12S	16S
Eurolophosaurus divaricatus	Alagoado	4	3	3	1
	Queimadas/Ibiraba	3	2	2	2
	Mocambo do Vento	3	1	2	1
Eurolophosaurus amathites	Santo Inácio	2	2	1	1
	Gameleira do Assuruá	1	-	-	1
Eurolophosaurus nanuzae	Caetité	2	1	1	1
	Rio de Contas	2	1	1	1
	Pedra Menina	2	1	1	1
	Serra do Cipó	2	1	1	1
Uranoscodon superciliosus	Manaus	1	1	1	1
Microlophus quadrivittatus	Punta Blanca	1	1	1	1
Tropidurus hispidus	Santo Inácio	1	1	1	1

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Table 2. List of PCR and sequencing primers, and a summary of the PCR conditions for all four gene segments. Incubation times are given in parentheses in minutes:seconds.

Primer label	Sequence (5'-3')	PCR conditions: denaturation/ annealing/extension x 35 *
B1[a]	CCATCCAACATCTCAGCATGATGAAA	95ºC (1:00), 55ºC (1:00), 72ºC (0:30)
B2 [a] (Cyt *b*)	GCCCCTCAGAATGATATTTGTCCTCA	
L7354[b]	TACCAACACCTATTCTGATT	92°C (0:45-1:00), 47°C-53°C (0:45) or 54°C (1:00), 68°C (1:00) or 72°C (0:45)
H7794 [b] (COI)	ATAATGGCAAATACTGCCCC	
L1091[c]	AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT	95°C (1:00), 54°C (1:00), 72°C (0:30)
H1478 [c] (12S)	TGACTGCAGAGGGTGACGGGCGGTGTGT	
Ar-5'[d]	CAAACCCCGCCTGTTTACCAAAAACAT	95°C (1:00), 54°C (1:00), 68°C (1:00)
Br-3' [d] (16S)	CCGGTCTGAACTCAGATCACGT	

* At 92°C or 95°C for 5:00 before first cycle, and for 7:00 at 68°C or 72°C after the last cycle. Reference for primers are: [a] Fu et al. (1997); [b] Frost et al. (1998); [c] Kocher et al. (1989); [d] Palumbi (1996).

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Table 3. Mean base frequencies, X^2 and P values in homogeneity test for base composition among taxa for each DNA segment.

Gene	Cyt *b*	COI	12S	16S
Bp[a]	209	348-349	307-313	456-468
%A	27.1	28.7	36.2	32.8
%C	29.1	22.8	26	27.2
%G	15.7	19.8	19.2	20.1
%T	28.1	28.7	18.6	19.9
X^2	6.0346	10.9451	3.18	6.0138
P	1.00	0.99	1.00	1.00

[a] Number of bases.

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Table 4. Tree measures with different character weightings under the maximum parsimony (MP) criterion. CI (consistency index) values considering only informative characters. RI (retention index).

Character weighting	Length	CI	RI
All characters=1	574	0.644	0.654
3rd codon position=0	232	0.709	0.74

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Table 5. Percent genetic distances (GTR+Γ+I) for the combined data (below diagonal) and percent genetic distances (K2P) for cyt *b* data (above diagonal) among ingroup and outgroup taxa.

	U	M	Th	dAl	dQu	dMV	aSI	nCa	nRC	nPM	nSC
U	-	20.4	24.3	18.4	17.8	17.8	19.7	19.1	20.4	20.4	20.3
M	29.3	-	22.9	20.2	19.6	19.0	19.0	21.5	21.5	24.1	25.5
Th	27.6	21.9	-	21.1	19.1	20.4	19.8	21.1	22.5	21.6	20.9
dAl	25.4	21.4	18.3	-	8.7	8.8	17.3	14.3	14.3	16.7	19.7
dQu	24.2	20.8	16.9	6.0	-	1.9	18.5	15.5	16.7	18.5	19.1
dMV	24.7	19.9	17.1	5.5	1.4	-	17.9	15.5	16.8	19.1	20.4
aSI	29.1	22.9	19.7	15.5	16.3	15.8	-	7.6	8.7	16.8	16.7
nCa	26.7	22.9	20.1	15.2	15.3	15.1	4.2	-	1.0	15.0	13.2
nRC	27.0	22.6	20.3	15.0	15.5	15.3	4.3	0.2	-	15.0	13.2
nPM	29.0	28.1	21.2	16.6	15.7	16.7	9.8	9.8	9.6	-	7.1
nSC	29.6	28.9	22.1	18.9	16.0	17.3	10.4	10.3	10.2	3.3	-

U= *Uranoscodon superciliosus*; M= *Microlophus quadrivittatus*; Th= *Tropidurus hispidus*; d= *Eurolophosaurus divaricatus*; a= *E. amathites*; n= *E. nanuzae*; Al= Alagoado; Qu= Queimadas/ Ibiraba; MV= Mocambo do Vento; SI= Santo Inácio; Ca= Caetité; RC= Rio de Contas; PM= Pedra Menina; SC= Serra do Cipó.

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Table 6. Divergence times (my) based on combined data set (cyt *b*, COI, 12S and 16S) considering two different molecular clocks. Slower molecular clocks must also be considered (see text).

	2%/my	2.8%/my
dAl-aSI	7.7	5.5
dAl-nCa/RC	7.5	5.4
dAl-nPM	8.3	5.9
dAl-nSC	9.5	6.8
dQu/MV-aSI	8.0	5.7
dQu/MV-nCa/RC	7.7	5.5
dQu/MV-nPM	8.1	5.8
dQu/MV-nSC	8.3	5.9
aSI-nCa/RC	2.1	1.5
aSI-nPM	4.9	3.5
aSI-nSC	5.2	3.7
dAl-dQu/MV	2.9	2.1
nCa/RC-nPM	4.9	3.5
nCa/RC-nSC	5.1	3.7
nPM-nSC	1.7	1.2

d= *Eurolophosaurus divaricatus*; a= *E. amathites*; n= *E. nanuzae*; Al= Alagoado; Qu= Queimadas/ Ibiraba; MV= Mocambo do Vento; SI= Santo Inácio; Ca= Caetité; RC= Rio de Contas; PM= Pedra Menina; SC= Serra do Cipó.

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Figure 1

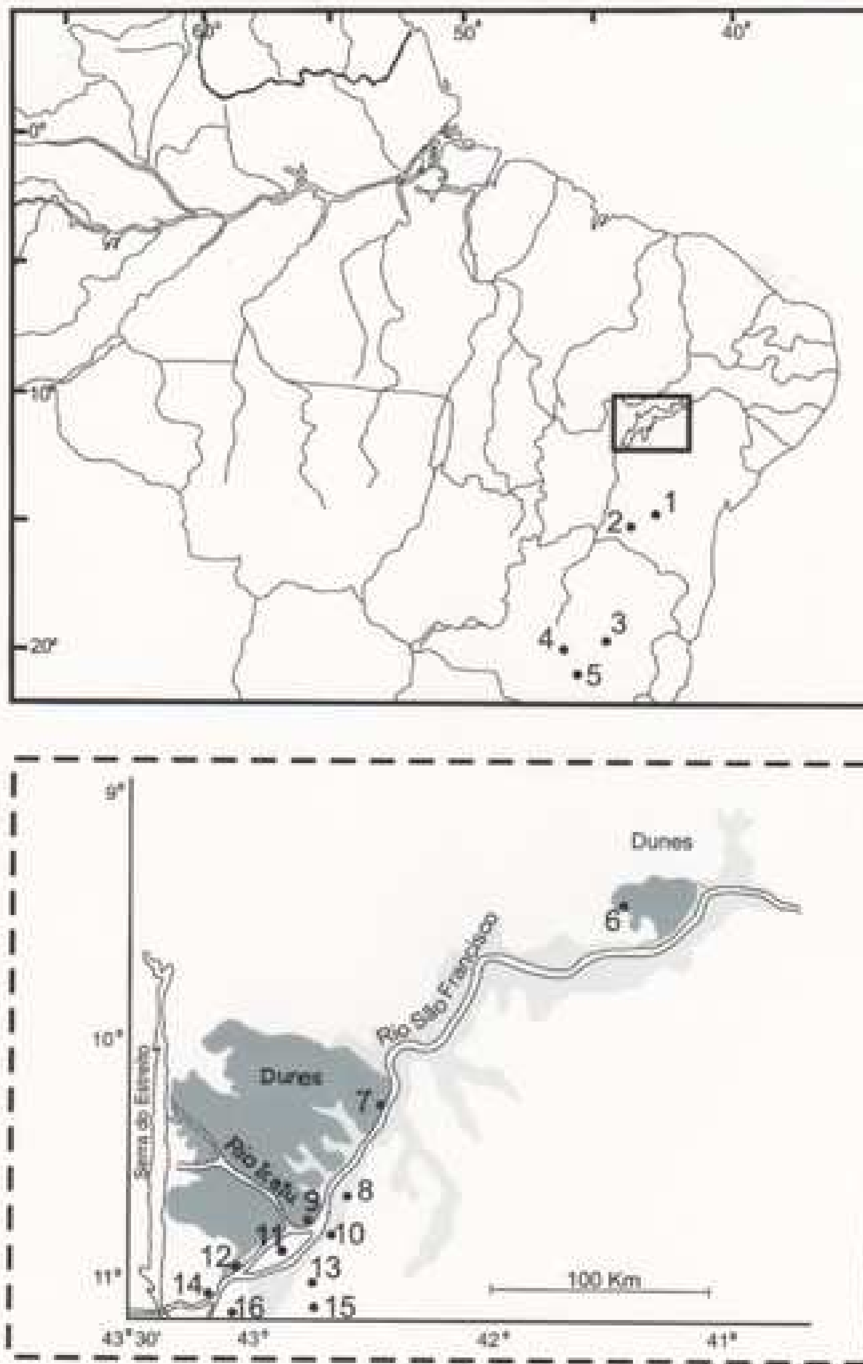

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Figure 2

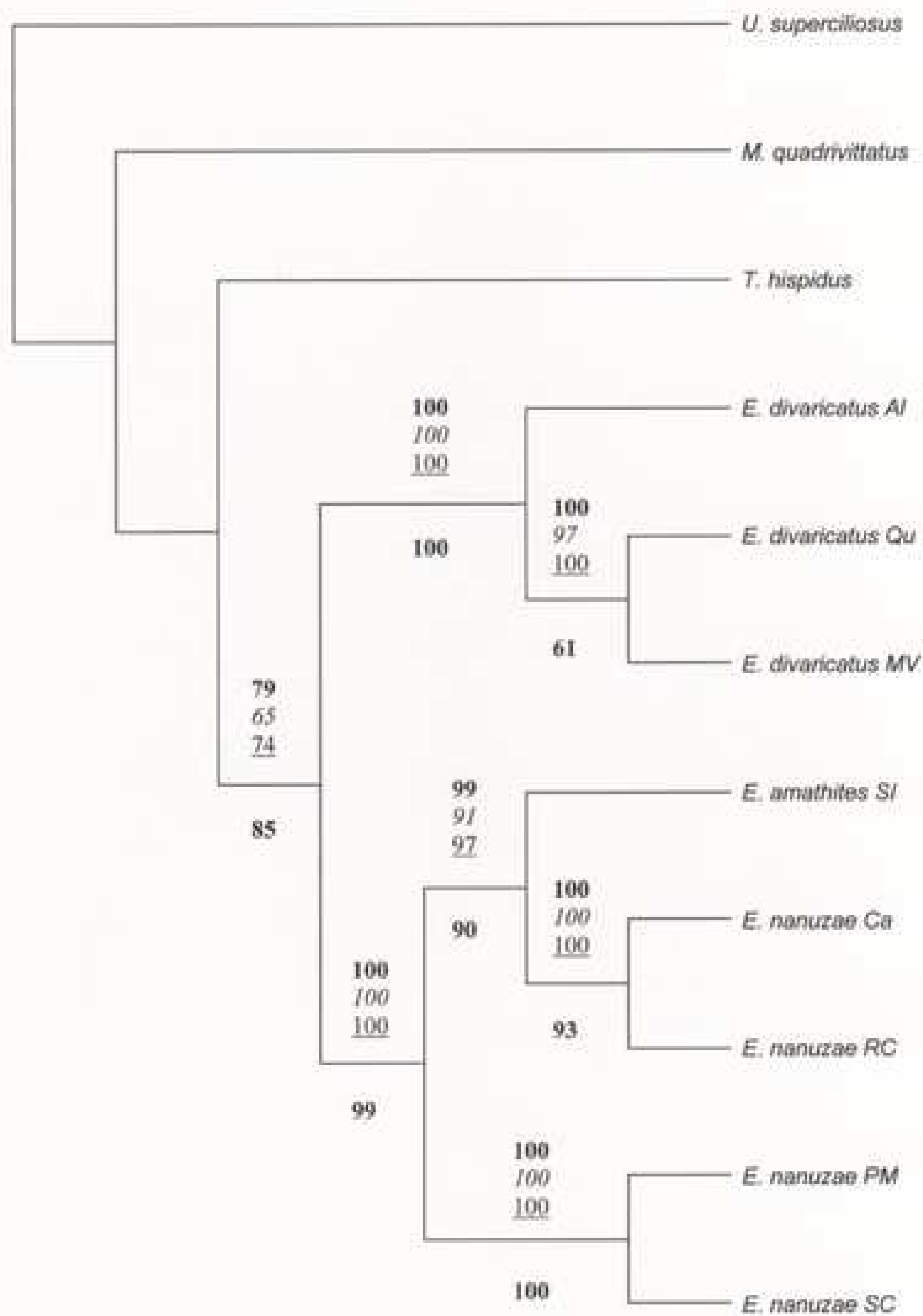

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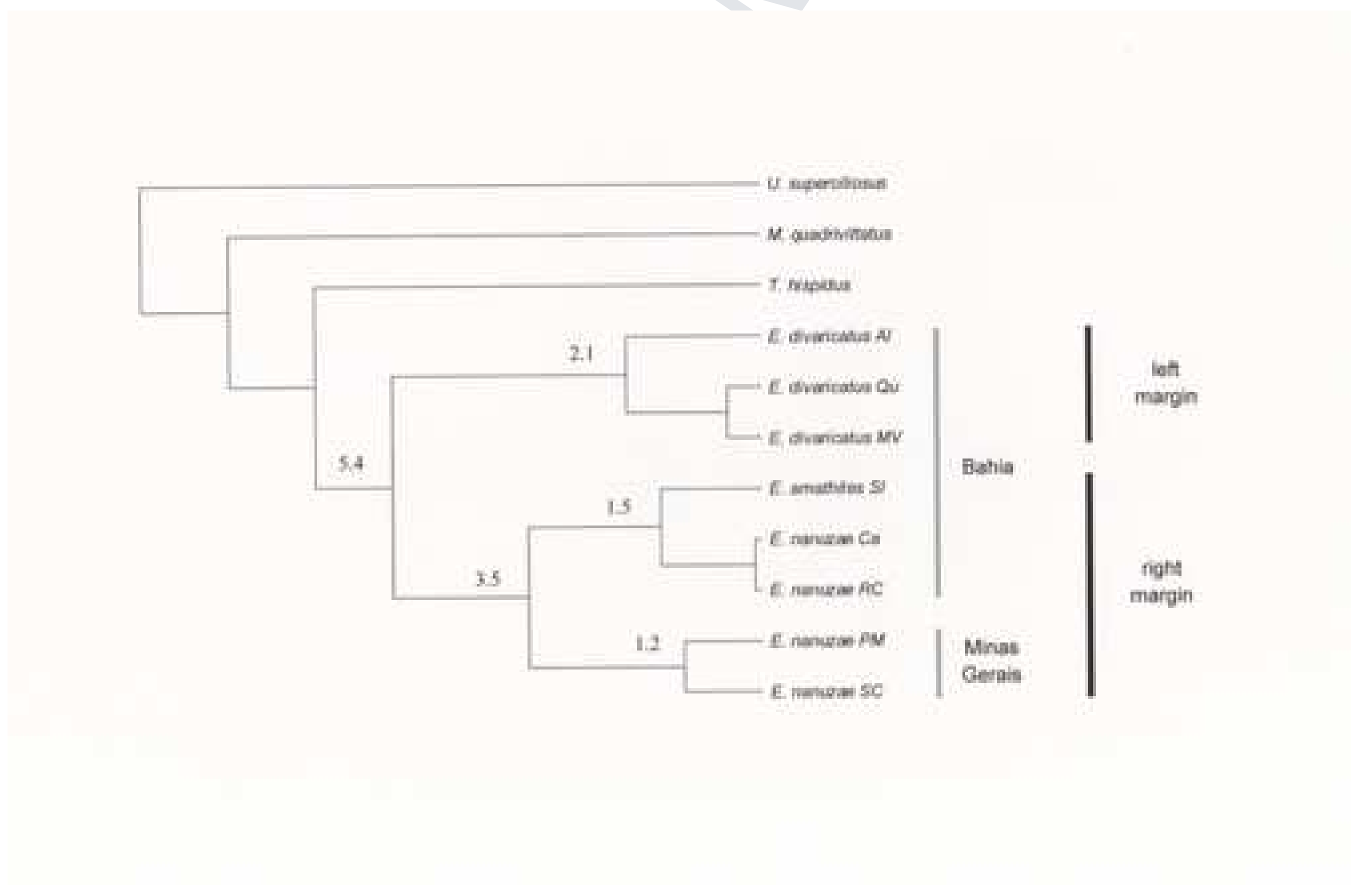
Figure 3



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Appendix I. Ingroup and outgroup 12S mtDNA sequences. Stem regions are numbered in bold, according to the secondary structure models in Hickson et al. (1996), Richards and Moore (1996) and Titus and Frost (1996). Underlined numbers indicate characters omitted from the phylogenetic analysis. *28' ?* follows Titus and Frost designation.

```
                  28'?  22'       31                  31'          2'           32
                            1 1111111122 22222 2223333333 33 4444 44 4444555555555 56666
                  123 4567890 1234567890 12345 6789012345 67 8901 23 4567890123456 78901
U. superciliosus  GTC CGCCAGA GAATTACAAGC GAAAA GCTTAAAACTC AA AAGA CT TGGCGGTGCCCCA CACTC
M. quadrivitatus  ... ....... ...C...... ..... .......... .. .G.. .. ........T.... .....
T. hispidus       ... ....... ...C.....T ..... A......... .. .... .. ............. ...C.
E. divaricatus Al ... ....... ...C...... ..... ..C.G..... .. .... .. ............. ...C.
E. divaricatus Qu ... ....... ...C...... ..... ..C.G..... .. .... .. ............. ...C.
E. divaricatus MV ... ....... ...C...... ..... ..C.G..... .. .... .. ............. ...C.
E. amathites SI   ... ....... ...C...... ..... ..C....... .. .... .. ............. ...C.
E. nanuzae C      ... ....... ...C...... ..... ..C....... .. .... .. ............. ...C.
E. nanuzae RC     ... ....... ...C...... ..... ..C....... .. .... .. ............. ...C.
E. nanuzae PM     ... ....... ...C..GG.T ..... ..C....... .. .... .. ............. A..C.
E. nanuzae SC     ... ....... ...C...G.T ..... ..C....... .. .... .. ............. A..C.
```

```
                        33          34          35          35'          36
                                                             111 11111111 1111111 1111111111
                  666 66677 77 7777778888 888 8889 99999999 9000 00000001 1111111 1122222222
                  456 78901 23 4567890123 456 7890 12345678 9012 34567890 1234567 8901234567
U. superciliosus  AAC TTAGA GG AGCCTGTCCT ATA ATCG ATAACCCA CGAT AAACCTGA CCACCTT TTGCC--ACC
M. quadrivitatus  ... ..... .. .......... ... .... ..G.T... .... .....CA. .....C. .....CA.A.
T. hispidus       G.. ..... .. .......... ... .... ....T... .... .....CA. .....C. .C...CC...
E. divaricatus Al G.. ..... .. .......... ... .... ........ .... .....C.. ..G..C. .....-A.T.
E. divaricatus Qu G.. ..... .. .......... ... .... ........ .... .....C.. ..G..C. .....-A.T.
E. divaricatus MV G.. ..... .. .......... ... .... ........ .... .....C.. ..G..C. .....-A.T.
E. amathites SI   G.. ..... .. .......... ... .C.. ....T... .... .....C.. ..G.T.. .....AA...
E. nanuzae C      G.. ..... .. .......... ... .C.. ....T... .... .....C.. ..G.T.. .....CA...
E. nanuzae RC     G.. ..... .. .......... ... .C.. ....T... .... .....C.. ..G.T.. .....CA...
E. nanuzae PM     G.. ..... .. .......... ... .... ....T... .... .....C.. ..G.T.. .....CT...
E. nanuzae SC     G.. ..... .. .......... ... .... ....T... .... .....C.. ..G.T.. .....CT...
```

```
                              38                39  40        40′           39′        42
                  111 11111111111111111 1111 111111 111 11111 111 1111111 111111 1111 1111
                  223 33333333344444444 4455 555555 556 66666 666 6777777 777788 8888 8888
                  890 12345678901234567 8901 234567 890 12345 678 9012345 678901 2345 6789
U. superciliosus  ACA GCCTATATACCGCCGTC ACCA ATCTAC CTC TACAA GAG ACACACA GTAGGT AAAA TAGT
M. quadrivitatus  T.C ................. G.A. .C.C.. ..- ..... A.. .ACA... ..G... .C.. ....
T. hispidus       --. ................. G.A. .C.... ..- C.... ... .A.AG.C ...... T... ....
E. divaricatus Al --. ................. G.A. .C.... ..- C.... ... .ACA.TC ...... .T.. ...-
E. divaricatus Qu --. ................. G.A. .C.... ..- C.... ... .ACA.TC ...... .... ...-
E. divaricatus MV --. ................. G.A. .C.... ..- C.... ... .ACT.TC ...... .T.. ...-
E. amathites SI   C-. ................. G.A. .C.... ..- C.... ... .AC..TC ...... .... C..C
E. nanuzae C      C-. ................. G.A. .C.... ..- C.... ... .AC..TC ...... .... ....
E. nanuzae RC     C-. ................. G.A. .C.... ..- C.... ... .AC..TC ...... .... ....
E. nanuzae PM     --. ................. G.A. ...... ..- C.... ... .AC..TC ...... .... C...
E. nanuzae SC     --. ................. G.A. ...... ..- C.... ... .AC..TC ....A. .... C...
```

```
                          42′               38′               36′        34′           45
                  111111 1111 22222222 22222222222222 2222 2222222 2 22222222222 222 2222222
                  999999 9999 00000000 00111111111122 2222 2222333 3 33333344444 444 4455555
                  012345 6789 01234567 89012345678901 2345 6789012 3 45678901234 567 8901234
U. superciliosus  GTTT-C ACTA AAACATCA GGTCAAGGTGTAGC TTAT AAGGTGG A AGAGATGGGCT ACA TTCTTTA
M. quadrivitatus  T.AAA. .... ....GA.. .............. .A.. GG..... . ........... ... .......
T. hispidus       -AAAA. .... ....G... .............. .A.. GG..... G ........... ... .......
E. divaricatus Al -CCAA. .... ....G... .............. .A.. .GT.C.. . ........... ... .......
E. divaricatus Qu -CCAA. .... ....G... .............. .A.. .GT.C.. . ........... ... .......
E. divaricatus MV -CCAA. .... ....G... .............. .A.. .GT.C.. . ........... ... .......
E. amathites SI   -CCAA. .... ....G... .............. .A.C .GA.C.. . ........... ... .......
E. nanuzae C      -CCAA. .... .G..G... .............. .A.. ..A.C.. . ........... ... .......
E. nanuzae RC     -CCAA. .... ....G... .............. .A.. ..A.C.. . ........... ... .......
E. nanuzae PM     -CAAA. .... ....G... .............. .A.. ..A.C.. . ........... ... .......
E. nanuzae SC     -.AAA. .... ....G... .............. .A.. ..A.C.. . ........... ... .......
```


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```
                               45'              47               47'               33'       48
                      22222 2222222 2222222 2222222 22222222 2222222 222233333 33333 3333 33
                      55555 6666666 6667777 7777778 88888888 8999999 999900000 00000 1111 11
                      56789 0123456 7890123 4567890 12345678 9012345 678901234 56789 0123 45
U. superciliosus      ACCAA TAAAGCA -CACAAA AAGAAGC ATGAAAAA GCCCCCC AAAGGAGGA TTTAA CAGT AA
M. quadrivitatus      .TTCC C...... -A...G. ...GGC. ......C. .G.T... ......... ..... .... ..
T. hispidus           .A--C .....A. -...G.. .....CA G.....C. C.TT.TA .....C.A. ..... .... ..
E. divaricatus Al     .A.-C ....... -A..... .....CA ......C. C.TT.TA ..C..T... ..... .... ..
E. divaricatus Qu     ...-C ....... -G..... .....TA ......C. C.TT.TA ..C..T... ..... .... ..
E. divaricatus MV     ...-C ....... -A..... .....TA ......C. C.TT.TA ..C..T... ..... .... ..
E. amathites SI       ..A-T ....... AA..... .....C. C......G T.TT..A .....T... ..... .... ..
E. nanuzae C          ..G-C ....... AA..... .....C. C......G T.TT..A .....T... ..... .... ..
E. nanuzae RC         ..G-C ....... AA..... .....C. C......G T.TT..A .....T... ..... .... ..
E. nanuzae PM         .A.-C .....T. GA..... .-...CT C......C T.TT..A .....T... ..... .... ..
E. nanuzae SC         .T.-C .....T. GA..... .-...CT C......C T.TT..A .....T... ..... .... ..
```

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Appendix II. Ingroup and outgroup 16S mtDNA sequences. Stem regions in the sequences are numbered in bold, according to the secondary structure models in Gutell and Fox (1988) and Burk et al. (2002). Underlined position numbers indicate characters omitted from the phylogenetic analysis.

```
                   32        33            33′       34            34′     35               35′
                              11111 111 112222222  222333 333333 344444 44 444 555555555566 666
                   123 4 5678901234 567 890123456  789012 345678 901234 56 789 012345678901 234
U. superciliosus   GCC T GCCCAGTGAA A-C TTCAACGGC  CGCGGT ATCCTA ACCGTG CA AAG GTAGCGTAACCA CTT
M. quadrivitatus   ... . .......... .T- .........  ...... ...... ...... .. ... .........T.. ...
T. hispidus        ... . .......... .CT .........  ...... ...... ...... .. ... .........T.. T..
E. divaricatus Al  ... . .......... .CT .........  ...... ...... ...... .. ... .........T.. T..
E. divaricatus Qu  ... . .......... .CT .........  ...... ...... ...... .. ... .........T.. T..
E. divaricatus MV  ... . .......... .CT .........  ...... ...... ...... .. ... .........T.. T..
E. amathites SI    ... . .......... .A. ..A......  ...... ...... ...... .. ... .........T.. T..
E. nanuzae Ca      ... . .......... .A. ..A......  ...... ...... ...... .. ... .........T.. T..
E. nanuzae RC      ... . .......... .A. ..A......  ...... ...... ...... .. ... .........T.. T..
E. nanuzae PM      ... . .......... .A. ..A......  ...... ...... ...... .. ... .........T.. T..
E. nanuzae SC      ... . .......... .A. ..A......  ...... ...... ...... .. ... .........T.. T..
```

```
                     36           36′             32′         29′                 28′
                                                            11111111 11111 1111111111111111
                   666667 77777 777788 8888888899 999 9999 900000000 00111 1111111222222222
                   567890 12345 678901 2345678901 234 5678 901234567 89012 3456789012345678
U. superciliosus   GCCTCC TAAAT AGAGGC TAGTATGAAC GGC TAAA TGAGGACTA ACCTG TCTCCTTTAACCAATC
M. quadrivitatus   .T.CT. ..... .AG.A. CG.......T ... .... .....G.C. ..... ......C...TT....
T. hispidus        .T.CT. ..... ..G.A. C......... ... .... .....G.C. ..... .....CC.....T...
E. divaricatus Al  .T.C.. ..... ..G.A. CG........ ... .... .....G... .T... ............C...
E. divaricatus Qu  .T.C.. ..... ..G.A. C......... ... .... .....G... ..... ............C...
E. divaricatus MV  .T.C.. ..... ..G.A. C......... ... .... .....G.C. ..... ........G...C...
E. amathites SI    .T.CT. ..... .AG.A. CT.......T ... .... .....G.C. ..... ............C...
E. nanuzae Ca      .T.CT. ..... .AG.A. CT.......T ... .... .....G.C. ..... ............C...
E. nanuzae RC      .T.CT. ..... .AG.A. CT.......T ... .... .....G.C. ..... ............C...
E. nanuzae PM      .T.CT. ..... .AG.A. CT........ ... .... .....G.C. ..... ............CC..
E. nanuzae SC      .T.CT. ..... .AG.A. CT........ ... .... .....G.C. ..... ............CC..
```

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```
                    22′      A′       37             37′              38                39
                  1 11 11111 1 11111 11111 11111111 11111 1111111 1111111111 11111 1111111111111
                  2 33 33333 3 33444 44444 44555555 55556 6666666 6677777777 77888 8888888999999
                  9 01 23456 7 89012 34567 89012345 67890 1234567 8901234567 89012 3456789012345
U. superciliosus  A AT GAAAC T GATCT ACCAG TCAAAAAG CTGGT ATAAAAC CATAAGACGA GAAGA CCCTGTGGAGCTT
M. quadrivitatus  . G. ..... . ..... ..... .AC..... ..... .....C. .......... ..... .............
T. hispidus       . G. ..... . ..... ..... .AC..... ..... ....TC. T......... ..... .............
E. divaricatus Al . G. ..... . ..... ..... .AC..... ..... .....C. ..C....... ..... .....C.......
E. divaricatus Qu . G. ..... . ..... ..... .AC..... ..... .....C. ..C....... ..... .............
E. divaricatus MV . G. ..... . ..... ..... .AC..... ..... ....GC. ..C....... ..... .....C.......
E. amathites SI   . G. ..... . ..... ..... .AC..... ....C .....C. .......... ..... .............
E. nanuzae Ca     . G. ..... . ..... ..... .AC..... ..... ....GC. .......... ..... .............
E. nanuzae RC     . G. ..... . ..... ..... .AC..... ..... ....GC. .......... ..... .............
E. nanuzae PM     . G. ..... . ..... G.... .AC..... ....C ..GCCTA .......... ..... .............
E. nanuzae SC     . G. ..... . ..... G.... .AC..... ....C ....CTA .......... ..... .............
```

```
                             40
                   1 1112222222222222222 22222222222222222222222222222222222
                   9 9990000000000111111 11112222222222333333333344444444445
                   6 7890123456789012345 67890123456789012345678901234567890
U. superciliosus   C AAGCTTCA----ATCAACA CA----C-CCAC--ACACCATG-----CCC-----
M. quadrivitatus   T ..ATCAA.AATC.A..C.. G.ACTA.CA.CTC----TA.ACCA--C..TGATT-
T. hispidus        T ..AT.AA.A---G.....G GT----A-A.TT-----...ACAC--C...GTCAC
E. divaricatus Al  . ..ATCAA.----GC..... G.----.-A.CA----....A.AC--C...-----
E. divaricatus Qu  T ..ATCAA.----GC..... GG----.-..TAT----.A.A--A--C...TGCC-
E. divaricatus MV  T ..ATCAA.----GC..... GG----.-...AC----.A.A.C----...TGCC-
E. amathites SI    A ..AT..AC----G...... G.----.-.AG.-----...ACAA--A.T.TACT-
E. nanuzae Ca      A ..AT..AC----G...... G.----.-A..AC----...ATAA--A...TACT-
E. nanuzae RC      A ..AT..AC----G...... G.----.-A..AC----...ATAA--A...TACT-
E. nanuzae PM      . ..A-CCT.---CG.....G G.----.-A..AT----TA.ACCGGCC...CCCT-
E. nanuzae SC      T ..A.C.AC----G.....G G.----.-AT.ATT.A....AC-C--C..T-----
```

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```
                              40'          41        41'    42                  42'
                        22222222222222222 22 22222 222 22222 222222 22222222222 333333 333333333333333
                        55555555566666666 66 77777 777 77888 888888 89999999999 000000 000011111111112
                        12345678901234567 89 01234 567 89012 345678 90123456789 012345 678901234567890
U. superciliosus        TGATC--CATAG--CTT TA AGTTG GGG CGACT TCGGAA ACAAGCCAAAC TTCCGA GCACAAG--ACCACA
M. quadrivitatus        ...C-----G.---T.. .C ..... ... ..... ...... TA..AGAC... ...... .T.A...AG...CT-
T. hispidus             ...C.--.-..A-AT.. .T ..... ... ..... ...... CA..A...... ...... ...A.C.--...TT.
E. divaricatus Al       ..CCTGG.C..AGAT.. .C ..... ... ..... ...... CA..A..C... ...... ...-.G.--...TT.
E. divaricatus Qu       ..GC.----..AGAT.. .T ..... ... ..... ...... CA..A..C... ...... ...AGG---...TT.
E. divaricatus MV       ..GC.----..AGAT.. .C ..... ... ..... ...... CA..A..C... ...... ...AGG---...TT.
E. amathites SI         ...C-----..ACAT.. .C ..... ... ..... ...... CA..A...... ...... ...AGG.--..-T..
E. nanuzae Ca           ...C-----..ACAT.. .C ..... ... ..... ...... CA..A...... ...... ...AGG.--.A.TA.
E. nanuzae RC           ...C-----..ACAT.. .C ..... ... ..... ...... CA..A...... ...... ...AGG.--.A.TA.
E. nanuzae PM           ...C-----.CCTGT.. .T ..... ... ..... ...... .A..A...... ...... ...AG---G...TT.
E. nanuzae SC           ...CT--.C.G---T.. .T ..... ... ..... ...... .A..A...... ...... ...-.G.--...TT.
```

```
                                44        D      44'                 45       45'        D'
                        333333 333333333 333 3 33 33333333 3333333333 333 333333 333 333333 3 3333
                        222222 222333333 333 3 44 44444444 5555555555 666 666666 677 777777 7 7888
                        123456 789012345 678 9 01 23456789 0123456789 012 345678 901 234567 8 9012
U. superciliosus        G-TCTA ACCA-CGAC TAA C AT GTCTAGAT ATAATTGACC CAG TA-TCA CTG ATAAAC G AACC
M. quadrivitatus        --CTCT ....CA... CG. . .A ...A.C.A .ACC...... ... .CCA.. ... ..C... . ....
T. hispidus             AA...T .AA.-A... C.. . .A .....A.C CA...-.... ... ..-AA. ... ...... . ....
E. divaricatus Al       AA..CC ....-A... .G. . .A ...A.A.. .AC..-.... ... ..-AT. ... ...... . ....
E. divaricatus Qu       AA..CC ...C-A... CG. . .G ...-.A.C .AC..-.... ... ..-A.. ... ...... . ....
E. divaricatus MV       AA..CC ...C-A... CG. . .G ...-.A.C .AC..-.... ... ..-A.. ... ...... . ....
E. amathites SI         AAC.CT .A..-A... CC. . .A ...C.A.. .AC..-.... ... C.-AG. ... ...... . ...T
E. nanuzae Ca           AAC.CT .A..-A... CC. . .A ...C.A.. .AC..-.... ... C.-AG. ... ...... . ....
E. nanuzae RC           AAC.CT .A..-A... CC. . .A ...C.A.. .AC..-.... ... C.-AG. ... ...... . ....
E. nanuzae PM           AAC.CT ....-A... CC. . .A ...--C.A .AC..-.... ... ..-A.. ... .C.... . ....
E. nanuzae SC           AAC.CT ....-A... CC. . .A ...C.A.A .AC..-.... ... ..-A.. ... .C.... . ....
```

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```
                         39′                     46                        46′
                  33333333333333 3334444 44444444444444444 4444444 444444444444444444
                  88888889999999 9990000 00000011111111112 2222222 223333333333444444
                  34567890123456 7890123 45678901234567890 1234567 890123456789012345
U. superciliosus  AAGTTACCCCAGGG ATAACAG CGCAATCTTCTTTAAGA GTCCCTA TCGACAAGAAGGTTTACG
M. quadrivitatus  .............. ....... ............C.... ....A.. ..................
T. hispidus       .............. ....... ............C.... ....A.. ..................
E. divaricatus Al .............. ....... .......C....A.... ....A.. .........G........
E. divaricatus Qu .............. ....... .......C....A.... ....A.. .........G........
E. divaricatus MV .............. ....... .......C....A.... ....... .........G........
E. amathites SI   .............. ....... .......C....C.... ....A.. .........G........
E. nanuzae Ca     .............. ....... .......C....C.... ....A.. .........G........
E. nanuzae RC     .............. ....... .......C....C.... ....A.. .........G........
E. nanuzae PM     .............. ....... .......C....C.... ....A.. .........G........
E. nanuzae SC     .............. ....... .......C....C.... ....A.. .........G........
```

```
                                  47            48           48′
                  4444444444 44444444444 44 4444444444 4444 44444
                  4444555555 55556666666 66 6777777777 7888 88888
                  6789012345 67890123456 78 9012345678 9012 34567
U. superciliosus  ACCTCGATGT TGGATCAGGAC AC CCAATTGGTG CAGC CGCTA
M. quadrivitatus  .......... ........... .. .......... .... .....
T. hispidus       .......... ........... .. .......... .... .....
E. divaricatus Al .......... ........... .. .......... .... .....
E. divaricatus Qu .......... ........... .. .......... .... .....
E. divaricatus MV .......... ........... .. .......... .... .....
E. amathites SI   .......... ........... .A ....C..... .... .....
E. nanuzae Ca     .......... ........... .. ....C..... .... .....
E. nanuzae RC     .......... ........... .. ....C..... .... .....
E. nanuzae PM     .......... ........... .A ....C..... .... .....
E. nanuzae SC     .......... ........... .A ....C..... .... .....
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ACCEPTED MANUSCRIPT