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FULL LENGTH RESEARCH PAPER

DNA barcoding reveals species level divergence between populations of the microhylid frog genus *Arcovomer* (Anura: Microhylidae) in the Atlantic Rainforest of southeastern Brazil

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Abstract

The microhylid frogs belonging to the genus *Arcovomer* have been reported from lowland Atlantic Rainforest in the Brazilian states of Espírito Santo, Rio de Janeiro, and São Paulo. Here, we use DNA barcoding to assess levels of genetic divergence between apparently isolated populations in Espírito Santo and Rio de Janeiro. Our mtDNA data consisting of cytochrome oxidase subunit I (COI) nucleotide sequences reveals 13.2% uncorrected and 30.4% TIM2 + I + Γ corrected genetic divergences between these two populations. This level of divergence exceeds the suggested 10% uncorrected divergence threshold for elevating amphibian populations to candidate species using this marker, which implies that the Espírito Santo population is a species distinct from *Arcovomer passarellii*. Calibration of our model-corrected sequence divergence estimates suggests that the time of population divergence falls between 12 and 29 million years ago.

Keywords

Candidate species, COI calibration, cryptic species, Mitochondrial DNA, molecular clock, phylogeography

History

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Introduction

DNA barcoding has been proven to be a useful tool for uncovering cryptic species diversity in amphibians (e.g. Crawford et al., 2012; Murphy et al., 2013). Despite the caveats of using single genes to identify candidate amphibian species (reviewed in Vences et al., 2005; Vieites et al., 2009), the relative ease and low cost of acquiring mitochondrial DNA (mtDNA) sequences nonetheless enables researchers to perform rapid preliminary population surveys to identify so-called ''candidate species,'' which can lead the way to more thorough species delimitation studies (Reilly et al., 2012; Vences et al., 2005). Indeed, for amphibian barcode studies that rely on the ''Folmer'' region of the mtDNA cytochrome oxidase subunit I gene or ''COI'' (Folmer et al., 1994), which is the standard DNA barcode of life sequence (Hebert et al., 2003), Vences et al. (2005) suggested that an uncorrected sequence divergence threshold of 10% might be used to elevate populations to candidate species status, i.e. units that might represent undescribed species (Vieites et al., 2009).

The Brazilian Atlantic Rainforest contains an exceptionally rich amphibian fauna but is under severe threat due to human-caused alteration and destruction of the landscape (Carnaval et al., 2009; Morellato & Haddad 2000). Studies using morphological and/or molecular data continue to document new cryptic species of amphibians from this highly endangered ecosystem (e.g. Tonini et al., 2014). A small microhylid species, which has been reported as inhabiting the leaf litter and temporary ponds in the lowland coastal region of the Atlantic Forest of southeastern Brazil, is *Arcovomer passarellii* Carvalho, 1954 (Figure 1; Giaretta & Martins, 2009; Malagoli et al., 2012). A recent study of this species' geographic distribution using museum specimen records revealed that populations of *Arcovomer* are distributed as disjunct populations stretching from the central coast region of the state of Espírito Santo southwards to the northeastern coastal region of the state of São Paulo (Malagoli et al., 2012). Some workers have expressed the opinion that the populations found in São Paulo and Espírito Santo states are undescribed species (Pombal & Bastos, 1992; p. 251 de Sá et al., 2012).

The type locality for *A. passarellii* is Duque de Caxias in the state of Rio de Janeiro (Izecksohn & Carvalho-e-Silva, 2001). Recent field collecting of adult *A. passarellii* individuals from nearby coastal areas (Maricá and Búzios, Rio de Janeiro) and at the northernmost known locality for this species (Barra do Riacho in the central coast of Espírito Santo), together with an ongoing DNA barcoding study of Brazilian vertebrates by the National Museum (Museu Nacional) of the Universidade Federal do Rio de Janeiro (UFRJ), afforded us an opportunity to make an assessment on the genetic distinctness of the Espírito Santo and Rio de Janeiro populations. Our results, which are based on mtDNA COI sequence data, provide compelling evidence that these two populations are indeed separate species.

Methods

Genetic samples

Tissue samples from adult specimens of *Arcovomer passarellii* deposited in the herpetological collection at the Museu Nacional, Universidade Federal do Rio de Janeiro (MNRJ) were used in this study (Table 1). At the time of collection, tissue samples (muscle, toe, or skin) were preserved in 95% ethanol for later use in genetic analyses. A total of 12 individuals were included in this study: 10 from the central coast region of the state of Espírito

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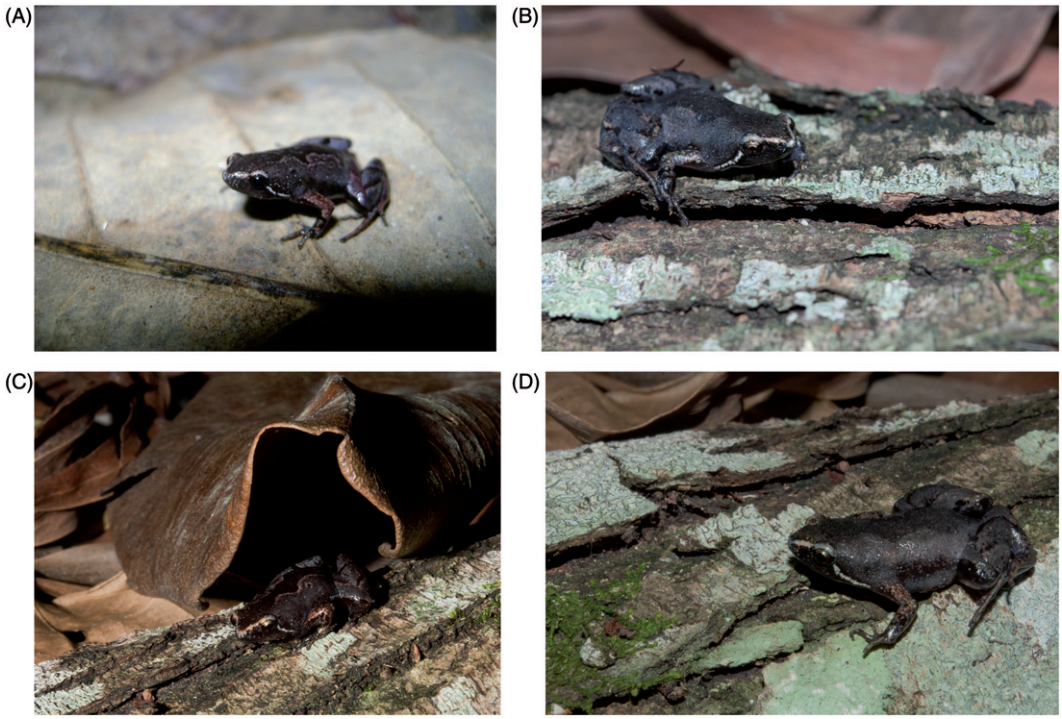

Figure 1. (A) *Arcovomer passarellii* from Barra do Riacho in the state of Espírito Santo, Brazil. Photo credit: Rodrigo de O. L. Salles. (B) *Arcovomer passarellii* from Barra do Riacho in the state of Espírito Santo, Brazil. Photo credit: Marcos Bilate. (C) *Arcovomer passarellii* from Barra do Riacho in the state of Espírito Santo, Brazil. Photo credit: Marcos Bilate. (D) *Arcovomer passarellii* from Barra do Riacho in the state of Espírito Santo, Brazil. Photo credit: Marcos Bilate.

Table 1. List of specimens used in this study including voucher numbers for specimens deposited in the Museu Nacional, Universidade Federal do Rio de Janeiro (MNRJ), laboratory sample numbers in the DNA Extract Collections of the Laboratório de Pesquisa em Biodiversidade Molecular, Museu Nacional (MNLM), locality information, BoldSystems Process ID numbers, and GenBank accession numbers.

Voucher MNRJ	Sample MNLM	Collection locality	BoldSystems process ID	GenBank accession
76810	4877	Barra do Riacho, ES	MNRJ082-14	KP037045
76812	4878	Barra do Riacho, ES	MNRJ083-14	KP037046
80702	4879	Ponta Negra, Maricá, RJ	MNRJ084-14	KP037042
82111	4880	Búzios, RJ	MNRJ085-14	KP037047
86572	4881	Barra do Riacho, ES	MNRJ086-14	KP037048
86573	4882	Barra do Riacho, ES	MNRJ087-14	KP037051
86574	4883	Barra do Riacho, ES	MNRJ088-14	KP037050
86575	4884	Barra do Riacho, ES	MNRJ089-14	KP037049
86576	4885	Barra do Riacho, ES	MNRJ090-14	KP037043
86577	4886	Barra do Riacho, ES	MNRJ091-14	KP037040
86578	4887	Barra do Riacho, ES	MNRJ092-14	KP037044
86579	4888	Barra do Riacho, ES	MNRJ093-14	KP037041

RJ = state of Rio de Janeiro and ES = state of Espírito Santo, Brazil.

Santo (hereafter referred to as the ''ES'' population) and two from the state of Rio de Janeiro (hereafter referred to as the ''RJ'' population).

Molecular data

Genomic DNA was extracted from each tissue sample using the Wizard DNA extraction kit (Promega, Madison, WI). We used PCR to amplify a 658 base pair fragment at the 5′ end (i.e. ''Folmer fragment'') of the mtDNA cytochrome oxidase subunit I gene (COI) using universal amphibian primers by Che et al. (2012), which we modified by adding M13 sequencing primers:

Chmf4M13-21 5′-TGTAAAACGACGGCCAGTTYTCWAC WAAYCAYAAAGAYATCGG-3′
Chmr4M13-29 5′-CAGGAAACAGCTATGACCACYTCRGG RTGRCCRAARAATCA-3′

The following thermocycle profile was used to generate the PCR products: [(94 °C for 1:30) × 1 cycle], [(94 °C for 0:30, 50 °C for 45 s, 70 °C for 1:00) × 35 cycles], and (70 °C for 10:00). The presence of single target PCR bands in each reaction was confirmed using agarose gel electrophoresis. PCR products were treated with Exo-SAP before being sequenced in both directions by the Sanger method at the High-Throughput Genomics Laboratory, University of Washington, WA. Collection localities, including geospatial coordinates, sequence data, trace files, primer details, and photographs of specimens are available in the Barcode of Life Data Systems (BOLDSYSTEMS, http://www.boldsystems.org/; Ratnasingham & Hebert, 2007), under project ''Gestão de vouchers e capacitação institucional para geração de DNA BarCodes – Museu Nacional/UFRJ'' (project code: MNRJ). Sequences have also been deposited in GenBank. BOLD Process ID and GenBank accession numbers are listed in Table 1.

In addition, we obtained outgroup sequences from GenBank for purposes of rooting the ingroup. These outgroup sequences are from representative species in the following closely-related microhylid genera (see de Sá et al., 2012): *Hamptophryne boliviana* (GenBank #KF621252), *Dermatonotus muelleri* (GenBank #KF621249), *Elachistocleis ovalis* (GenBank #FJ766753, FJ766754), and *Gastrophryne olivacea* (GenBank #AB611900).

DNA sequence analyses

We used the software FinchTV v.1.4.0 (Geospiza Inc., Seattle, WA) to evaluate the quality of base calls in each chromatogram. Sequences were then aligned by eye using the program Se-Al (Rambaut, 1995) and translated into amino acids for purposes of determining whether or not our sequences are likely derived from the mtDNA COI gene or from copies inserted into the nuclear genome. Summary statistics including numbers of variable sites, invariable sites, and distinctive haplotypes were obtained using DNAsp (Rozas et al., 2003).

We conducted phylogenetic analyses using maximum likelihood (ML) and maximum parsimony (MP) using the program PAUP* (Swofford, 2000). The highest-scoring gene tree under ML was generated using the best-fitting substitution model for our sequence dataset, which was found using the program jModeltest (Darriba et al., 2012; Guindon & Gascuel, 2003; Posada, 2008). We evaluated clade support in ML and MP trees by using non-parametric bootstrapping (Felsenstein, 1985) and considered any clade with a bootstrap proportion >70% as being strongly supported (Hillis & Bull, 1993).

In order to assess the robustness of the inferred root position for the *Arcovomer* clade, we conducted different rooting analyses using PAUP*. First, in our ML and MP trees we examined the position of the root as determined by outgroup sequences. In a second analysis, we used the midpoint rooting method on ML and MP trees that only contained the ingroup sequences (i.e. outgroup sequences were not included in the tree search) and on trees containing all sequences (i.e. outgroup sequences were included in the tree search). Besides looking for stability in the root position, another motivation for us using the midpoint method is that we wanted to estimate the rooted phylogeny for all sequences (ingroup + outgroup), then compare our rooted tree to the inferred relationships among microhylid genera presented by de Sá et al. (2012), who did not use the COI gene. The third method consisted of using a molecular clock to infer the root of the ingroup clade in ML trees (Huelsenbeck et al., 2002). We clock-rooted our ML tree that did not contain outgroup sequences and the ML tree containing outgroup sequences. Because conducting an unconstrained heuristic tree search under a clock-enforced ML criterion is computationally intensive, we used our previously inferred unrooted ingroup topology, which never varied among analyses (see ''Results'' section), as a topological constraint in the analysis. The assumption of a molecular clock was tested using a likelihood ratio test (Felsenstein, 1981). This test is performed in the following manner: (1) ML scores from the non-clock and clock analyses (done separately but on the same tree) are first obtained; (2) the test statistic δ, which is equal to two times the difference of the two likelihood scores [i.e. $\delta = 2(\ln L_{\text{clock}} - \ln L_{\text{non-clock}})$] is computed; and (3) the statistical significance of δ is determined by comparison to a χ^2 distribution with an α level of 0.05 and s – two degrees of freedom, where s is the number of sequences (Huelsenbeck & Crandall, 1997; Posada & Crandall, 2001). A significant result would suggest that the sequences have not evolved in a strict clock-like manner.

We also conducted analyses aimed towards evaluating the degree of genetic divergence between the ES and RJ populations of *Arcovomer*. In the first analysis, we used PAUP* (Swofford, 2000) to compute uncorrected pairwise sequence divergences. In order to avoid potential problems associated with sequence saturation, which can be especially problematic in mtDNA sequences (Arbogast et al., 2002; Brown et al., 1979), we also calculated model-corrected distances based on the chosen nucleotide substitution model. In a second genetic divergence analysis, we estimated the timing of divergence between the ES and RJ populations. To convert our corrected % sequence divergence estimate between the ES and RJ populations, we first estimated the rate of sequence evolution of our COI sequences (% divergence/million years). Using information found in our sequence dataset and in the time-calibrated phylogeny of de Sá et al. (2012), we estimated two sets of rates (time calibrations). The first rate set, which we call ''Calibration Point 1,'' was estimated by using the age of the *Arcovomer-Hamptophryne* clade (in millions of years or ''MY'') together with the average corrected % distance between the *Arcovomer* and *Hamptophryne* COI sequences in our dataset. Using this information, we calculated rates based on the estimated actual, minimum, and maximum clade ages for the *Arcovomer* and *Hamptophryne* clade, which are 34.50, 21.26, and 48.89 MY, respectively (see Table 3 and Figure 5 in de Sá et al., 2012). These clade ages correspond to the Bayesian-estimated posterior mean and 95% error margins around the nodal ages in the time tree of de Sá et al. (2012). The second evolutionary rate set, which we call ''Calibration Point 2,'' was estimated by using the age of the Gastrophryninae Subclade III in de Sá et al. (2012), a clade that includes *Arcovomer*, *Hamptophryne*, *Dermatonotus*, *Gastrophryne*, and *Elachistocleis*, together with the average corrected % distance of *Arcovomer* versus each of our outgroup sequences (excluding *Hamptophryne*). With this information, we calculated rates based on the estimated actual, minimum, and maximum clade ages for the Gastrophryninae Subclade III clade, which are 41.30, 27.91, and 59.65 MY, respectively (see Table 3 and Figure 5 in de Sá et al., 2012). Once obtained, we then applied these time calibrations to our average corrected distance between the ES and RJ populations of *Arcovomer* in order to obtain estimates for the timing of divergence.

Results

The multiple sequence alignment of 12 ingroup and five outgroup haplotypes contained a total of 658 sites. Evidence that our sequences represent the orthologous mtDNA sequences, as opposed to nuclear paralogues, comes from the observations that they translate into amino acid sequences containing no unexpected stop codons and no indels. Among the *Arcovomer* sequences, 86 sites were variable and three distinctive haplotypes were observed. Only a single variable site was found among the ten ES individuals and no variable sites were observed between the two RJ individuals. The two populations are distinguishable by 86 fixed differences.

A TIM2 + I + Γ substitution model was chosen as the best fitting model for our dataset, with the following parameter estimates (output from jModeltest into PAUP block format): Lset base = (0.2546 0.2979 0.1597) nst = 6 rmat = (18.1796 34.9046 18.1796 1.0000 131.3764) rates = gamma shape = 0.6630 ncat = 4 pinvar = 0.5170. Results of the likelihood ratio test were non-significant, which suggests that the COI sequences evolved in a clock-like manner [$\delta = 2(\ln L_{\text{clock}}$ 2387.77 $- \ln L_{\text{non-clock}}$ 2385.76) = 4.02, $\chi^2_{[df=15,0.05]} = 25$, $p = 0.99$).

All phylogenetic analyses regardless of optimality criterion used, and whether outgroup sequences were included or excluded, produced the same unrooted ingroup topology with the *Arcovomer* haplotypes being segregated into ES and RJ

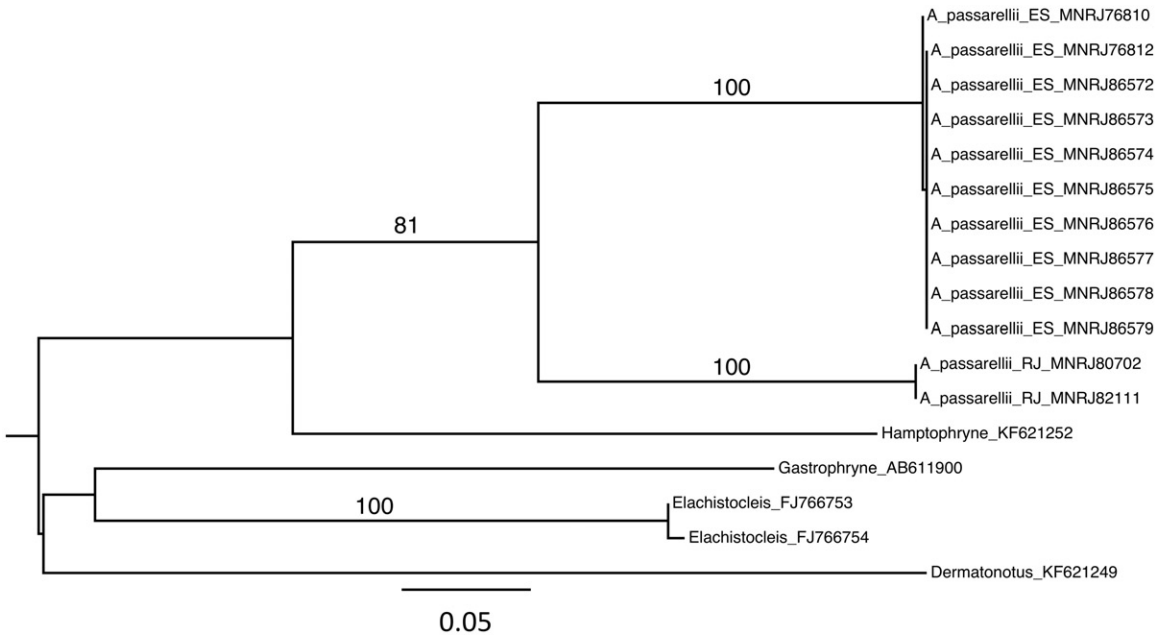

Figure 2. ML phylogram showing the relationships among 12 mtDNA haplotypes of *Arcovomer passarellii* collected from the states of Espírito Santo (ES) and Rio de Janeiro (RJ), Brazil. Sequences from several closely related microhylid genera including *Hamptophryne*, *Dermatonotus*, *Elachistocleis*, and *Gastrophryne* were also included in the analysis. Numbers above branches represent bootstrap proportions and only values >70 are shown. Note, because the ML and MP trees have congruent ingroup topologies, and all rooting analyses identified the same root position for the *Arcovomer* clade, only the midpoint-rooted ML tree is shown. The scale bar below the tree is in units of substitutions/site.

bifurcation groups. The location of the root on the ingroup haplotype group was also stable, as all rooting analyses inferred a root location that creates monophyletic ES and RJ haplotype clades (Figure 2). These two haplotype clades within *Arcovomer* are also strongly supported as evidenced by their high bootstrap values (Figure 2).

In the MP analysis, the only topological variation we observed within the ingroup consisted of three equally parsimonious trees resulting from the instability in the position of specimen MNRJ76810 relative to the remaining samples of *Arcovomer* from Espírito Santo. This occurred because these alternative hypotheses depend on the ambiguous interpretation of nucleotide 619. Accelerated character state optimization (ACCTRAN) using *Hamptophryne*, *Dermatonotus*, *Elachistocleis*, and *Gastrophryne* as outgroups produced a highly supported hypothesis of *Arcovomer* monophyly as well as highly supported hypotheses of monophyly of the ES and RJ populations (384 steps, consistency index 0.76, retention index 0.79, consistency index excluding uninformative characters 0.72, retention index 0.60, 159 parsimony-informative characters). Our hypothesis implies a minimum of 11 molecular synapomorphies that are diagnostic for *Arcovomer*, 34 autapomorphies for the ES population, and 31 autapomorphies for the RJ population. If alternative (DELTRAN) optimizations methods are used to resolve ambiguous characters, as many as 41, 56, and 66 character-state transformations may be assigned to each of these clades.

Our data suggest the existence of low levels of sequence divergence within each population of *Arcovomer* though we note that our sampling of haplotypes from the RJ population is low (Table 2). In contrast, however, the uncorrected distance between ES and RJ populations is surprisingly high and even approaches the level of divergence observed between *Arcovomer* and other sampled microhylid genera (Table 2). Correcting these distances using the $TIM2+I+\Gamma$ substitution model did little to change the within *Arcovomer* population distances, but did increase the distance between the ES and RJ populations from 13.2% to 30.4% (Table 2). Likewise, the distances between *Arcovomer* and other genera were also elevated when taking into account multiple substitutions (Table 2).

Our estimates for the rate of sequence divergence in the COI gene in these frogs ranged from 1.5% per MY to 1.6% per MY for the Calibration Point 1 and 2 methods, respectively (Table 3). The minimum and maximum bounds for the Calibration Point 1 rate were 1.0 and 2.4% per MY, whereas the range for Calibration Point 2 was 1.1–2.4% per MY (Table 3). Using these rates to calibrate the ES versus RJ corrected sequence divergence of 30.4% from Table 2 suggests a divergence time between these two populations of 18.4–20.5 million years ago (Table 4). The minimum and maximum divergence times around these estimates are 12.5–29.1 million years ago (Table 4). All pairwise (model-corrected) sequence divergences and calculations used to estimate rates and divergence times are provided in Appendix 1.

Discussion

The microhylid frog *Arcovomer passarellii* is endemic to lowland Brazilian Atlantic Forest and is only known to occur in several widely separated locations near the coasts of Espírito Santo, Rio de Janeiro, and São Paulo (Malagoli et al., 2012). The northern-most populations in the state of Espírito Santo are separated from those to the south in the state of Rio de Janeiro by hundreds of kilometers and from the São Paulo state populations by nearly a thousand kilometers. Although future searches for this diminutive litter-dwelling frog may reveal new localities, it is still of interest to know whether or not these populations are genetically distinct from each other. The results in the present study, which included samples from central Espírito Santo and Rio de Janeiro, support the hypothesis by Pombal & Bastos (1992) and de Sá et al. (2012), according to which at least one of the ES populations of *A. passarellii* represents a new species.

Our inferred mtDNA gene tree provides three perspectives on the level of genetic divergence between the ES and RJ populations of *A. passarellii*. First, the mtDNA haplotypes are reciprocally

Table 2. Average uncorrected and model-corrected % sequence divergences between RJ and ES populations of *Arcovomer* and *Arcovomer* versus each outgroup genus.

	Average uncorrected	Average TIM2 + I + Γ
COI sequences compared	Pairwise distances (%)	Pairwise distances (%)
Arcovomer ES versus ES	0.0	0.0
Arcovomer RJ versus RJ	0.0	0.0
Arcovomer ES versus RJ	13.2	30.4
Arcovomer versus *Hamptophryne*	16.4	51.1
Arcovomer versus other genera[a]	17.3	68.1

The corrected distances are based on a TIM2 + I + Γ substitution model. [a]Includes *Gastrophryne*, *Dermatonotus*, and *Elachistocleis.*

Table 3. Rates of DNA sequence divergence (% divergence per million years) in the mtDNA COI gene observed in some New World microhylid frogs.

	Calibration Point 1 *Arcovomer-Hamptophryne* clade	Calibration Point 2 Gastrophryninae subclade III
Rates based on estimated clade age	1.5	1.6
Rates based on minimum clade age	2.4	2.4
Rates based on maximum clade age	1.0	1.1

Calibration Point 1 refers to the age of the clade containing the microhylid genera *Arcovomer* and *Hamptophryne*, whereas Calibration Point 2 refers to the age of the Gastrophryninae Subclade III (see Table 3 and Figure 5 in de Sá et al. 2012). Further details on data and calculations can be found in Appendix 1.

Table 4. Estimated time since divergence (in millions of years) between the Espírito Santo and Rio de Janeiro populations of *Arcovomer passarellii* using rates of sequence evolution for the mtDNA COI gene obtained from Table 3.

	Calibration Point 1 *Arcovomer-Hamptophryne* clade	Calibration Point 2 Gastrophryninae subclade III
Divergence time	20.5	18.4
Minimum divergence time	12.6	12.5
Maximum divergence time	29.1	26.6

Further details on data and calculations can be found in Appendix 1.

monophyletic with respect to the two populations suggesting that they have been isolated for a significant amount of time (Avise, 2000). Second, the uncorrected average pairwise divergence estimate between the ES and RJ populations is 13.2%, a value that exceeds the suggested threshold value of 10% for delimiting candidate amphibian species using mtDNA COI sequences (Vences et al., 2005; see also Vieites et al., 2009). Finally, our molecular clock analysis suggests that the most recent common ancestor for the ES and RJ populations existed between the middle Miocene to early Oligocene (12–29 million years ago). These observations support the hypothesis that the ES and RJ *Arcovomer* populations have diverged from each other long enough ago to become separate species.

A comprehensive revision of the morphological and taxonomic status of the various populations of *Arcovomer* is beyond the scope of the current study. Nonetheless, our study raises the issue of which populations can be associated with the nominal taxon *A. passarellii* described by Carvalho (1954) from the municipality of Duque de Caxias situated in the Baixada Fluminense lowlands. Our sample MNRJ 80702 was collected only 60 km away from the type locality of *A. passarellii*. The precise coordinates of the type-locality are unknown, and the extent of urban encroachment in the Rio de Janeiro metropolitan area make collection of fresh voucher specimens and associated genetic samples closer to the type-locality suitable very unlikely. The distance separating MNRJ from the type-locality, however, is small enough to ensure that the haplotype of sample MNRJ 80702 is a representative barcode of *A. passarellii*. That distance is smaller than the distance separating RJ samples that have identical COI haplotypes. In contrast, the distance separating the ES and RJ populations is 370 km, which far exceeds the range of known RJ populations. Although mitochondrial DNA sequences from topotypes of *A. passarellii* are currently unavailable, it is, therefore, likely that the RJ samples are conspecific with the holotype of *A. passarellii*, and, therefore, the genetically distinct ES represents an undescribed species.

Pombal & Bastos (1992) and de Sá et al. (2012) have previously suggested that the São Paulo and ES populations represent different species, but those authors did not provide any morphological or molecular evidence to support that hypothesis. de Sá et al. (2012) did not have access to samples from Rio de Janeiro, and we are unaware of publically available COI sequences from *Arcovomer* specimens from São Paulo. However, the geographic distance between the type locality of *A. passarellii* and the São Paulo population studied by de Sá et al. (2012) is 170 km and also exceeds the range of known RJ populations. We predict that, when available COI sequence data from the São Paulo populations will reveal levels of genetic differentiation comparable to those reported here for the ES and RJ populations.

Although our observations of reciprocal monophyly and exceptional level of sequence divergence in our data are compelling findings by themselves, the estimated divergence time between the ES and RJ populations is largely dependent on our time calibrations. However, our calibrations, which were derived from two different nodes in the time-calibrated microhylid phylogeny of de Sá et al. (2012), did yield similar estimates. Our use of divergence time information obtained from de Sá et al. (2012) is justifiable because the Gastrophryninae Subclade III in Figure 4 of their paper was well supported and, although the *Arcovomer-Hamptophryne* clade was not well supported in their study (see their Figure 4), we recovered the same relationship in our midpoint-rooted tree using a different mtDNA gene (see our Figure 2). Future studies should include additional individuals from Rio de Janeiro and use multi-locus coalescent methods to evaluate our mtDNA gene divergence time (Edwards & Beerli, 2000).

The distribution of *Arcovomer* may coincide with the ranges of other vertebrate species that are historically confined to the coastal plain regions scattered along the lowland Atlantic Rainforests of Brazil. For example, Weitzman et al. (1988) analyzed the distribution of *Mimagoniates microlepis*, a freshwater Characiform fish, and found that this species may actually represent a complex of incipient or full species that occur in a series of isolated small populations in the lowland Atlantic Rainforest near the coast. These authors pointed out that a branch of the Serra do Mar juts into the Atlantic Ocean at Ponta da Trindade between the towns of Parati (also spelled ''Paraty'') in the state of Rio de Janeiro and Ubatuba in the state of São Paulo.

This formidable mountain range forms a barrier to lowland-inhabiting vertebrates found to the southwest and northeast. Both *Arcovomer* (Malagoli et al., 2012) and *M. microlepis* (Weitzman et al., 1988) have morphologically distinctive populations that have apparently been kept separate by this montane-oceanic barrier. The situation at the northern extent of their respective ranges is less obvious. The morphotype of *M. microlepis* found in the south from Angra dos Reis, Rio de Janeiro state, is distributed north to the boundary between Espírito Santo and Bahia states (Weitzman et al. 1988), whereas the population of *Arcovomer* found in Rio de Janeiro state is known from several sites along the southwestern and central coastal regions (Izecksohn & Carvalho-e-Silva, 2001; Malagoli et al., 2012; this study). To the north, *Arcovomer* is known from only a few scattered coastal populations in southern and central lowland areas in Espírito Santo state (de Sá et al., 2012; Malagoli et al., 2012; this study). In contrast to the montane-oceanic barrier postulated at the southern end of *Arcovomer*'s range, the mountain ranges situated between the ES and RJ populations are relatively low and represent only partial barriers between adjacent lowland areas. Any of the rivers that terminate in the ocean along this coastline, which includes the Rio Itapemirim, Rio Itabapoana, and Rio Paraíba do Sul, could have played a more important role in isolating one or more populations of *Arcovomer.* Further sampling in coastal lowland areas between Barra do Riacho and Búzios is required in order to refine the knowledge of the geographic limits between the ES and RJ populations.

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Declaration of interest

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References

Arbogast BS, Edwards SV, Wakeley J, Beerli P, Slowinski JB. (2002). Estimating divergence times from molecular data on phylogenetic and population genetic timescales. Ann Rev Ecol Syst 33:707–40.

Avise J. (2000). Phylogeography, the history and formation of species. Cambridge, MA: Harvard Univ Press.

Brown WM, George M, Wilson AC. (1979). Rapid evolution of animal mitochondrial DNA. PNAS 76:1967–71.

Carnaval A, Hickerson MJ, Haddad CFB, Rodrigues MT, Moritz C. (2009). Stability predicts genetic diversity in the Brazilian Atlantic forest hotspot. Science 323:785–9.

Carvalho AL. (1954). A preliminary synopsis of the genera of American microhylid frogs. Occ Pap Mus Zool Univ Mich 555:1–19.

Che J, Chen H, Yang J, Jin J, Jiang K, Yuan Z, Murphy RW, Zhang Y. (2012). Universal COI primers for DNA barcoding amphibians. Mol Ecol Res 12:247–58.

Crawford AJ, Cruz C, Griffith E, Ross H, Ibáñez R, Lips KR, Driskell AC, et al. (2012). DNA barcoding applied to *ex situ* tropical amphibian conservation programme reveals cryptic diversity in captive populations. Mol Ecol Res 2012:1–14.

Darriba D, Taboada GL, Doallo R, Posada D. (2012). jModelTest 2: More models, new heuristics and parallel computing. Nat Methods 9:772.

de Sá RO, Streicher JW, Sekonyela R, Forlani MC, Loader SP, Greenbaum E, Richards S, Haddad CFB. (2012). Molecular phylogeny of microhylid frogs (Anura: Microhylidae) with emphasis on relationships among New World genera. BMC Evol Biol 2012:12:241.

Edwards SV, Beerli P. (2000). Perspective: Gene divergence, population divergence, and the variance in coalescent time in phylogeographic studies. Evol 54:1839–54.

Felsenstein J. (1981). Evolutionary trees from DNA sequences: A maximum likelihood approach. J Mol Evol 17:368–76.

Felsenstein J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783–91.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol 3:294–9.

Giaretta A, Martins L. (2009). Notes on the call and behavior of *Arcovomer passarellii* (Anura: Microhylidae). Herpetol Notes 2:91–3.

Guindon S, Gascuel O. (2003). A simple, fast and accurate method to estimate large phylogenies by maximum-likelihood. Syst Biol 52: 696–704.

Hebert PDN, Ratnasingham S, de Waard JR. (2003). Barcoding animal life: Cytochrome c oxidase subunit 1 divergences among closely related species. Proc Royal Soc Lond B: Biol Sci 270:S96–9.

Hillis DM, Bull JJ. (1993). An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. Syst Zool 42: 182–92.

Huelsenbeck JP, Crandall KA. (1997). Phylogeny estimation and hypothesis testing using maximum likelihood. Ann Rev Ecol Syst 28:437–66.

Huelsenbeck JP, Bollback JP, Levine AM. (2002). Inferring the root of a phylogenetic tree. Syst Biol 51:32–43.

Izecksohn E, Carvalho-e-Silva SP. (2001). Anfíbios do município do Rio de Janeiro. Rio de Janeiro: Editora UFRJ.

Malagoli LR, Condez TH, Haddad CFB. (2012). *Arcovomer passarellii* Carvalho, 1954 (Amphibia: Anura: Microhylidae): Distribution extension in São Paulo state, Brazil and geographic distribution map. Check List 8:505–6.

Morellato LPC, Haddad CFB. (2000). Introduction: The Brazilian Atlantic Forest. Biotropica 32:786–92.

Murphy RW, Crawford AJ, Bauer AM, Che J, Donnellan SC, Fritz U, Haddad CFB, et al. (2013). Cold Code: The global initiative to DNA barcode amphibians and nonavian reptiles. Mol Ecol Res 13: 161–7.

Pombal Jr JP, Bastos RP. (1992). Geographic distribution: *Arcovomer passarellii* (NCN). Lawrence: SSAR Herpetol Rev 23:85.

Posada D. (2008). jModelTest: Phylogenetic model averaging. Mol Biol Evol 25:1253–6.

Posada D, Crandall KA. (2001). Selecting the best-fit model of nucleotide substitution. Syst Biol 50:580–601.

Rambaut A. (1995). Se-Al: Sequence alignment program. Ver. 2.0. Oxford, UK: Oxford University.

Ratnasingham S, Hebert PDN. (2007). BoLD: The barcode of life data system. Mol Ecol Notes 7:355–64. Available at: http://www.barcodinglife.org (Accessed 6 November 2014).

Reilly SB, Marks SB, Jennings WB. (2012). Defining evolutionary boundaries across parapatric ecomorphs of Black Salamanders (*Aneides flavipunctatus*) with conservation implications. Mol Ecol 21:5745–61.

Rozas J, Sanchez-DelBarrio JC, Messeguer X, Rozas R. (2003). DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics 19:2496–7.

Swofford DL. (2000). PAUP*: Phylogenetic analysis using parsimony (*and other methods). Sunderland, MA: Sinauer.

Tonini JFR, Forlani MC, de Sá RO. (2014). A new species of *Chiasmocleis* (Microhylidae, Gastrophryninae) from the Atlantic Forest of Espírito Santo State, Brazil. ZooKeys 428:109–32.

Vences M, Thomas M, Bonett RM, Vieites DR. (2005). Deciphering amphibian diversity through DNA barcoding: Chances and challenges. Philos Trans R Soc B 360:1859–68.

Vieites DR, Wollenberg KC, Andreone F, Köhler J, Glaw F, Vences M. (2009). Vast underestimation of Madagascar's biodiversity evidenced by an integrative amphibian inventory. PNAS 106:8267–72.

Weitzman SH, Menezes NA, Weitzman MJ. (1988). Phylogenetic biogeography of the Glandulocaudini (Teleostei, Characiformes, Characidae) with comments on the distributions of other freshwater fishes in Eastern and Southeastern Brazil. Proceedings of a workshop on neotropical distribution patterns. Academia Brasileira de Ciências, Rio de Janeiro.

Appendix 1

This appendix shows calibrations, corrected distances, and calculations of divergence time in millions of years (MY) between the Rio de Janeiro (RJ) and Espírito Santo (ES) populations of *A. passarellii* using mtDNA COI sequence data. The dataset consists of a multiple alignment of $n = 2$ sequences for the RJ population, $n = 10$ for the ES population, and outgroup sequences from closely-related microhylid genera: *Hamptophryne* ($n = 1$), *Gastrophryne* ($n = 1$), *Dermatonotus* ($n = 1$), and *Elachistocleis* ($n = 2$).

A. Estimate calibrations for the divergence time estimates

To estimate the rates of evolution for the COI gene in these frogs, we used information obtained from Table 3 and Figure 5 of de Sá et al. (2012). From their Table 3, we obtained the following two clade ages including their associated 95% Bayesian confidence intervals:

Calibration Point 1 (Node 12. Origin of *Arcovomer* + *Hamptophryne* clade)

Note: divergence corresponds to the timing of divergence that separates *Arcovomer* from *Hamptophryne*. See Figure 5 in de Sá et al. (2012):

Estimated age (MY) = 34.50
Minimum age (MY) = 21.26
Maximum age (MY) = 48.89

Calibration Point 2 (Node 14. Origin of Gastrophryninae subclade III)

Note: this divergence corresponds to the timing of divergence that separates the *Arcovomer-Hamptophryne* clade from the clade containing *Gastrophryne*, *Dermatonotus*, *Elachistocleis* clade. See Figure 5 in de Sá et al. (2012):

Estimated age (MY) = 41.30
Minimum age (MY) = 27.91
Maximum age (MY) = 59.65

B. Calculation of model-corrected relative sequence divergences for each node of interest

The program jModeltest selected the TIM2 + I + Γ model for these COI sequences (see Methods) with the following parameters (in PAUP block format):

Lset base = (0.2546 0.2979 0.1597) nst = 6 rmat = (18.1796 34.9046 18.1796 1.0000 131.3764) rates = gamma shape = 0.6630 ncat = 4 pinvar = 0.5170)

Using the corrected pairwise distances (listed below), we calculated the following five average model-corrected sequence divergences:

1. All *Arcovomer* ES (within population) pairwise comparisons. This yielded an average corrected (relative) divergence = 0.03%
2. All *Arcovomer* RJ (within population) pairwise comparisons. This yielded an average corrected (relative) divergence = 0.00%
3. All *Arcovomer* ES versus RJ populations pairwise comparisons. This yielded an average corrected (relative) divergence = 30.40%
4. All pairs of sequences relevant to Calibration Point 1. This yielded an average corrected (relative) divergence = 51.09%
5. All pairs of sequences relevant to Calibration Point 2. This yielded an average corrected (relative) divergence = 68.06%

Below are the pair-wise model-corrected distances:

***Arcovomer* (ES only)** Pairwise comparison	**TIM2 + I + Γ distances**
Arcovomer (ES only)	0
Arcovomer (ES only)	0
Arcovomer (ES only)	0
Arcovomer (ES only)	0
Arcovomer (ES only)	0
Arcovomer (ES only)	0
Arcovomer (ES only)	0
Arcovomer (ES only)	0
Arcovomer (ES only)	0
Arcovomer (ES only)	0
Arcovomer (ES only)	0
Arcovomer (ES only)	0
Arcovomer (ES only)	0
Arcovomer (ES only)	0
Arcovomer (ES only)	0
Arcovomer (ES only)	0
Arcovomer (ES only)	0
Arcovomer (ES only)	0
Arcovomer (ES only)	0
Arcovomer (ES only)	0
Arcovomer (ES only)	0
Arcovomer (ES only)	0
Arcovomer (ES only)	0
Arcovomer (ES only)	0
Arcovomer (ES only)	0
Arcovomer (ES only)	0
Arcovomer (ES only)	0
Arcovomer (ES only)	0
Arcovomer (ES only)	0
Arcovomer (ES only)	0
Arcovomer (ES only)	0
Arcovomer (ES only)	0
Arcovomer (ES only)	0
Arcovomer (ES only)	0
Arcovomer (ES only)	0
Arcovomer (ES only)	0
Arcovomer (ES only)	0.00153431
Arcovomer (ES only)	0.00153431
Arcovomer (ES only)	0.00153431
Arcovomer (ES only)	0.00153431
Arcovomer (ES only)	0.00153431
Arcovomer (ES only)	0.00153431
Arcovomer (ES only)	0.00153431
Arcovomer (ES only)	0.00153431
Arcovomer (ES only)	0.00153765
Average distance	0.000306936
Average % divergence	0.03%

***Arcovomer* (RJ only)** Pairwise comparison	**TIM2 + I + Γ distances**
Arcovomer (RJ only)	0
Average distance	0
Average % divergence	0%

***Arcovomer* (ES) versus *Arcovomer* (RJ)** Pairwise comparison	**TIM2 + I + Γ distances**
Arcovomer (ES) versus *Arcovomer* (RJ)	0.3049078
Arcovomer (ES) versus *Arcovomer* (RJ)	0.29866478
Arcovomer (ES) versus *Arcovomer* (RJ)	0.29866478
Arcovomer (ES) versus *Arcovomer* (RJ)	0.3016426
Arcovomer (ES) versus *Arcovomer* (RJ)	0.3016426
Arcovomer (ES) versus *Arcovomer* (RJ)	0.3049078
Arcovomer (ES) versus *Arcovomer* (RJ)	0.3049078
Arcovomer (ES) versus *Arcovomer* (RJ)	0.3049078
Arcovomer (ES) versus *Arcovomer* (RJ)	0.3049078
Arcovomer (ES) versus *Arcovomer* (RJ)	0.3049078
Arcovomer (ES) versus *Arcovomer* (RJ)	0.3049078
Arcovomer (ES) versus *Arcovomer* (RJ)	0.3049078
Arcovomer (ES) versus *Arcovomer* (RJ)	0.3049078
Arcovomer (ES) versus *Arcovomer* (RJ)	0.3049078

(*continued*)

Continued

***Arcovomer* (ES) versus *Arcovomer* (RJ) Pairwise comparison**	**TIM2 + I + Γ distances**
Arcovomer (ES) versus *Arcovomer* (RJ)	0.3049078
Arcovomer (ES) versus *Arcovomer* (RJ)	0.3049078
Arcovomer (ES) versus *Arcovomer* (RJ)	0.3049078
Arcovomer (ES) versus *Arcovomer* (RJ)	0.3049078
Arcovomer (ES) versus *Arcovomer* (RJ)	0.3049078
Arcovomer (ES) versus *Arcovomer* (RJ)	0.3049078
Average distance	0.303956978
Average % divergence	30.40%

***Arcovomer* versus *Hamptophryne* Pairwise comparison**	**TIM2 + I + Γ distances**
Arcovomer versus *Hamptophryne*	0.5029524
Arcovomer versus *Hamptophryne*	0.51325321
Arcovomer versus *Hamptophryne*	0.50457168
Arcovomer versus *Hamptophryne*	0.50457168
Arcovomer versus *Hamptophryne*	0.51325321
Arcovomer versus *Hamptophryne*	0.51325321
Arcovomer versus *Hamptophryne*	0.51325321
Arcovomer versus *Hamptophryne*	0.51325321
Arcovomer versus *Hamptophryne*	0.51325321
Arcovomer versus *Hamptophryne*	0.51325321
Arcovomer versus *Hamptophryne*	0.51325321
Average distance	0.510947888
Average % divergence	51.09%

***Arcovomer* versus others Pairwise comparison**	**TIM2 + I + Γ distances**
Arcovomer versus *Gastrophryne*	0.68048829
Arcovomer versus *Gastrophryne*	0.69440699
Arcovomer versus *Gastrophryne*	0.6117546
Arcovomer versus *Gastrophryne*	0.6117546
Arcovomer versus *Gastrophryne*	0.69440699
Arcovomer versus *Gastrophryne*	0.69440699
Arcovomer versus *Gastrophryne*	0.69440699
Arcovomer versus *Gastrophryne*	0.69440699
Arcovomer versus *Gastrophryne*	0.69064498
Arcovomer versus *Gastrophryne*	0.69440699
Arcovomer versus *Gastrophryne*	0.69440699
Arcovomer versus *Gastrophryne*	0.69440699
Arcovomer versus *Dermatonotus*	0.66252363
Arcovomer versus *Dermatonotus*	0.6761027
Arcovomer versus *Dermatonotus*	0.66999835
Arcovomer versus *Dermatonotus*	0.66999835
Arcovomer versus *Dermatonotus*	0.6761027
Arcovomer versus *Dermatonotus*	0.6761027
Arcovomer versus *Dermatonotus*	0.6761027
Arcovomer versus *Dermatonotus*	0.6761027
Arcovomer versus *Dermatonotus*	0.67234159
Arcovomer versus *Dermatonotus*	0.6761027
Arcovomer versus *Dermatonotus*	0.6761027
Arcovomer versus *Dermatonotus*	0.6761027
Arcovomer versus *Elachistocleis*	0.66668141
Arcovomer versus *Elachistocleis*	0.68067634
Arcovomer versus *Elachistocleis*	0.70489311
Arcovomer versus *Elachistocleis*	0.70489311
Arcovomer versus *Elachistocleis*	0.68067634
Arcovomer versus *Elachistocleis*	0.68067634
Arcovomer versus *Elachistocleis*	0.68067634
Arcovomer versus *Elachistocleis*	0.68067634

(*continued*)

Continued

***Arcovomer* versus others Pairwise comparison**	**TIM2 + I + Γ distances**
Arcovomer versus *Elachistocleis*	0.68263263
Arcovomer versus *Elachistocleis*	0.68067634
Arcovomer versus *Elachistocleis*	0.68067634
Arcovomer versus *Elachistocleis*	0.68067634
Arcovomer versus *Elachistocleis*	0.66687667
Arcovomer versus *Elachistocleis*	0.6808638
Arcovomer versus *Elachistocleis*	0.7165572
Arcovomer versus *Elachistocleis*	0.7165572
Arcovomer versus *Elachistocleis*	0.6808638
Arcovomer versus *Elachistocleis*	0.6808638
Arcovomer versus *Elachistocleis*	0.6808638
Arcovomer versus *Elachistocleis*	0.6808638
Arcovomer versus *Elachistocleis*	0.68281901
Arcovomer versus *Elachistocleis*	0.6808638
Arcovomer versus *Elachistocleis*	0.6808638
Arcovomer versus *Elachistocleis*	0.6808638
Average distance	0.680579445
Average % divergence	68.06%

C. Calculation of rates of COI sequence divergence

	Rates	
Calibration Point 1		
Using estimated clade age:	1.48	% corrected sequence divergence/ MY
Using minimum clade age:	2.40	% corrected sequence divergence/ MY (maximum rate)
Using maximum clade age:	1.05	% corrected sequence divergence/ MY (minimum rate)
Calibration Point 2		
Using estimated clade age:	1.65	% corrected sequence divergence/ MY
Using minimum clade age:	2.44	% corrected sequence divergence/ MY (maximum rate)
Using maximum clade age:	1.14	% corrected sequence divergence/ MY (minimum rate)

D. Calculation of divergence time between *Arcovomer* ES and RJ populations on a time scale using the obtained rates of sequence divergence:

Estimation of divergence times based on Calibration Point 1 rates:	
Divergence time:	20.52 MY
Minimum divergence time:	12.65 MY
Maximum divergence time:	29.08 MY
Estimation of divergence times based on Calibration Point 2 rates:	
Divergence time:	18.45 MY
Minimum divergence time:	12.47 MY
Maximum divergence time:	26.64 MY