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Multilocus phylogeny and evolution of a Sky Island species complex reveal Pliocene diversification and unexpected incongruence between morphological and molecular data

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MULTILOCUS PHYLOGENY AND EVOLUTION OF A SKY ISLAND SPECIES COMPLEX REVEAL PLIOCENE DIVERSIFICATION AND UNEXPECTED INCONGRUENCE BETWEEN MORPHOLOGICAL AND MOLECULAR DATA

Dissertação apresentada ao Programa de Pós Graduação em Ecologia, Conservação e Manejo da Vida Silvestre da Universidade Federal de Minas Gerais, como parte das exigências para obtenção do título de Mestre

Orientador: Prof. Dr. Fabrício R. dos Santos

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No dia 25 de abril de 2019, às 14:00 horas, na sala 162, bloco B2 do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, teve lugar a defesa de dissertação de mestrado no Programa de Pós-Graduação em Ecologia, Conservação e Manejo da Vida Silvestre, de autoria do(a) mestrando(a) Fernando Leal Ferreira, intitulado: "**Multi-locus phylogeny and evolution of a Sky Island species complex reveal Pliocene diversification and unexpected incongruence between morphological and molecular data**". Abrindo a sessão, o(a) orientador(a) e Presidente da Comissão, Doutor(a) Fabrício Rodrigues Santos, após dar a conhecer aos presentes o teor das normas regulamentares do trabalho final, passou a palavra para o(a) candidato(a) para apresentação de seu trabalho. Estiveram presentes a Banca Examinadora composta pelos Doutores: Pedro Paulo Goulart Taucce (UNESP RIO CLARO), Adalberto José dos Santos (ICB-UFMG) e demais convidados. Seguiu-se a arguição pelos examinadores, com a respectiva defesa do(a) candidato(a). Após a arguição, apenas os senhores examinadores permaneceram no recinto para avaliação e deliberação acerca do resultado final, sendo a decisão da banca pela:

☒ Aprovação da dissertação, com eventuais correções mínimas e entrega de versão final pelo orientador diretamente à Secretaria do Programa, no prazo máximo de 30 dias;

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Nada mais havendo a tratar, o Presidente da Comissão encerrou a reunião e lavrou a presente ata, que será assinada por todos os membros participantes da Comissão Examinadora.

Belo Horizonte, 25 de abril de 2019.

Comissão Examinadora	Assinatura
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Doutor(a) Pedro Paulo Goulart Taucce	
Doutor(a) Adalberto José dos Santos	

Resumo

Ecossistemas de ilhas de altitude (Sky Islands) são conhecidos por promover altas taxas de especiação e, consequentemente, conter um grande número de espécies endêmicas. A perereca *Ololygon machadoi* habita ilhas de altitude que ocorrem nas montanhas brasileiras das cadeias do Espinhaço e da Canastra. Coletamos espécimes adultos morfologicamente semelhantes a *O. machadoi*, mas com girinos apresentando diferenças conspícuas no padrão de cor, o que levantou dúvidas sobre a identidade taxonômica dessas populações. Para esclarecer essas incertezas taxonômicas, nós redescrevemos *O. machadoi* com base na série de tipos e espécimes de topótipo usando análises de caráter refinadas e análises filogenéticas multi-locus de amostras de toda a faixa de distribuição de espécies. A árvore filogenética recuperou *O. machadoi* como parafilético devido ao posicionamento de populações da Serra da Canastra. Dentro do grande clado formado por amostras do Espinhaço, descobrimos que as diferenças conspícuas no padrão de cor do girino correspondem a uma variação intraespecífica notável, uma vez que um dos morfotipos distintos está aninhado dentro do outro na filogenia. Por outro lado, as análises morfológicas de indivíduos adultos revelaram importantes diferenças de caracteres entre os exemplares tipo e topótipo de O. machadoi e aqueles coletados no Quadrilátero Ferrífero (QF), um maciço ferrífero da região Sul da Serra do Espinhaço, sugerindo uma segunda espécie ainda não descrita. Para investigar a estrutura populacional e os padrões evolutivos dos táxons irmãos O. machadoi e Ololygon sp. QF, realizamos três métodos de delimitação de espécies (GMYC, BPP e STACEY) e também obtivemos uma filogenia datada. O método mais conservador foi o STACEY, que recuperou 12 populações altamente estruturadas dentro de O. machadoi e três dentro do Ololygon sp. QF. A maioria dessas populações está claramente de acordo com a configuração das ilhas de autitude, sugerindo que eventos climáticos vicáriantes têm desempenhado um papel importante na estrutura populacional atual. Essa hipótese também foi apoiada pela filogenia datada, que sugere que a maior parte da diversificação populacional ocorreu durante o Plioceno, um período 2–3 ° C mais quente do que hoje. Este período mais quente provavelmente restringiu as populações ao topo das montanhas, onde a temperatura permaneceu mais amena.

Palavras-chave: Biogeografia; Campo Rupestre; Delimitação de espécies; Espinhaço; Glândulas de pele especializadas; *Ololygon*.

Abstract

Sky islands ecosystems are known to promote high speciation rates and consequently hold high numbers of endemic species. The treefrog *Ololygon machadoi* is a sky island dweller that occurs in the Brazilian highlands of the Espinhaço Range and the Canastra Range. We have collected adult specimens morphologically similar to *O. machadoi* but with tadpoles presenting conspicuous differences in color pattern, which raised doubts about the taxonomic identity of these populations. To clarify these taxonomic uncertainties, we redescribed *O. machadoi* based on the type series and topotype specimens using a refined character analyses and used multi-locus phylogenetic analyses of samples from all the species distribution range and five other *Ololygon* species to test its monophyly. The phylogenetic tree recovered *O. machadoi* as paraphyletic due to the placement of populations from the Canastra Range. Within the large clade formed by samples from the Espinhaço, we have found that the conspicuous differences in the tadpole color pattern correspond to a remarkable intraspecific variation since one of the distinct morphotypes is nested within the other in the phylogeny. On the other hand, the morphological analyses of adult individuals revealed important character differences between the type and topotype specimens of *O. machadoi* from the specimens collected at the Quadrilátero Ferrífero (QF), an iron mountain massif in the South region of the Espinhaço Range, suggesting a second undescribed species. To investigate population structure and evolutionary patterns of the sister taxa *O. machadoi* and *Ololygon* sp. QF, we performed three species delimitation methods (GMYC, BPP and STACEY) and also obtained a dated phylogeny. The most conservative method was the STACEY, which recovered 12 highly structured populations within *O. machadoi* and three within the *Ololygon* sp. QF. Most of these populations are clearly in accordance with the sky island configuration, suggesting that climatic vicariant events have played a major role in the current population structure. This hypothesis was also supported by the dated phylogeny, which suggests that the majority of the populational diversification occurred during the Pliocene, a period 2–3°C warmer than today. This warmer period probably restricted the populations to mountaintops, where the temperature remained cooler.

Keywords: Biogeography; Campo Rupestre; Espinhaço Range; Sepecialized skin gland; *Ololygon*; Species delimitation.

Sumário

1. INTRODUCTION

Understanding the processes driving the geographic distribution of organisms is a key issue to infer their evolutionary mechanisms (Gaston, 2000; Kerkhoff et al., 2014). In insular archipelagos, the dynamics of immigration and extinction are crucial to determine the structure of their communities, as postulated by the theory of island biogeography of MacArthur and Wilson (1967). Oceanic islands are surrounded by a water matrix that acts as a physical barrier to dispersion and gene flow for most of their terrestrial dwellers, promoting population isolation and genetic structuring within each island (Shaw and Gillespie, 2016). In terrestrial areas, patches of mountains separated from each other by lowlands with environmental conditions conspicuously distinct from those in the mountaintops may behave as a continental archipelago, named "sky islands" (Warshall, 1995). The degree of population confinement caused by sky islands will depend on its historical environmental changes, mainly in the intervening lowlands, and the ecology of the species inhabiting the mountaintops (Knowles, 2000; DeChaine and Martin, 2005). These highly dynamic and continuous processes play important roles on species distribution and their populations structure, promoting elevated levels of endemism on sky islands ecosystems (Lomolino et al., 1989; Burgess et al., 2007; Leite et al., 2008; Fjeldså et al., 2012; Chaves et al., 2015).

A remarkable example of sky island diversification is the biota of the Espinhaço Range in eastern Brazil. This Precambrian orogenic formation is the second most extensive mountain range in South America, extending for over 1200 km from the central portion of the Minas Gerais state (MG) to the northern portion of the Bahia state (BA) and harboring an extraordinary amount of endemic species of both animals and plants (Leite et al., 2008; Chaves et al., 2015; Silveira et al., 2016). Besides acting as a single sky island, isolated from others mountain ranges of its surroundings, the presence of many altitudinal discontinuities along the Espinhaço Range makes it also a sky island archipelago, with many endemic species restricted to small areas (Leite et al., 2008; Vasconcelos, 2008; Echternacht et al., 2011). The Espinhaço Range is predominantly composed of quartzite formations with sandy soil and rocky outcrops but it also has ironstone

massifs, mostly on its southernmost portion, in a region known as Quadrilátero Ferrífero (QF) translated as “iron quadrangle” (Silveira et al., 2016). Although having along its range a phytophysiognomy predominantly composed of Campo Rupestre (rupestrian grassland), the vast latitudinal extension of the Espinhaço Range associated with its location between two biomes recognized as global hotspots of biodiversity (Atlantic Forest to the east and Cerrado to the west) and also inserted northwards in a semi-arid biome (Caatinga), provide a large variety of microhabitats and environmental features (Alves and Kolbek, 2010). All these singularities are important factors that contribute to the differential species composition and populational structure among the Espinhaço Range’s sky islands (Rapini et al., 2008).

Among the animal groups inhabiting the mountains of the Espinhaço Range, anuran amphibians present one of the most remarkable rates of endemism. Thirty-nine (27%) of the 145 described species known to occur along its range are endemic, many of them restricted to a very small areas (Leite et al., 2008; Barata et al., 2013; Carvalho et al., 2013; Silva et al., 2013; Araujo-Vieira et al., 2015; Pimenta et al., 2015; Juncá et al., 2015; Walker et al., 2015; Rocha et al., 2017; Leal et al. 2020). This high endemism rate may be related to the limited dispersion ability of amphibians, caused by factors such as their low individual mobility, marked philopatry to birth sites, specialization in the usage of specific habitats and deep dependence of water (Zeisset and Beebee, 2008). These factors, along with the great environmental discontinuity of the Espinhaço Range, probably lead to the highly genetically structured populations of some frog species endemic to the Espinhaço (Ramos et al., 2017; Magalhães et al., 2017; Nascimento et al., 2018). Such richness of endemic species, associated with a high degree of genetic structuring within populations make the Espinhaço Range an area of extreme importance for conservation and a particularly interesting region for studies on species phylogeography and evolution.

Werner C. A. Bokermann was one of the pioneers in the study of the anuran fauna of the Espinhaço Range, being responsible for the description of many of its endemic species since the late 1950s (*e.g.* Bokermann, 1956; 1964; 1967a). In 1973, together with Ivan Sazima, he described *Ololygon machadoi* (as *Hyla machadoi* at that time) for the highlands of the Serra do Cipó, on the Mid-south region of the Espinhaço Range. This species, which belongs to the *O. catharinae* species group (*sensu* Faivovich, 2002), is a small sized tree frog, with adult males measuring around 17

mm, and inhabits streams running over quartzite rocks and sandy soil in the Campo Rupestre (Bokermann and Sazima, 1973). Tadpoles of *O. machadoi* have a very conspicuous coloration, with a dark body transversally crossed by two bright yellow bars and are generally found in backwaters with rocky substratum (Bokermann and Sazima, 1973; Eterovick and Sazima, 2004).

After being described, *O. machadoi* was recorded in a few areas outside its type locality. Eterovick and Barata (2006) and Canelas and Bertoluci (2007) reported *O. machadoi* from the Serra do Caraça, in the QF region, approximately 90 km south from the type locality. Leite et al. (2008) reported the species to the municipality of Conceição do Mato Dentro in the eastern slopes of the Espinhaço, approximately 25 km northeast of the type locality. Although some authors considered *O. machadoi* as endemic to the Espinhaço Range (*e.g.* Leite et al., 2008), Haddad et al. (1988) reported this species in the Canastra Range, another quartzite massif of Southeast Brazil. The identification was based on adults and tadpoles with very similar morphology to some paratypes of *O. machadoi* analyzed by those authors. This population from the Canastra Range it is the only known for occurring out of the Espinhaço Range, being separated from the Espinhaço by approximately 250 km of lowlands. This disjunct distribution may have interesting biogeographic implications as, although both Espinhaço and Canastra ranges have many endemic anuran species, besides *O. machadoi* only another anuran species is currently known to occur strictly in both mountain ranges (*Scinax maracaya*; Cardoso and Sazima, 1980).

In the past 15 years, we have been investigating the herpetofauna of the Espinhaço Range along all of its extension. During field expeditions, we have collected many individuals of *Ololygon* with adult individuals morphologically similar to *O. machadoi*. However, we found that all populations northwards of the type locality have tadpoles with a distinct color pattern. Furthermore, while making a field survey to the Parque Estadual Pico do Itambé, a site also within the Espinhaço Range, we identified a population of what could be *O. machadoi*, but with its tadpoles presenting a third and very distinct morphotype. The morphological variation found in tadpoles from distinct populations related to *O. machadoi* lead us to hypothesize the existence of two undescribed species within the Espinhaço. Taking it in consideration, the present study aims to clarify the taxonomy of populations related to *O. machadoi*, using detailed morphological analyses and with the addition of multilocus phylogenetic inferences to test the species monophyly. In view of the

interesting distribution pattern of this species complex, which is restrict to mountaintops and occurs in two distinct mountain ranges, we also aim to investigate the evolution of the *O. machadoi* species complex, through phylogeographical approaches and dated phylogeny.

2. MATERIAL AND METHODS

2.1. Genetic and taxon sampling

We used genetic data acquired from 106 individuals of *Ololygon machadoi* species complex from 25 localities, covering all the species known distribution and additional populations first reported in this study (Table S1). To test the monophyly of the species, we also sequenced other species of *Ololygon* and other genera of the subfamily Scinaxinae, as fallows: *O. canastrensis* (n = 2), *O. carnevallii* (n = 1), *O. longilinea* (n = 1), *O. pombali* (n = 1), *O. tripui* (n = 1), *Scinax x-signatus* (n = 2) and *Sphaenorhynchus prasinus* (n = 1). We choose these *Ololygon* species based on morphological similarity and geographical proximity to *O. machadoi*. *Sphaenorhynchus* has been repeatedly recovered as the sister taxon of *Ololygon* plus *Scinax* (see Wiens et al., 2010; Pyron and Wiens, 2011; Duellman et al., 2016) and was used to root the tree. We checked the identification of all sequenced samples through morphological analysis of their vouchers. Tissue samples and respective vouchers are deposited in the Centro de Coleções Taxonômicas da Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil (CCT-UFMG) and in the collection of the Museu de Ciências Naturais da Pontifícia Universidade Católica de Minas Gerais, Belo Horizonte, MG, Brazil (MCN-PUC Minas).

We amplified and sequenced the mitochondrial gene COI (Cytochrome c oxidase subunit I) and the nuclear genes POMC (Pro-opiomelanocortin), RAG-1 (Recombination activating gene 1), RHOD (Rhodopsin) and βFib7 (Beta fibrinogen intron 7). First, we extracted genomic DNA from tissue samples of liver and thigh muscle from adult specimens and from tail muscle of tadpoles, all preserved in 95–100% ethanol, following a modified phenol–chloroform protocol (Sambrook and Russel, 2001). Then we performed PCR reactions with a final volume of 15 µl containing 1 µl (20 ng/µl) of genomic DNA, 1.5 µl (10X) of Taq buffer (Thermo Fisher Scientifc), 0.6 µl (50 mM) $MgCl_2$, 1.2 µl (2.5 mM) dNTPs, 0.3 µl (25µM) of each primer, 0.06 µl (5u/tube) of Platinum Taq DNA polymerase (Thermo Fisher Scientifc) and 10.04 µl of Milli-Q water. We followed Santos-

Junior et al. (2015) for PCR product purification, sequencing reactions and DNA precipitation. Forward and reverse sequences were acquired for all samples using an ABI 3120xl/GeneticAnalyser sequencer (Thermo Fisher Scientifc) following the manufacturer specification, at Laboratório de Biodiversidade e Evolução Molecular, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil (see Table S2 for PCR procedures, and Table S3 for primer details).

Sequences were assembled, and chromatograms were visually checked with the software SeqScape v2.6 (Applied Biosystems) and then aligned using the ClustalW algorithm of the software MEGA 7.0 (Tamura et al., 2011) with default parameters. We phased the nuclear genes of all individuals using the PHASE algorithm (Stephens et al., 2001) implemented in DnaSP v5.10.1 (Librado and Rozas, 2009) under default settings.

2.2. Phylogenetic analysis

We performed Bayesian inference (BI) and Maximum Likelihood (ML) analyses, both using a dataset with all genes concatenated. We used the software PartionFinder v2.1.1 (Lanfear et al., 2017) to find the best partitioning scheme and best evolutionary model for the mitochondrial and each of the nuclear genes (Table S4) and used them for both BI and ML analyses. We conducted BI using the software MrBayes 3.1.2 (Ronquist et al., 2012) with two independent runs of 100 million generations, each starting with four Markov chains (one cold), sampling every 10,000 generations. The first 25% generations were discarded as burnin. We used the software Tracer v1.7.1 to visually check for convergence. The ML analysis was conducted in the software IQ-TREE and branch support was assessed using Ultrafast Bootstrap Approximation (UFB; Hoang et al., 2017) of 10,000 bootstrap replicates. The resulting trees of both BI and ML analyses were visualized using FigTree 1.4.3 (Rambaut, 2014).

2.3. Taxonomic assessment and morphological analyses

To obtain an accurate taxonomic assessment of *Ololygon machadoi*, we analyzed its type series and also collected specimens at the type locality. We used the morphological data acquired from the type series and topotypic specimens to find out if the sequenced specimens and the specimens from populations that we could not access genetic data correspond to *O. machadoi*.

For tadpole analyses and comparisons, we used as reference information from the original description of the species (Bokermann and Sazima, 1973) and from larvae collected at the type locality.

We took eight morphometric measurements of adult specimens under a stereomicroscope with a digital caliper to the nearest 0.1 mm, as follow: snout-vent length (SVL – from the tip of the snout to the cloacal opening), head length (HL – from the tip of the snout to the posterior corner of the jaw), head width (HW – between the jaw edges at tympanum level), eye-snout distance (ESD – from the anterior margin of the eye to the tip of the snout), tympanum diameter (TD – between anterior and posterior outer margins of the tympanic annulus), thigh length (THL – took with the thigh flexed approximately at an angle of 45º with the body, from the center of the cloacal opening to the outer edge of the knee), tibia length (TIL – from the outer edge of the knee to the outer edge of the heel), and foot length (FL –from the outer edge of the heel to the tip of the fourth toe).

We analyzed the following morphological traits of the examined specimens and, when relevant, used them for comparisons: color pattern (in preserved specimens) of head, dorsum of body and limbs, flank, inguinal region, hidden surfaces of thigh and tibia, ventral region of limbs, gular region, chest and belly; snout format in dorsal and lateral views; canthus rostralis format; loreal region format; presence of vocal slit in adult males; development of vocal sac in adult males; presence and development of nuptial pad in adult males; color of nuptial pad; presence of mental gland; presence of inguinal gland; presence of chest gland; presence of pectoral fold; forearm robustness of males in relation to females; heelpiece tubercle; dorsal texture; color in life of dorsum of the body and head, inguinal region, hidden surfaces of thigh and tibia, gular region, chest and belly. We also assessed characteristics of live specimens of both adults and tadpoles of the *O. machadoi* species complex through photographs and/or personal observation of the collectors (most of them from F.L. and F.S.F.L., authors of this study; see Table S5 for examined specimens belonging to *Ololygon machadoi* species complex).

Moreover, we analyzed adult individuals of 18 species from the *O. catharinae* group to use for comparison, as fallow: *O. angrensis*, *O. argyreornata*, *O. aromothyella*, *O. berthae*, *O.*

canastrensis, *O. carnevallii*, *O. catharinae*, *O. flavoguttata*, *O. heyeri*, *O. littoralis*, *O. longilinea*, *O. luizotavioi*, *O, melanodactyla*, *O. obtriangulata*, *O. pombali*, *O. ranki*, *O. rizibilis* and *O. tripui*. Specimens are housed in the following Brazilian institutions: Amphibian Collection of Centro de Coleções Taxonômicas da Universidade Federal de Minas Gerais, Belo Horizonte, MG (CCT-UFMG), Museu de Zoologia João Moojen of Universidade Federal de Viçosa, Viçosa, MG (MZUFV) and Museu de Zoologia, Universidade de São Paulo, São Paulo, SP (MZUSP) (see Table S6 for examined specimens used for comparison). Morphological data of the remaining specimens of the *O. catharinae* group were acquired from bibliography.

2.4. Population assignment

Multispecies coalescent methods were already demonstrated to fail on the purpose of delimiting species but to succeed in recovering population structure (Sukumaran and Knowles, 2017). Therefore, to delimit population boundaries of *Ololygon machadoi* we conducted a sequence of three species delimitation approaches: first we used the Generalized Mixed Yule Coalescence (GMYC; Fujisawa and Barraclough, 2013) to discover lineages, then we used the Bayesian Phylogenetics and Phylogeography (BPP; Yang, 2015) software and the STACEY algorithm implemented in BEAST2 version 2.5.1 (Bouckaert et al., 2014) to validate the lineages delimited by the GMYC. We followed the population boundaries obtained by the most conservative analyses (the one with minimum number of validated populations).

For the GMYC analysis we used unique haplotypes of COI to obtain an ultrametric tree using BEAST2. We set the molecular clock under lognormal relaxed model and used Yule speciation process as prior, running 100 million generations and sampling every 10,000 generations. The first 10% generations were discarded as burnin. We used the resulting tree to perform a single-threshold GMYC analysis using the web server https://species.h-its.org/gmyc/ that runs the original R implementation of the GMYC model.

For the BPP analysis we used the complete dataset of molecular markers, assigning a priori each individual to its population accordingly to the GMYC results. We used the A10 algorithm (species delimitation with user-specified guide tree) running 5 million generations, sampling every five generations and 25% generations were discarded as burnin. We checked the robustness of

speciation probabilities by running three different prior combinations that represent different ancestral population size (θ) and root age (τ): (i) large ancestral population sizes and deep divergence: both θ and τ set to G(1,10); (ii) large ancestral population sizes and recent divergences: θ and τ set to G (1,10) and G(2,2000) respectively; and (iii) small ancestral population sizes and recent divergences: both θ and τ set to G(2,2000).

For the STACEY analysis, implemented in BEAST2, we also used our complete dataset of molecular markers. We assigned a priori each individual to its population according to the results of the GMYC. Nucleotide substitution models were inferred with BEAST Model Test option of the program with all-reversible mutation rate (Bouckaert and Drummond, 2017). We set the molecular clock under lognormal relaxed model and used the Fossilized Birth Death Model as prior. We ran 100 million generation sampling every 10,000 generations and discarded the first 10% generations as burnin. We then access the statistical support of the species (population) delimitation with the SpeciesDelimitationAnalyser (Jones et al., 2014).

2.5. Genetic distance of COI

We calculated uncorrected pairwise distances of COI in MEGA7 (Kumar et al., 2016). Pairwise distances were calculated both between delimited populations of the *Ololygon machadoi* and between all *Ololygon* species included in our phylogenetic analyses.

2.6. Divergence times

We inferred a dated species tree in the software BEAST2 using the complete dataset of both genes and samples. Individuals were grouped according to the clades inferred by the BI analyses. We used the Yule speciation process as prior, set a relaxed lognormal clock model for the mitochondrial partitions and strict clock model for the nuclear partitions. Nucleotide substitution model was inferred with BEAST Model Test package with all-reversible mutation rate (Bouckaert and Drummond, 2017) implemented in BEAST2. For the COI gene, we used a mean substitution rate of 0.020321 mutation/site/million with the standard deviation of 0.15, as estimated by Nascimento et al. (2018) for *Hyla*, a genus belonging to the same family of *Ololygon.* Substitution rates of the nuclear genes were coestimated by the program. We ran 800 million generations sampling every 20,000 generations of which 25% were discarded as burnin. We used the software

Tracer v1.7.1 to visually check for convergence. Finally, we summarized the species tree with TreeAnnotator 2.5.1 (Bouckaert et al., 2014) using a maximum clade credibility tree and median heights as node ages. The resulting tree was visualized using FigTree 1.4.3 (Rambaut, 2014).

3. RESULTS

3.1 Genetic data collection

We obtained sequence data of five loci from 115 individuals, including: 106 individuals belonging to the *Ololygon machadoi* species complex from 25 localities covering all of its known distribution and many additional localities; six individuals from five species belonging to the *O. catharinae* species group; two individuals of a species of *Scinax* and one individual of *Sphaenorhynchus*. We obtained 2304 bp with the complete dataset of the mitochondrial gene and the four nuclear genes. The COI dataset covered all individuals and resulted in sequences of 630 bp of which 240 (~38 %) were polymorphic sites, 24 of them being singletons. The POMC dataset was obtained for all but one individual and resulted in sequences of 522 bp of which 128 (~25 %) were polymorphic sites, 27 of them being singletons. The RHOD dataset was also obtained for all but one individual and resulted in sequences of 315 bp of which 45 (~14 %) were polymorphic sites with only one singleton. The RAG1 dataset was obtained for 112 individuals (three missing) and resulted in sequences of 426 bp of which 45 (~13 %) were polymorphic sites, 15 of them being singletons. The βFib7 dataset was obtained for 104 individuals (11 missing) but still covered all localities, it resulted in sequences of 410 bp of which 67 (~16 %) were polymorphic sites, 10 of them being singletons. GenBank accession numbers and voucher detail are presented in Table S1.

3.2. Phylogenetic analyses

The BI (Fig. 1) and ML analysis of all genes concatenated recovered a very similar topology (see Fig. S1 and Fig. S2 for BI and ML trees respectively, both with referenced terminals and node probabilities). The following values presented in parenthesis are the BI posterior probability and ML bootstrap, respectively; a single value refers to clades recovered only in one or other indicated analyses. Both analyses recovered *Ololygon* as monophyletic (1.0, 100), sister to *Scinax*. *Ololygon machadoi*, however, was recovered as paraphyletic due to the placement of populations from

Canastra Range (referred as *Ololygon* sp. Canastra from now on). *Ololygon* sp. Canastra was recovered as monophyletic (1.0, 98), with the three localities with sequenced samples being divided in two highly supported main clades, one (1.0, 100) composed of individuals from the municipality of São Roque de Minas and other (1.0, 99) grouping individuals from the municipalities of Capitólio and Delfinópolis.

Ololygon sp. Canastra was placed in a highly supported clade (1.0, 100) as the sister taxon to a clade (1.0, 100) composed of *O. canastrensis* and *O. longilinea*. The relationships of the clade composed of *Ololygon* sp. Canastra, *O. canastrensis* and *O. longilinea* with the remaining species was incongruent between BI and ML analyses. The BI recovered this clade as sister group to a poorly supported clade (0.78) composed of *O. tripui*, *O. carnevallii*, *O. pombali* and these three species as sister group to all the remaining populations assigned to *O. machadoi*. On the other hand, the ML recovered this clade within a poorly supported clade (68) as sister group to *O. tripui*, *O. carnevallii* and *O. pombali*. The relationship among these last three species, however, was well supported and congruent between the BI and ML analyses, with *O. tripui* being the sister taxon to *O. canevallii* (1.0, 83) and both being sister to *O. pombali* (0.99, 83).

The clade formed by all populations of *O. machadoi* from the Espinhaço Range was highly supported (1.0, 99). Relationships within this clade were mostly well supported in both BI and ML analysis and were fairly congruent between both analyses. The only exception was the placement of the clade with individuals from the type locality. The BI recovered this clade as fully supported (1.0) and sister to all the remaining sampled localities of *O. machadoi*, whereas the ML analysis recovered this clade as poorly supported (52) and sister to a clade composed of individuals from Serra do Cipó (Atlantic slope), Serra do Ambrósio, Pico do Itambé, Serra D'anta and Acauã.

3.3. Taxonomic assessment

3.3.1. Morphological analyses

We analyzed external morphology of 547 adult individuals belonging to the *Ololygon machadoi* species complex, including its holotype, 42 paratypes,12 topotypes and 492 individuals from other localities. We also analyzed tadpoles from 113 lots (Table S5) and assessed characteristics of live specimens of both adults and tadpoles from all localities used in the molecular analyses

and from most of the remaining localities with available specimens of the *O. machadoi* species complex.

From the *Ololygon* sp. Canastra we could only access morphological and genetic data from individuals in larval stages. Although this population does not form a monophyletic group with *O. machadoi,* its tadpoles (Fig. 2A) have exactly the same color pattern found in tadpoles of *O. machadoi* from the type locality (Fig. 2B) and from the QF region, which is composed of a dark body transversally crossed by two bright, dorsal yellow bars.

Populations of *O. machadoi* from the QF region presented conspicuous morphological differences from those of other populations from the Espinhaço Range. Adult males from the QF (referred as *Ololygon* sp. QF from now on) lack vocal slits whereas adult males of *O. machadoi* present well developed vocal slits; adult males of *Ololygon* sp. QF lack a closely packed and protruded white or brown chest gland, a structure present in *O. machadoi* (Fig. 3); adult females of *Ololygon* sp. QF present glandular acini scattered over mental region, chest, flanks and inguinal region (Fig. 4) whereas adult females of *O. machadoi* lack glandular acini over these areas. Despite those differences, as in *Ololygon* sp. Canastra, *Ololygon* sp. QF also has tadpoles presenting dark body transversally crossed by two bright yellow bars, the same color pattern found in tadpoles of *O. machadoi* from its type locality.

Among the remaining analyzed populations of *O. machadoi*, we did not find any character of adult external morphology that could be useful to distinguish these populations from the type series. All the variation that we found presented a large overlap among and within populations (see the next section for details of variation). On the contrary, the color pattern of tadpoles of *O. machadoi* from different localities presented population consistent variation. The color pattern found in tadpoles from the type locality (Fig. 2B) also occurs in a very few localities close to the type locality, mainly in the mountaintops and western slopes, at municipalities of Morro do Pilar and Santana do Riacho. All other sampled localities and sequenced populations have light brown tadpoles bearing the posterior half of the tail abruptly darkened or with irregularly shaped dark blotches (Fig. 2C). The single exception to this color pattern is the population from the Pico do Itambé State Park, which presents yellow tadpoles with only the posterior region of the body dark

(Fig. 2D). Although the color pattern of tadpoles presents population structured morphological variation, the phylogenetic placement of the population from Pico do Itambé State Park makes this character problematic to be used as diagnosis to putative new species since it would imply in the species with black tail tadpoles being paraphyletic. For this reason, we will consider these color patterns of tadpoles as populational variation of *O. machadoi* (see also Fig. 9 to visually check the phylogenetic distribution of the color patterns of tadpoles).

3.3.2. Species accounts

Ololygon machadoi (Bokermann and Sazima 1973). Redescription (Figs. 3A–C, 5, 6, 7, S3, S4)

Hyla machadoi – Bokermann and Sazima, 1973

Scinax machadoi – Duellman and Wiens, 1992

Ololygon machadoi – Duellman, Marion and Hedges, 2016

Holotype: MZUSP 73669 (WCAB 46847), adult female (specimen examined; Figs. 5, 6), 29 April 1972, Serra do Cipó, Alto Palácio region at km 121 (-19.2631044°, -43.5285244°; 1318 m above sea level; datum WGS84) of the road (currently MG-10), Municipality of Morro do Pilar (previously belonging to the Municipality of Jaboticatubas), Minas Gerais state, Brazil, collected by Ivan Sazima and Marlies Sazima.

Paratypes: All specimens from the same region of the holotype at Serra do Cipó, Alto Palácio, Municipality of Morro do Pilar, Minas Gerais state, Brazil: WCAB 2455–2458, collected in the same locality of the holotype by Angelo B. M. Machado in February 1957. WCAB 14701 (specimen examined, now under the number MZUSP 73724), collected in the same locality of the holotype by Werner C. A. Bokermann e Angelo B. M. Machado on 10 February 1964. WCAB 46250–46252, 47448–47449, collected at km 120 (-19.255587°, -43.538206°; 1385 m above sea level; datum WGS84) by Werner C.A. Bokerman, Ivan Sazima and Marlies Sazima in February 1972. WCAB 46253–46258, 47450–47456, collected at km 126 (-19.231204°, -43.507044°; 1343 m above sea level; datum WGS84) by Werner C.A. Bokerman, Ivan Sazima and Marlies Sazima in February 1972.WCAB 47457-58, collected in the same locality of the holotype by Ivan Sazima, Marlies Sazima on 1st May 1972. WCAB 47419 (examined specimen, now under the number MZUSP 73675), collected at km 126 by Ivan Sazima and Marlies Sazima in July 1972. WCAB 47459, collected at km 126 by Ivan Sazima and Marlies Sazima in September 1972. WCAB 47460–47476,

collected at km 126 by Ivan Sazima and Marlies Sazima in January 1973. WCAB 47477-47534, collected at km 126 by Ivan Sazima, Marlies Sazima and O. C. Oliveira in February 1973 (examined specimens: WCAB 47481, 47482, 47484–47488, 47491, 47493–47495, 47500, 47501, 47503, 47505, 47506, 47508, 47509, 47513–47520, 47522, 47523, 47525, 47527–47534, now under the numbers MZUSP 74109–74145; WCAB 47483, 47489, 47496 and 47498, now under the numbers MZUSP 73763–73766).

Diagnosis: (1) adult male SVL = 14.3–26.3 mm (2) dorsal pattern composed of four blotches with variable size (Figs. 3A–C, 5A); (3) presence of yellow flash color on inguinal region and hidden surfaces of thighs in live individuals (Fig. 7); (4) presence of vocal slits in adult males; (5) presence of glandular nuptial pads, not hypertrophied, in adult males; (6) presence of macroscopically evident chest gland in adult males (Figs. 3A–C); (7) absence of inguinal gland; (8) absence of pectoral fold (Figs. 3A–C, 5B); (9) forearms not hypertrophied in adult males (Figs. 3A–C).

Comparison with other species: (1) By having adult males with SVL = 14.3–26.3 mm, *Ololygon machadoi* is set apart from *O. angrensis*, *O. brieni*, *O. canastrensis*, *O. catharinae*, *O. flavoguttata*, *O. jureia*, *O. kautskyi* and *O. muriciensis* (combined SVL = 27.0–34.5 mm; Heyer et al., 1990; Pombal and Gordo, 1991; Carvalho-e-Silva and Peixoto, 1991; Cruz et al., 2011). (2) Dorsal pattern composed of four blotches with variable size distinguishes *O. machadoi* from *O. angrensis* (which has anastomosed dorsal pattern), from *O. aromothyella*, *O. berthae*, *O. littoreus*, *O. obtriangulata* and *O. strigilata* (a longitudinal stripe from the upper eyelid to the inguinal region on those species; Pimenta et al., 2007). (3) The presence of yellow flash color on inguinal region and hidden surfaces of thighs in live individuals differentiates *O. machadoi* from *O. albicans*, *O. angrensis*, *O. caissara*, *O carnevallii*, *O. kautskyi*, *O. luizotavioi*, *O. melanodactyla* and *O. obtriangulata* (inguinal region and hidden surfaces of thighs without flash color on those species; Bokermann, 1967b; Lutz, 1973; Caramaschi and Kisteumacher, 1989; Carvalho-e-Silva and Peixoto, 1991; Lourenço et al., 2013, 2014, 2016), from *O. ariadnae* (which has inguinal region and hidden surfaces of thighs with violet or pink color; Lourenço et al., 2014), from *O. brieni*, *O. catharinae*, *O. humilis* and *O. trapicheiroi* (inguinal region and hidden surfaces of thighs with blueish color on those species; Lourenço et al., 2013, 2014), from *O. flavoguttata*, *O. heyeri* and *O. skuki* (inguinal region and hidden surfaces of thighs with orange color on those species; Lima et al., 2011; Lourenço et al.,

2014) and from *O. hiemalis*, *O. jureia*, *O. littoralis*, *O. muriciencis*, *O. ranki*, *O. skaios*, *O. strigilata* and *O. tripui* (inguinal region and hidden surfaces of thighs with greenish color on those species; Andrade and Cardoso, 1987; Haddad and Pombal, 1987; Pombal and Gordo, 1991; Pimenta et al., 2007; Pombal et al., 2010; Cruz et al., 2011; Lourenço et al., 2014). (4) The presence of vocal slits in adult males distinguishes *O. machadoi* from *O. ariadne* and *O. skaios* (vocal slits absent in adult males of those species; Lourenço et al., 2014). (5) The presence of glandular nuptial pads, not hypertrophied, in adult males distinguishes *O. machadoi* from *O. pombali* (which has glandular acini not closely packed forming a pad) and from *O. rizibilis* (which has hypertrophied nuptial pad). (6) The presence of macroscopically evident chest gland in adult males distinguishes *O. machadoi* from *O. angrensis*, *O. argyreornata*, *O. aromothyella*, *O. berthae*, *O. canastrensis*, *O. carnevallii*, *O. catharinae*, *O. flavoguttata*, *O. heyeri*, *O. littoralis*, *O. longilinea*, *O. luizotavioi*, *O, melanodactyla*, *O. obtriangulata*, *O. pombali*, *O. ranki*, *O. rizibilis* and *O. tripui* (chest gland absents in those species). (7) The absence of inguinal gland distinguishes *O. machadoi* from *O. ariadne*, *O. brieni*, *O. caissara*, *O. canastrensis*, *O. catharinae*, *O. centralis*, *O. flavoguttata*, *O. hiemalis*, *O. jureia*, *O. longilinea*, *O. luizotavioi*, *O. obtriangulata*, *O. ranki* and *O. rizibilis* (inguinal gland present in adult males of those species; Pombal and Bastos, 1996; Lourenço et al., 2014, 2016). (8) The absence of pectoral fold distinguishes *O. machadoi* from *O. agilis* and *O. melanodactyla* (pectoral fold present in those species; Lourenço et al., 2014); (9) By having forearms not hypertrophied in adult males O. machadoi is distinguished from *O. aromothyella*, *O. longilinea*, *O. melanodactyla*, *O. rizibilis* and *O. goya* (forearms hyperthophied in adult males of those species; Faivovich, 2005; Lourenço et al., 2014; Andrade et al., 2018).

Redescription of holotype: Sub-adult female, SVL 20.6 mm. Head longer than wide, its width 87% of its length; head width 34 % of SVL and length 39% of SVL. Snout sub-elliptical in dorsal view, slightly mucronate; protruding in lateral view. *Canthus rostralis* distinct, slightly concave; loreal region slightly concave. Snout expanding beyond lower jaw. Nostril dorsolaterally oriented, slightly protruding. Internarial region concave; top of the head flat. Eyes prominent, antero-laterally oriented. Tympanum distinct externally, rounded. Supratympanic fold absent. Dentigerous process of vomer distinct, in two contiguous slightly curved series making an obtuse angle, located between the choanae; each series bears 3 teeth. Premaxillary and maxillary teeth

present. Choanae oval, separated between each other by a distance as large as four times its maximum diameter. Tongue rounded, attached overall (narrowly free around lateral and posterior margin). Vocal slits and vocal sac absent. Forearms not hypertrophied, bearing some scattered tubercles on its external edge; upper arms slender. Fingers slender, relative lengths I = II = III < IV; fingers tip slightly elliptical, laterally expanded. Webbing absent between fingers II and III, extremely reduced between fingers III and IV, and IV and V. Subarticular tubercles present, simple, prominent and rounded; supernumerary tubercles discrete. External metacarpal tubercles ovoid, large and prominent, bilobed on its distal portion. Inner metacarpal tubercles ovoid, large and prominent. Nuptial pad absent. Tibia length 54 % SVL; foot length 70 % SVL. Toes slender, relative lengths I < II < V = III < IV; toes tip slightly elliptical, laterally expanded. Webbing formula $I2^{+}$ - $2^{+}II1^{1/3}$ – $2^{1/2}III1^{1/3}$ -$2^{+}IV2^{+}$ - $1^{+}V$. Subarticular tubercles distinct, simple, prominent and rounded; supernumerary tubercles present, small and rounded. Tarsal fold absent. External edge of foot crenulated; external edge of tarsus bearing scattered tubercles. Inner metatarsal tubercles distinct, ovoid; outer metatarsal tubercle distinct, rounded. Inguinal gland absent. Cloacal opening directed posteriorly at upper level of thighs. Region below the cloaca granular, tubercles reach the thighs at its posteroventral edge, where tubercles become increasingly smaller and lower. Texture of hidden and ventral surface of the limbs smooth; belly, chest and gular region granular. Pectoral fold absent. Dorsal surfaces of the head and body rugose and presenting scattered tubercles; limbs are slightly rugose.

Measurements (mm) of the holotype are: SVL 20.6; HL 8.1; HW 7.0; ESD 3.8; TD 1.3; THL 10.3; TL 11.1; FL 14.4.

Remarks: The presence of a sexually dimorphic chest gland in adult males of *Ololygon machadoi* is a remarkable character, being reported to *Ololygon* for the first time in the present work. We have found this structure in all analyzed populations of *O. machadoi,* including its type series. The level of external discernibility of the chest gland varied among individuals and populations, mainly accordingly to its ventral color and, apparently, to the time that the specimen was preserved, being more difficult to be identified in specimens preserved for a very long time. In preservative the gland is distinctly discernible through the skin as a closely packed and protruded white or brownish structure (Figs. 3A–C, S3).

In the original species description, Bokermann and Sazima (1973) identified the holotype as being a male. We examined the specimen and noticed that it lacks secondary sexually dimorphic structures such as nuptial pads, chest gland and vocal slits. For these reasons we redetermined the sex of the holotype as a female. Additionally, due to the lack of visible developed oocytes and the smaller size in relation to other female paratypes (holotype SVL = 20.6 mm; female paratypes with visible developed oocytes SVL = 22.17–25.4 mm; n = 3), the holotype is probably an immature specimen. Additionally, it is noteworthy that the original description of *O. machadoi* did not present any photograph or detailed drawing of the holotype, the only photograph of this species presented in that article is of a paratype. In this way, the present work presents for the first time a detailed photograph of the holotype of *O. machadoi* (Figs. 5, 6).

Color pattern of the holotype in preservative: General color in shades of brown. Interorbital blotch trapezoidal, outlined by a lighter edge, extending from the superior border of one eyelid to the other, its short base posteriorly directed and with a division making it resembles a W-shape. *Chanthus rostralis* with a faintly evident longitudinal dark stripe over it. Irregular blotches over the loreal region and upper lips. A dorsolateral pale white stripe from the posterior corner of the eyes to the posterior portion of the flank. Dorsum of the body with four distinct blotches; the anterior two are triangular-shaped, covering the posterior portion of each eyelid, slightly transversally oriented and reaching the middle of the flank, bordered by a distinct light stripe on their external edge; the two posterior blotches are also transversally oriented, from the middle of the dorsum to the posterior region of the flanks. Distinct transversal dark bars over the dorsal surface of forearms, thighs, tibias, tarsi and feet, invading the hiding surfaces of the thighs and tibia, where they become more irregular-shaped. Inguinal region with irregularly-shaped light blotches, tending to be rounded. Ventral surfaces without distinct blotches only with scattered melanophores, more concentrated on palmar and plantar regions.

Variation within type series: Measurements and main proportions of 33 adult male paratypes and three adult female paratypes are presented in Table 1. Snout format may be acute in lateral view, subovoid to rounded in dorsal view (Figs. 3A–C); mucronation may varies from absent (Fig. 3A) to very distinct (Fig. 6A). Adult males present sexual dimorphism by having vocal slits, glandular nuptial pads and chest gland. Nuptial pads and chest gland may vary in development

degrees. Interorbital blotch may be trapezoidal with no division on the short base; may have division on its short base in different levels (Figs. 3A–C, 6A), resembling a W-shape; may be triangular; may be an elongated bar, may be elongated but irregularly shaped and may be fused to the two anterior dorsal blotches. Upper lips may display a distinct light spot below the eyes and a distinct transversal light stripe from the inferior margin of eyes to the corner of the mouth. The dorsolateral pale white stripe may be more or less distinct, almost indistinct in some individuals. Dorsal color pattern is highly variable, the four blotches on dorsum of body may vary in size, from very small and sparsed, to large and occupying almost all the dorsum of the body or even being in contact to each other. Dorsal blotches tend to be elongated, longitudinally or diagonally oriented toward the flanks (Figs. 3A–C, 6A). Two small longitudinal stripes may be present margin the urostyle. Transversal dark bars over the dorsal surface of forearms, thighs, tibias, tarsi and feet may be more or less distinct; blotches on anterior and posterior surface of thighs may vary in shape, from being an extension of dorsal bars to being diffuse and irregularly shaped. Ventral surface may have dark irregularly shaped blotches, mainly on belly and throat.

Color in life: When in activity, males have dorsal color pattern ranging from pale yellow to beige for both blotches and background color, making the blotches poorly contrasted and almost indistinct in some individuals (Figs. S4A,C,D); females are darker and with dorsal blotches more distinct. Ventral color of males ranges from pale yellow to white (Fig. 7); some individuals may have black and white vermiculation mainly over the chest and gular region, yellow spots scattered over the belly, chest and/or gular region; belly and chest may be slightly translucent and/or present iridophores scattered. In live individuals, chest gland is usually less discernible and not protrude from surround skin as in preserved individuals; when visible, are covered by white and/or yellow coloration (Fig. 7B,C). Females have ventral color homogenously pale white (Fig. 7A) to light brown. After being manipulated, both males and females rapidly change their color, getting darker shades mainly on their dorsal blotches, becoming much more contrasted (Fig. 7, S4B); background color may be pale beige, brownish, yellowish, golden or greenish. Males and females have hidden surface of thighs, tibia and inguinal region with yellow flashy color, also present on axillary region of some individuals (Fig. 7). Iris color ranges from silver-grey to orange, with black vermiculation, being bicolored in some specimens with upper portion yellowish orange

and lower portion grey. Black vermiculation of iris may be more concentrated on the corners, above and below pupil, resembling a cross shaped.

Adult morphology across distribution: There are a great variation in morphological measurements among adult individuals (Table 1). Individuals from P.E. Pico do Itambé seem to become more silver colored in preservative; have gular, pectoral and belly regions whiter and have pectoral gland less distinct than other populations. The granularity degree of dorsum varies among individuals and within individuals, which usually become much less granular after being manipulated. No other significant variation was found.

Distribution and natural history: *Ololygon machadoi* is latitudinally distributed through 800km along the Espinhaço Range (Fig. 8), from the Serra do Cipó National Park in its Mid-south portion (MG state) to the Chapada Diamantina National Park in its North portion (BA state). It has a wide range of altitudinal distribution, occurring from 550 m above sea level (masl) at Acauã Ecological Station, municipality of Leme do Prado, MG state, to 1711 masl at Pico do Itobira, municipality of Rio de Contas, BA state. The mean occurrence elevation of the species is of 1048 masl (n = 148 distinct occurrence points).

Ololygon machadoi is mainly associated with permanent streams, occurring very rarely in temporary ones. This may be due to the long time that its tadpoles take to metamorphose, probably not being fast enough to complete its larval stage during the rainy season, when temporary streams still exist. In a few cases we have found tadpoles in puddles on the side of the streams, very close to it. Although its breeding activity peak occurs during the rainy season, from November to February, it is possible to find tadpoles and even a few adult individuals in calling activity during most part of the year. Despite its preference for permanent streams, *O. machadoi* presents a notable plasticity in relation to other characteristics of habitat usage. It can be found in streams in: open areas of Campo Rupestre (Fig. S5A) with rocky, sandy and/or mud substrate, gallery forest (Fig. S5B), nebular forest (Fig. S4C) and vereda formations (Fig. S4D). Furthermore, some localities in which we have found this species were impacted by anthropic activities such as cattle raising, and human settlement, being even apparently polluted.

When in calling activity, males of *O. machadoi* use to be perched on branches and leaves (Figs. S4A,C,D) at stream banks, mainly in backwater areas. Males may call singly or in chorus, generally not exceeding around eight males per calling site, but we have seen situations with an elevated degree of aggregation, such as up to 20 calling males in an area smaller than two square meters.

3.4. Population assignment

The GMYC analysis delimited 18 mitochondrial clusters within the highly supported clade containing samples of *Ololygon machadoi* from 18 localities along the Espinhaço Range plus three localities from the QF region (Fig. 1). The BPP analyses with three prior combinations of different ancestral population size and divergence times resulted in three distinct clusterings. When assuming small ancestral population size and recent divergence time, all the 18 clusters delimited by the GMYC were recovered with values of posterior probability higher than 0.95. When assuming large ancestral population sizes and recent divergence time, the two clusters of the Pico do Itambé were grouped (posterior probability of 0.84). The prior considering large ancestral population size and deep divergence time (Fig. 1) grouped the two clusters of Pico do Itambé (posterior probability of 0.65) and also the clusters referring to Grão Mogol and Serra Nova (posterior probability of 0.93). Finally, the STACEY analysis recovered 15 clusters with posterior probability of 0.98 (Fig. 1). It grouped the two clusters from the Pico do Itambé in a single one and also the clusters from Grão Mogol, Serra Nova and Botumirim. All 15 clusters correspond to well supported monophyletic groups found by both BI and ML analysis. From now on we will treat each of these clusters as different populations numbered from 1 to 15 (Fig. 1) to facilitate their referencing along the text (see also Fig. 8 to check geographical distribution of the populations).

3.5. Genetic distance of COI

The uncorrected pairwise distance of COI among *Ololygon machadoi* populations (populations 4 to 15) showed high values for most of the pairwise population distances, ranging from 2.5% between population 13 and 14, to 9.7% between populations 7 and 14 (Table 2). The mean distance within *O. machadoi* was 7.4% (SD = 0.5%). Among the populations of *Ololygon* sp. QF the distances were overall smaller, ranging from 2.7% between populations 2 and 3, to 5.6% between populations 1 and 3 (Table 2). The mean distance within *Ololygon* sp. QF was 3% (SD = 0.4%).

Between species, the uncorrected pairwise distance presented great variation (Table 3). The mean distance between *O. machadoi* and the *Ololygon* sp. QF was 9.5% (SD = 0.7%). and between *O. machadoi* and *Ololygon* sp. Canastra was 14.9% (SD = 1.0 %). The lowest value among species pairs was 4.4% (SD = 0.07%) between *O. longilinea* and *O. canastrensis*, and the highest was 20.3% (SD = 1.3%) between *O. carnevallii* and *O. pombali*.

3.6. Divergence time

Our results of divergence time estimation suggest that *Ololygon machadoi* diverged from *Ololygon* sp. QF around 11.2 million years ago (mya) during the late Miocene (Fig. 9). Within *O. machadoi*, the first split occurred around 7.9 mya, also during the late Miocene, separating the lineage of its type locality at the highland of the Serra do Cipó (pop. 04), in the Mid-south of the Espinhaço Range, from the one that originated all the remaining populations. The late Miocene marked two other major splits among the remaining populations of *O. machadoi*. First, around 6.3 mya a split occurred originating a lineage comprising populations 5 to 9, which are distributed in the Mid-south of the Espinhaço Range. Then, around 5.6 mya a split occurred in the remaining lineage originating a clade composed of populations 10 to 12, distributed in the mid-north of the Espinhaço Range, and other clade composed of populations 13 to 15, occurring in the north of the Espinhaço Range. The diversification of all populations within the main lineages of *O. machadoi* occurred during the Pliocene, between 2.9 and 4.8 mya.

The Pliocene also marked the diversification of lineages of *Ololygon* sp. QF and *Ololygon* sp. Canastra. Around 2.9 mya, the population of *Ololygon* sp. QF from Serra da Cambota (pop. 03) diverged from the lineage that originated the populations from Serra do Gandarela and Serra do Caraça (pop. 01 and pop. 02 respectively). The origin of *Ololygon* sp. Canastra is dated to around 4.6 mya and the split between the clade composed of individuals from São Roque de Minas and the ones from Capitólio and Delfinópolis occurred around 3.8 mya.

4. DISCUSSION

4.1. Phylogeny and morphology of the *Ololygon machadoi* species complex

Our analyses recovered some remarkable and unexpected relations among populations belonging to the *Ololygon machadoi* species complex. Although tadpole color pattern has been recurrently used in species diagnosis (Garcia et al., 2008; Silva and Alves-Silva 2008; Anstis et al., 2010; Silva and Alves-Silva 2011; Lourenço et al., 2013), our results have shown that distinct and even not closely related species may have tadpoles sharing exactly the same conspicuous color pattern and that different populations of a single species may present tadpoles with very distinct color patterns (see Figs. 2 and 9). As far as we know, this study is the first to report, in anurans, such level of incongruence. Below we discuss peculiarities found in *O. machadoi* and in each of its related putative species.

4.1.1. *Ololygon* sp. Canastra

The assignment of populations from Canastra Range to *O. machadoi* was proposed by Haddad et al. (1988) based on morphological similarities of both adults and tadpoles. Our morphological analyses of tadpoles from these populations agreed with those authors by finding that tadpoles of *Ololygon* sp. Canastra and the ones from the type locality of *O. machadoi* share a very similar external morphology and the conspicuous color pattern composed of a dark body transversally crossed by two bright yellow bars. This color pattern is also shared by all analyzed populations of *Ololygon* sp. QF. Contrary to what would be expected from the tadpoles' color pattern, our phylogenetic inference recovered the populations from Canastra Range as not forming a monophyletic group with the populations from the Espinhaço Range.

The phylogenetic placement of *Ololygon* sp. Canastra, combined with its tadpoles' color pattern being shared only with distantly related species, are indicatives that it is a new species. Although adult morphology of this taxon was also analyzed by Haddad et al. (1988), a more refined analysis is still to be done and it may also contribute to diagnose the species.

4.1.2. *Ololygon* sp. QF

Our phylogenetic analyses recovered all populations from the Espinhaço Range as monophyletic, with populations from the QF region (*Ololygon* sp. QF) being the sister taxon to the remaining

populations. *Ololygon* sp. QF has been assigned to *O. machadoi* by many authors (Leite et al., 2008; Eterovick and Barata, 2006; Canelas and Bertoluci, 2007; Eterovick et al., 2018; Gontijo et al., 2018). Indeed, this association is plausible as populations from the QF region and topotypes of *O. machadoi* have tadpoles sharing an identical color pattern (also shared by *Ololygon* sp. Canastra) and adult specimens with similar external morphology. However, a more accurate analysis of adult individuals revealed differences between *Ololygon* sp. QF and *O. machadoi*. For instance, the lack of both vocal slits and macroscopically evident chest gland in adult males of *Ololygon* sp. QF (structures present in adult males of *O. machadoi*) and the presence of glandular acini scattered over flanks, inguinal, chest and mental regions in adult females of *Ololygon* sp. QF (structures absent in adult females of *O. machadoi*). These characters have been used to diagnose many Hylidae species, including some *Ololygon* (Napoli 2005; Leite et al., 2012; Lourenço et al., 2014, 2016). Therefore, in spite of having similar tadpoles, these morphological singularities in adult individuals of *Ololygon* sp. QF is a noticeable evidence that it could be recognized as a species distinct from *O. machadoi*.

4.1.3. Ololygon machadoi

Analyses of adult individuals could not find any consistent difference among populations of *Ololygon machadoi*. On the other hand, our analyses of tadpoles confirmed our field observations that there are consistent morphological variation within populations. However, our hypothesis that the different tadpole morphotypes could be associated with new species was not corroborated by the phylogeny. Due to the phylogenetic position of the population from Pico do Itambé State Park, which has a distinct and exclusive tadpole morphotype, its recognition as a distinct species would imply in the species that has tadpoles with light brown body and black tail being paraphyletic (Fig. 9). Another important point is the discordance between BI and ML analysis regarding the placement of the topotype population of *O. machadoi*. While the BI recovered this population as being sister to all the remaining populations of *O. machadoi*, the ML analysis recovered it as sister to other populations from the Mid-south region (Fig. S2). Although poorly supported (52 bootstrap value), this placement in the ML analysis would imply in the morphotype composed of light brown body with black tail being polyphyletic. For all these

reasons we considered the distinct color patterns as a remarkable intraspecific variation of *O. machadoi*.

4.1.4. Tadpole coloration within *Ololygon*

Among the species included in our phylogenetic inference, tadpoles of *O. canastrensis*, *O. carnevallii* and *O. longilinea* have an overall dull color pattern (Andrade and Cardoso 1991; Pezzuti et al., 2016), distinct from tadpoles of *Ololygon* sp. Canastra, *Ololygon* sp. QF and the topotype population of *O. machadoi.* On the other hand, tadpoles of *O. pombali* and *O. tripui* share some similarities with the later species. In the case of *O. pombali*, its tadpoles also have body intercalated by dark and gold colors. However, the golden areas are wider, occupying all the snout region (more concentrated between eyes and nostril) and almost all the posterior third of the body (Lourenço et al., 2013 and FL pers. obs.). In the case of *O. tripui*, its tadpoles also have dark and gold colors, but the gold color is present as fine dots homogenously scattered over the body, more concentrated between eyes and nostril (Lourenço et al., 2009). Additionally, tadpoles presenting a golden bar between eyes and nostril have also been reported to many species of the *O. catharinae* group (*O. albicans*, *O. angrensis*, *O. kautskyi*, *O. trapicheiroi*, *O. ariadne* and *O. flavoguttata*) and its absence to others (*O. argyreornata*, *O. berthae*, *O. catharinae*, *O. goya*, O*. hiemalis*, *O. humilis*, *O. littoralis*, *O. luizotavioi*, *O. melanodactyla*, *O. obtriangulata*, *O. ranki*, and *O. rizibilis*) (Lourenço et al., 2013; Abreu et al., 2015; Andrade et al., 2018). In this way, the presence of the same conspicuous color pattern in tadpoles of different and even not closely related species raises questions about its evolutionary origins and possible adaptative roles. For *O. machadoi*, it has already been demonstrated that its disruptive color pattern has no adaptative value against visually oriented predators (Espanha et al., 2016). However, most species are still to be studied regarding its ecology, and a more inclusive phylogeny and detailed morphological analysis are still necessary to elucidate the process driving the evolution and potential adaptative role of tadpoles' coloration within *Ololygon*.

4.1.5. Specialized skin glands (SSG's)

Amphibians SSG's are distinct glandular tissue concentrated on limited regions of the skin and are usually associated with protection against predators, intraspecific communication and

reproduction (Brizzi et al., 2003). The presence of SSG's has been reported to most genus of Hylidae, predominantly occurring on mental, lateral and inguinal regions as well as on thumb, where they usually form nuptial pads (*e.g.* Pombal and Bastos, 1996; Faivovitch, 2005; Brunetti et al., 2015). Within *Ololygon*, SSG's have been identified on fingers, forearms and inguinal region of adult males of many species and on mental region only in adult males of *O. caissara* (Lourenço et al., 2016). The present study reports for the first time these glands occurring on chest region of adult males and females of *Ololygon* and on mental region of females.

We have found chest gland in adult males of *O. machadoi*, closely packed and macroscopically discernible by a thick white or brownish structure protruding from surrounding skin. Individual acini and even acini lumen are generally possible to be identified under small magnifications and sometimes individual acini were also visible to the naked eye (Fig. 3A–C). The gland may vary in size, from occupying a small area on the chest to covering all the chest region and most part of the belly (Fig. S3). In some individuals, sparse acinar units may occur over the belly and flanks (Fig. 3A). Chest gland seems to be very uncommon among Hylidae and until now similar structure has been identified only in males of *Quilticohyla acrochord*a, *Q. sanctaecrucis* (Campbell and Duellman, 2000) and in males and females of the *Dendropsophus leucophyllatus* group, which have a pair of conspicuous pectoral glands (Cochran and Goin, 1970; Duellman, 1970; Faivovich et al., 2005; Dias et al., 2017). Another type of pectoral gland has also been reported to occur in adult males of *Scinax fuscovarius* as well as in adult males of some populations of *S. nasicus* and *S x-signatus* (Lutz, 1973; Cei, 1980). The pectoral gland of those species is composed of two medially separated brownish plaques, are generally poorly developed and is not frequent to be seen even in adult males, being probably deciduous (Lutz, 1973; FL pers. obs. on *S. fuscovarius* and *S. x-signatus*). In contrast, the chest gland of *O. machadoi* is composed of a single and well-developed structure, which is present in all adult males analyzed, being more similar to the chest gland described to *Quilticohyla*. Araújo-Vieira et al. (2016) identified in adult males of *S. haddadorum* a "white, slightly thickened area in the pectoral region that is apparently glandular". Even though those authors were uncertain regarding the slightly thickened white area corresponds or not to a SSG's, analyzing the plate of the holotype it does not seem to be the same structure that we have found in *O. machadoi*.

We have also found chest gland occurring in both males and females of *Ololygon* sp. QF, however, in this case, two types of gland were identified and both are morphological distinct from the one occurring in *O. machadoi*. The first type occurs only in adult males and its acini units have some similarities to the ones occurring in adult males of *O. machadoi*, being closely packed, pale white to light brown. However, acini units are less thick, being hardly discernible even under magnification, and were not possible to be identified in all specimens. The chest gland itself is mostly not macroscopically discernible, not protrude from surround skin and its limits are unclear. This type of glandular tissue was also present on inguinal region of *Ololygon* sp. QF adult males. This inguinal gland is similar to the ones we have found in adult males of *O. canastrensis*, *O. catharinae*, *O. flavoguttata*, *O. longilinea*, *O. luizotavioi*, *O. obtriangulata* and *O. rizibilis* and is apparently the same described as occurring in *O. ariadne*, *O. brieni*, *O. centralis*, *O. hiemalis*, *O. jureia* and *O. ranki* (Pombal and Bastos, 1996; Lourenço et al., 2014, 2016).

The second type of chest gland that we have found in *Ololygon* sp. QF was identified in both males and females. In this case, acini are smaller than the first type, are not closely pecked and has a distinct brownish orange color. In males, we found these acini in just a few specimens and they occur in low density together with the first type of chest gland (Fig. 3D). In females, these acini occur in greater densities and not only over the chest, but also over flanks, mental and inguinal regions (Fig. 4). Some females also have these acini scattered on anterior, ventral and posterior regions of thighs. This type of glandular tissue had already been reported to occur in *O. caissara*, however, in this species its distribution is restrict to mental, and inguinal regions of males (Lourenço et al., 2016).

The present study is the first to report SSG's occurring in females in Scinaxinae. Within Hylidae these glands had already been reported in females of some species of Cophomantinae and Dendropsophinae (Brunetti et al., 2015; Dias et al., 2017). Brunetti et al. (2012) demonstrated that although occurring in both males and females of *Boana punctata*, SSG's are histologically and externally distinct between sexes, similarly to what we have found with our external morphology analyses of *Ololygon* sp. QF. The physiological role of SSG's in anurans is still unclear. However, due to the sexually dimorphic nature of these glands and some behavioral observations of courtships in which males and females touch each other in areas that have these glands, it has

been hypothesized that SSG's may be involved in chemical communication, mainly for breeding (Brizzi et al. 2003; Brunetti et al., 2014). Further studies aiming to describe and understand the histological and histochemical characteristics of the distinct SSG's occurring in Scinaxinae are still necessary to a better comprehension of its biological role. Additionally, the find of such a conspicuous structure in a species described in 1973, and that has been collected and studied by many authors since then, reinforces the need of a more refined morphological analysis of already described species to better access the taxonomic distribution of the SSG's. Refined morphological analyses are crucial not only to a precise taxonomy but also to understand, in conjunction with phylogenetical studies, the evolution of morphological features.

4.2. Divergence times

Our dated species tree suggests that the split between ancestral lineages of *Ololygon* sp. QF and *O. machadoi* occurred during the Late Miocene, ca. 11.2 mya. The occurrence of *Ololygon* sp. QF restrict to the South portion of the Espinhaço Range and the sequential split pattern found in the larger clades within *O. machadoi* indicates that the species dispersion happened from south to north. Interestingly, the large majority of divergence events that originated the lineages of the current populations of *Ololygon* sp. Canastra, *Ololygon* sp. QF and *O. machadoi* appear to have occurred during the Pliocene. The Early Pliocene epoch marks both a break of the gradual cooling that started during the middle Miocene and the beginning of a warming trend that lasted until ca. 3.2 mya, in the Late Pliocene (Zachos et al., 2001). This epoch was also marked by climate stability, with average temperatures 2–3°C warmer than today, causing a significant range expansion of rainforests (Draut et al., 2003; Robinson et al., 2008; Willis and MacDonald, 2011). With this scenario, is likely that the suitable environments for *O. machadoi* became restrict to higher altitudes on the mountaintops, consequently leading to the remarkable populational structure that we have found.

4.3 Biogeography

The distribution pattern of the species belonging to the *Ololygon machadoi* species complex is congruent with biogeographic patterns known to other anurans occurring in the same region. The recognition of populations from Canastra Range as not being *O. machadoi* makes that only *Scinax*

maracaya remains to have occurrence known as restrict to both Canastra Range and Espinhaço Range. Even though these mountains present a very similar environment, both characterized by quartzite formations with sandy soil and a phytophysiognomy mostly composed of Campo Rupestre, the approximately 250 km of lowlands that separate them are probably functioning as a dispersion barrier of unsuitable environment to species adapted to mountainous regions. In this way, the Canastra Range and the Espinhaço Range are acting, on a large scale, as sky islands.

On a smaller scale, the many altitudinal discontinuities along the Espinhaço Range make it acts as a sky island archipelago. Besides many smaller altitudinal discontinuities, we have identified four main altitudinal breaks (Fig. 8): (A) between the Serra da Cambota in the South region (QF) and the Cipó plateau in the Mid-south region; (B) between the Serra do Cabral and the Diamantina plateau, both in the Mid-south region; (C) to the west, between the Diamantina plateau (Mid-south) and the Serra de Itacambira (Mid-north), and to the east, delimited by the Jequitinhonha River valley; and (D) between the narrow mountains extending from Caetité to Riacho de Santana, in the Mid-north region, and the Chapada Diamantina, in the North region. The distribution pattern of the sister taxa *Ololygon* sp. QF and *O. machadoi*, is a case in which the break A is the distributional limit of the species, separating them. This same pattern has parallels with other species pairs. For instance, *Hylodes uai* and *Physalaemus erythos* are endemic to the QF region and its most morphologically similar species *H. otavioi* and *P. deimaticus* (which is also sister to *P. erythros*), respectively, are endemic to the Mid-south region of the Espinhaço (Leite et al., 2008; Lourenço et al., 2015). Additionally, there are species endemic to the QF region without morphological correspondent known in the Mid-south region (*e.g. Bokermannohyla martinsi*, *Sphaenorhincus canga*; Pinheiro et al., 2014; Araújo-Vieira et al., 2015) and others endemic to the Mid-south region without morphological correspondent in the QF region (*e.g. Boana cipoensis, Proceratophrys cururu*, *Physalaemus* sp. nov.; Leite et al., 2008; Leal et al., submitted). On the other hand, there are endemic species occurring in both South and Mid-south regions (*e.g. Bokermannohyla alvarengai*, *Bokermannohyla. saxicola*, *Crossodactylus trachystomus*, *Scinax curicica*, *Thoropa megatympanum*; Leite et al., 2008, Nascimento et al., 2018). This pattern suggests that different vicariant events may have occurred, differentially affecting the distribution of species and in some of them promoting allopatric speciation.

For the *Ololygon* sp. QF, all of our analyses of species delimitation methods recovered three independently evolving populations, structured in three different sky islands in the South region: the Serra do Gandarela, Serra do Caraça and Serra da Cambota. The topology recovered by our phylogenetic inference reflects the geography of this region, in which Serra do Caraça and Serra do Gandarela are closer to each other than to Serra da Cambota.

The distribution range of *Ololygon machadoi*, until the present work known to be restricted to south regions of the Espinhaço, is actually one of the widest among anuran species endemic to this mountain range. Apart from *O. machadoi*, only *Odontophrynus juquinha* is known to cross all the main altitudinal breaks between the Mid-south and North region (Rocha et. al., 2017). Even though having such a wide distribution, our analyses found a remarkable populational structuring. Among the methods we have used to delimit populations, the most conservative recovered 12 independently evolving populations of *O. machadoi*, from 18 sampling localities. In contrast, a populational study of *Pithecopus megacephalus*, a species that has a distribution range very similar to *O. machadoi* and it is also endemic to the Espinhaço Range*,* delimited only three populations out of 10 sampling localities (Ramos et al., 2017). In spite of difference in number of delimited populations, there are interesting agreement between those authors' results and ours. For instance, they found that individuals of *P. megacephalus* from the Serra do Cabral, in the Mid-south region, are more closely related to the ones from Botumirim and Grão Mogol, both in the Mid-north region, than to the ones from the Diamantina plateau, which is also in the Mid-south region. We found this same pattern in *O. machadoi*, with population from Serra do Cabral also inferred as more phylogenetically related to populations from the Mid-north (*e.g.* Itacambira, Botumirim and Grão Mogol populations) than to the one much geographically closer, in the Mid-south region (*e.g.* Serra D'anta population, in the Diamantina plateau; Figs. 1,9,S1,S2). For *B. saxicola*, Nascimento et al. (2018) found similar results, but in this case individuals from the Diamantina plateau were phylogenetically closer to the ones from Serra de Itacambira than to the ones from Serra do Cabral. These results in conjunction with the phylogenetic position of the population of *O. machadoi* from the Serra do Cabral suggest that the vicariant events separating the Serra do Cabral from the Diamantina plateau occurred prior to the ones that isolated it from the mountains in the Mid-north region or were more intense.

In the Mid-north region, two populations were formed by individuals from more than one sky island. Population 12 grouped individuals from Botumirim, Grão Mogol and Serra Nova, whereas population 13 grouped individuals from Santo Antônio do Retiro, Jacaraci and Caetité. Even though the grouped sky islands are in apparent agreement with their geographic locations, which are relatively close to each other, the placement of individuals from Serra Nova in the same population (and clade) of the ones from Grão Mogol and Botumirim was unexpected both under a geographic and biogeographic point of view. First, there is a large altitudinal discontinuity between Grão Mogol and Serra Nova, with altitudes in the valley not exceeding 900 masl, whereas the mountainous massif of Serra Nova seems to be continuum with the one of Santo Antônio do Retiro, with minimum altitudes being always above 1000 masl between these localities. Second, in the studies regarding both *B. saxicola* and *P. megacephalus*, this break between Grão Mogol and Serra Nova appears to be an important barrier to gene flow since populations of those species are structured right northwards and southwards of it (Ramos et al., 2017; Nascimento et al., 2018). Thus, there were probably other significant factors, in addition to altitudinal discontinuity, promoting the structuring found in the population 13.

In the Mid-south region, all delimited populations occur in distinct sky islands, with the exception of populations 4 and 5, which lie in the same sky island of the Serra do Cipó. These populations were also recovered in both BI and ML analysis as not being closely related and also present differences between their tadpoles' morphology. Besides occurring in the same sky island, populations 4 and 5 are also very close to each other, being separated by only 6 km. In contrast, the distance between the population 5 and 6, which are sister clades, is 133 km. In this case, climatic vicariance appears to not be a reasonable explanation to both the population and phylogenetic structure we have found.

The population 4 is from the highlands of the Serra do Cipó, an area that has phytophysiognomy predominantly composed of open fields of Campo Rupestre. This highland region has great influence from the Cerrado biome, which is present at the western slopes of the Serra do Cipó. On the other hand, the east slopes, where lies the population 5, is a region under the heavy influence of the Atlantic Forest biome. Populations 6, 7 and 9 also lie in the east region of the Espinhaço Range, being also under influence of the Atlantic Forest biome. In this way, it is possible

that the geographic incongruence among populations structure, its phylogenetical relations and differences in tadpole's morphology can be explained by ecological vicariance. This type of vicariance occurs when there is ecological specialization of groups that share an ancestral lineage, which were probably polymorphic for this ecological trait (Hardy and Linder, 2005; Struwe et al., 2011). Therefore, the distinct environments occupied by populations in the mountain tops and the ones in the Atlantic slopes may reflect specialization of descendant lineages from an ancestor one that could easily disperse between such environments. As a result of the specialization, lineages that could have become better adapted to forest environment would have remained more restrict to these regions and dispersing more easily across the Atlantic slopes of the Mid-south region of the Espinhaço Range. However, further analyses with sampling of intermediate localities of both populations 5 and 7 as well as 4 and 8 may be necessary to better understand the diversification pattern of populations of *O. machadoi* in the Mid-south region.

4.4. COI genetic distances

In recent years, many studies have been published advocating in favor of the use of mtDNA genetic distance as a method to identify new species (*e.g.* Hebert and Gregory, 2005; Vences et al., 2005a; Fouquet et al., 2007; Crawford et al., 2010; Crawford et al., 2012; Lyra et al., 2016). However, there are many problems in using mtDNA genetic distance to access species boundaries, such as retentions of ancestral polymorphism, uniparental inheritance, introgression followed by hybridization and different selective pressures that may lead species to diverge more rapidly or slowly (Moritz and Cicero, 2004). For Neotropical frogs, Lyra et al. (2016) have suggested a threshold of 6% divergence of COI for intraspecific variation. Considering pairwise distance of *Ololygon machadoi* populations, 56 out of the 66 pairwise distance have values greater than 6%, many of them higher than 9% (Table 2). On the other hand, pairwise distance between *O. canastrensis* and *O. longilinea* was 4.4%, significantly smaller than the suggested threshold. These differences may be the case of distinct levels of selective pressures, in which strong selective pressures lead *O. canastrensis* and *O. longilinea* to morphologically diverge rapidly and, by contrast, similar selective pressures on populations of *O. machadoi* caused their morphology and ecology to remain mostly unaffected, even being geographically isolated for millions of years.

Additionally, when analyzing described species, it is frequently observed a marked overlap of intraspecific and interspecific divergence values, making it difficult to establish thresholds for candidate species identification (Vences et al. 2005b). We also found this issue exemplified in our results. The divergence between *O. longilinea* and *O. canastrensis*, *Ololygon* sp. QF and *O. machadoi*, and between *Ololygon* sp. Canastra and *O. longilinea* presented lower values than some of the pairwise distances among *O. machadoi* populations (Tables 2, 3). Therefore, our results reinforce that species boundaries should not be accessed on the basis of mtDNA divergence, instead, more efforts should be made to improve morphological and ecological analyses.

5. CONCLUSIONS

By combining phylogenetical and morphological data, the present study shows that *Ololygon machadoi* is a species complex composed of the nominal species and two undescribed ones. As currently defined in literature, the species complex is paraphyletic due to the phylogenetical placement of populations from Canastra Range. We consider these populations as a new species, but further morphological analyses of adult individuals are still to be done. The second undescribed species is from the Quadrilátero Ferrífero and was identified on the basis of morphological singularities of adult species, distinguishing it from the remaining populations of the Espinhaço Range. In this way, *O. machadoi* would be endemic to the Espinhaço Range, occurring throughout all of its extension, excluding the QF region.

Our results also demonstrate that the usage of morphological data concomitantly with molecular species delimitation methods is essential to avoid overestimation of species diversity. If our taxonomic conclusions had relied only on the results of the multispecies coalescent analyses, we could consider the existence of at least 14 species, all being morphologically and even ecologically indistinguishable taxa occurring in the same mountain range. The same overestimation of species diversity would have happened if we had considered molecular distance as intraspecific/interspecific variation threshold. On the other hand, we have shown that phylogenetical analyses may also be important to correctly access the existing species diversity. For instance, if we had considered only morphological data to infer species diversity within *O.*

machadoi, the populationally structured intraspecific variation that we have found regarding tadpoles' morphology could have also been erroneously interpreted as an interspecific variation (as it was in our initial hypothesis) and consequently paraphyletic and even polyphyletic species could had been described.

The Espinhaço Range has a remarkable amount of endemic anuran species and its sky island archipelago structure provides an interesting and didactic model to investigate evolutionary and biogeographic processes. The results of this study indicate that climatic vicariance was probably the main process shaping the populational structure currently found in the *O. machadoi* species complex. Our dated species tree shows that the diversification peak in which most of the *O. machadoi* populations diverged is likely to have occurred during the Pliocene. This diversification period may be explained by the climate changes known to have occurred at this epoch, in which temperatures got warmer and the distribution of forested areas expanded. Thus, populations of *O. machadoi* would have been trapped in the cooler and environmental suitable mountaintops. The present study is only the third to investigate an anuran species endemic to the Espinhaço under a populational and evolutionary approach and is the first to include morphological analyses. Further studies with similar approach are crucial to achieve a more accurate reconstruction of the evolutionary history of the Espinhaço Range.

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7. FIGURES

Fig. 1. Consensus tree of BI based on concatenated dataset of the mitochondrial gene COI and nuclear genes POMC, RAG1, RHOD and βFib7. Asterisks indicate clades not recovered in the ML analysis. Nodes with closed circles indicate posterior probability above 0.95 and bootstrap values above 80, in the case of the clade has also been recovered in the ML analysis. Open circle indicates node with posterior probability below 0.95 and bootstrap value above 80. Open squares indicate nodes with posterior probability above 0.95 and bootstrap values below 80. Bars on the right show populations delimited by GMYC, BPP and STACEY. Populations were named from 1 to 15 accordingly to the STACEY results. Clades highlighted with green are from localities within the Espinhaço Range, excluding the QF region; clades highlighted with purple are from the QF region and clades highlighted with yellow are from the Canastra Range. Localities in italic refer to region names, non-italic refer to municipality names. Abbreviations refer to conservation units: P.N (National Park), P.E. (State Park), E.E. (Ecological Station), RPPN (Private Reserve of Natural Patrimony).

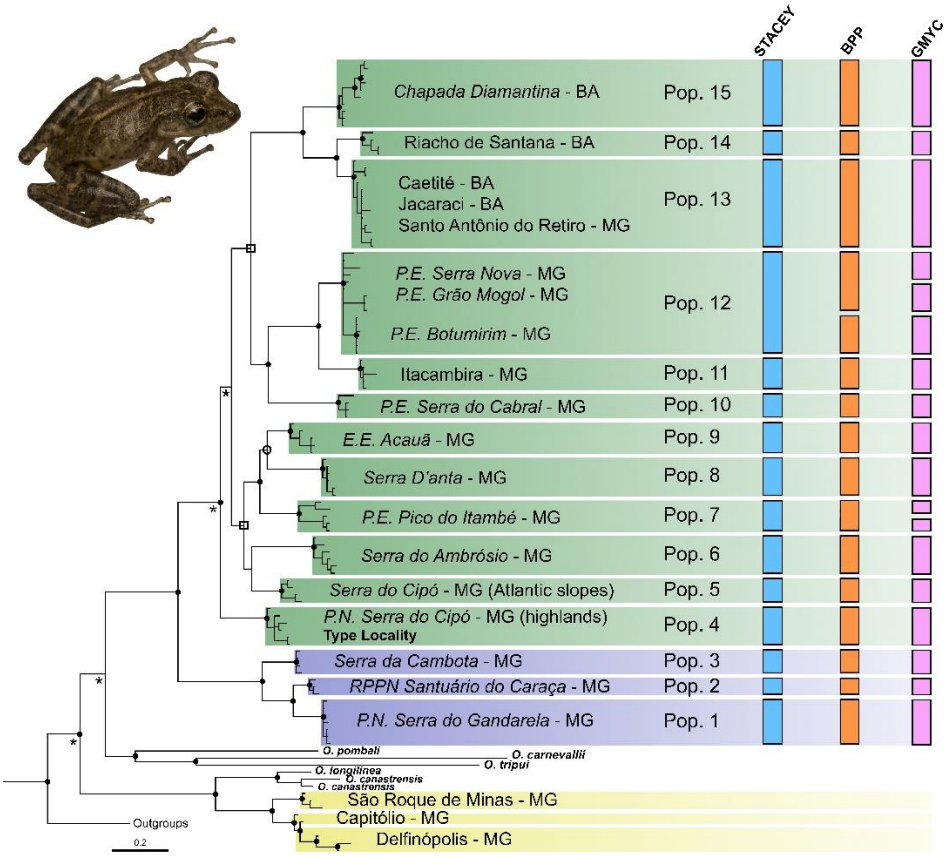


Fig. 2. Different color patterns of tadpoles found in populations of the *Ololygon machadoi* species complex: (A) tadpoles of *Ololygon* sp. Canastra, from Canastra Range, Municipality of Capitólio, MG state; (B) topotype tadpoles of *O. machadoi* from Serra do Cipó, municipality of Morro do Pilar, MG state; (C) tadpole of *O. machadoi* from Pico das Almas, municipality of Rio de Contas, BA state; (D) tadpole of *O. machadoi* from Parque Estadual Pico do Itambé, municipality of Santo Antônio do Itambé, MG state.

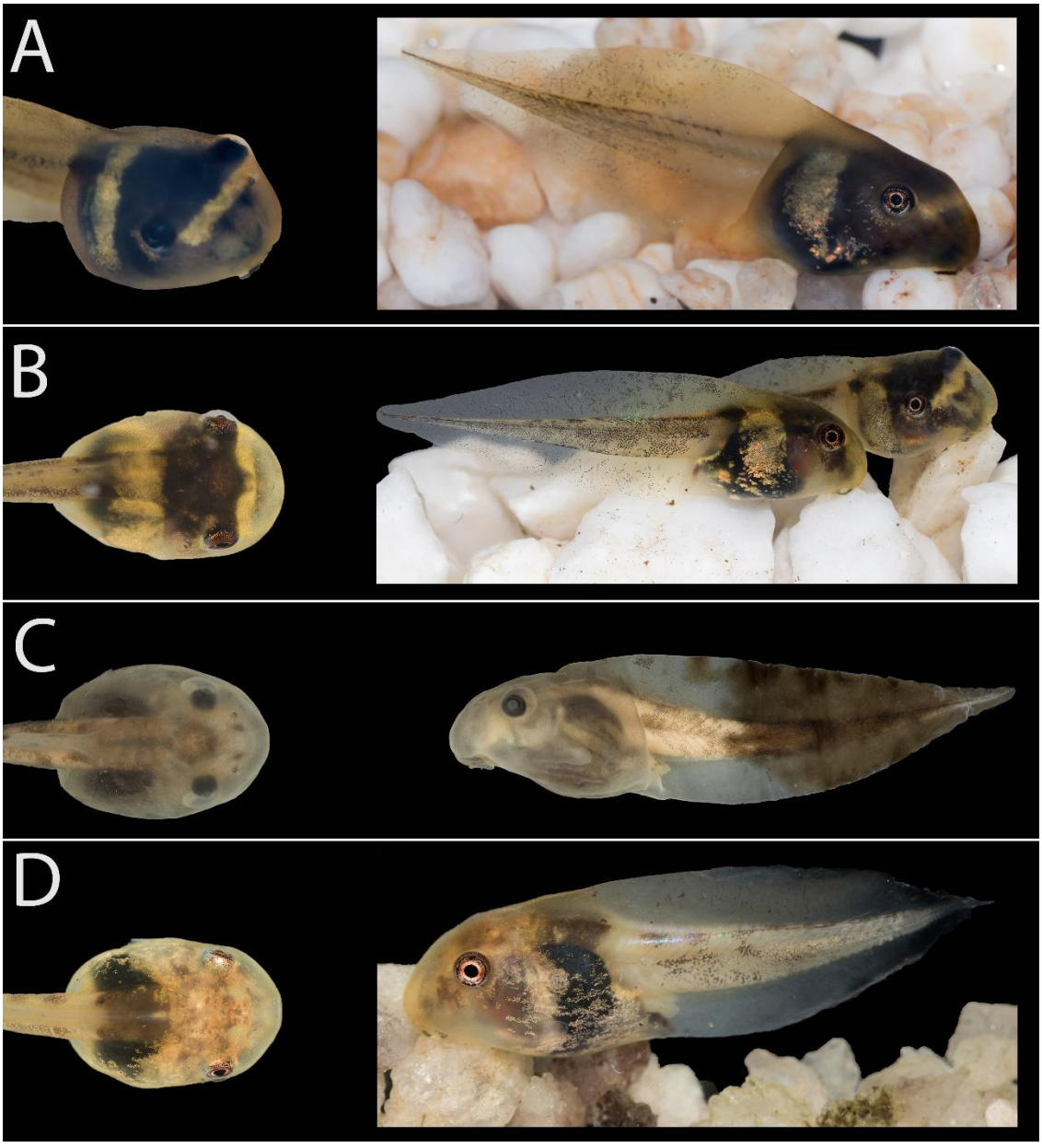


Fig. 3. Variation in size, color pattern, chest gland distinctiveness with details of acini morphology and aggregation in adult males of *Ololygon machadoi*: (A) UFMG 19603 from Serra D´anta, municipality of Diamantina, MG state; (B) UFMG 5821 from Serra do Cipó, munipality of Conceição do Mato Dentro; (C) UFMG 17682 from Serra do Cipó, municipality of Conceição do Mato Dentro, MG state and of *Ololygon* sp. QF (D): UFMG 19378, from Serra da Cambota, municipality of Barão de Cocais, MG state. Arrows point to individual acini. Notice that chest gland in *Ololygon* sp. QF is not externally discernible and even under magnification it displays very few acini units. Specimens are in the same scale; scale bar = 10 mm.

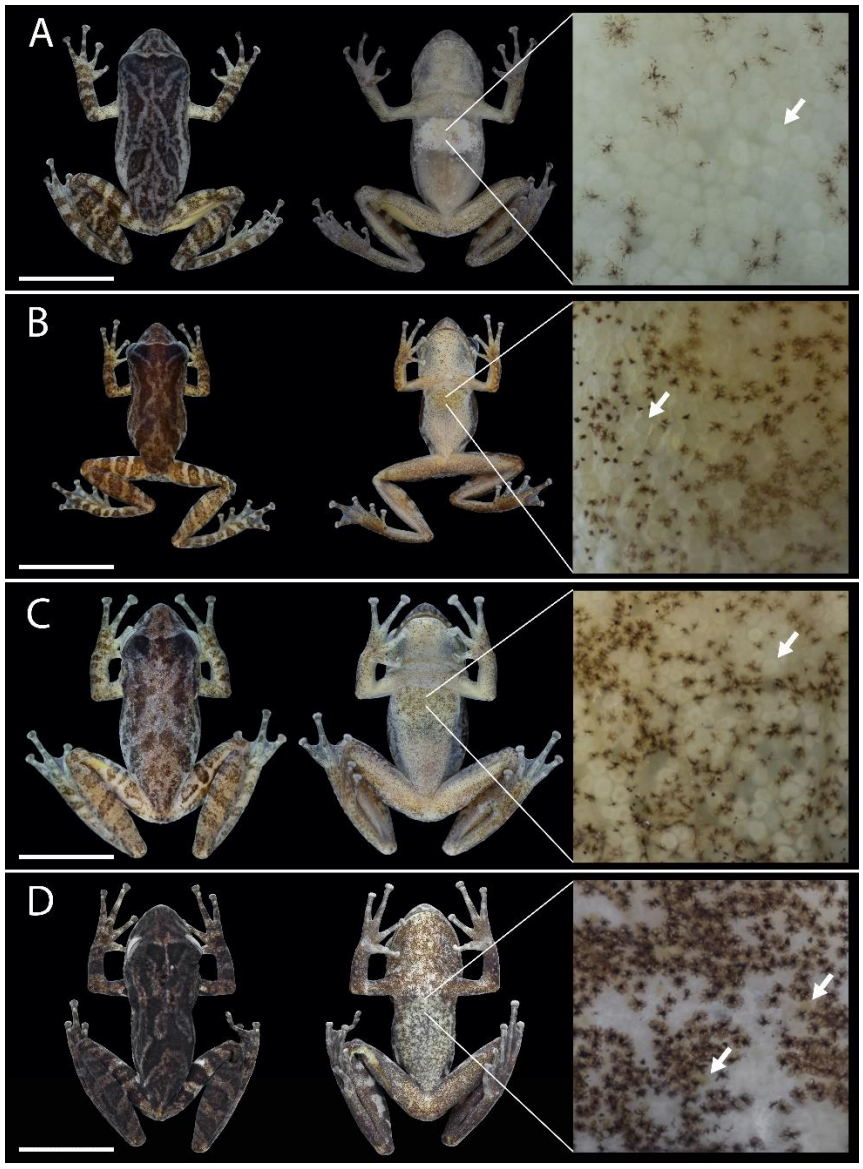


Fig. 4. Adult female of *Ololygon* sp. QF (UFMG-19294) from Serra da Cambota, municipality of Barão de Cocais, MG state: (A) ventral view and magnification of mental and chest region showing many glandular acini scattered. (B) Flank and magnification of the middle of the flank and of the inguinal region, displaying many glandular acini. Notice a distinct light brown coloration on mental region, chest and flank caused by acini aggregation, apart from the melanophores of the blotches. Arrows point to individual acini. Scale bar = 10 mm.

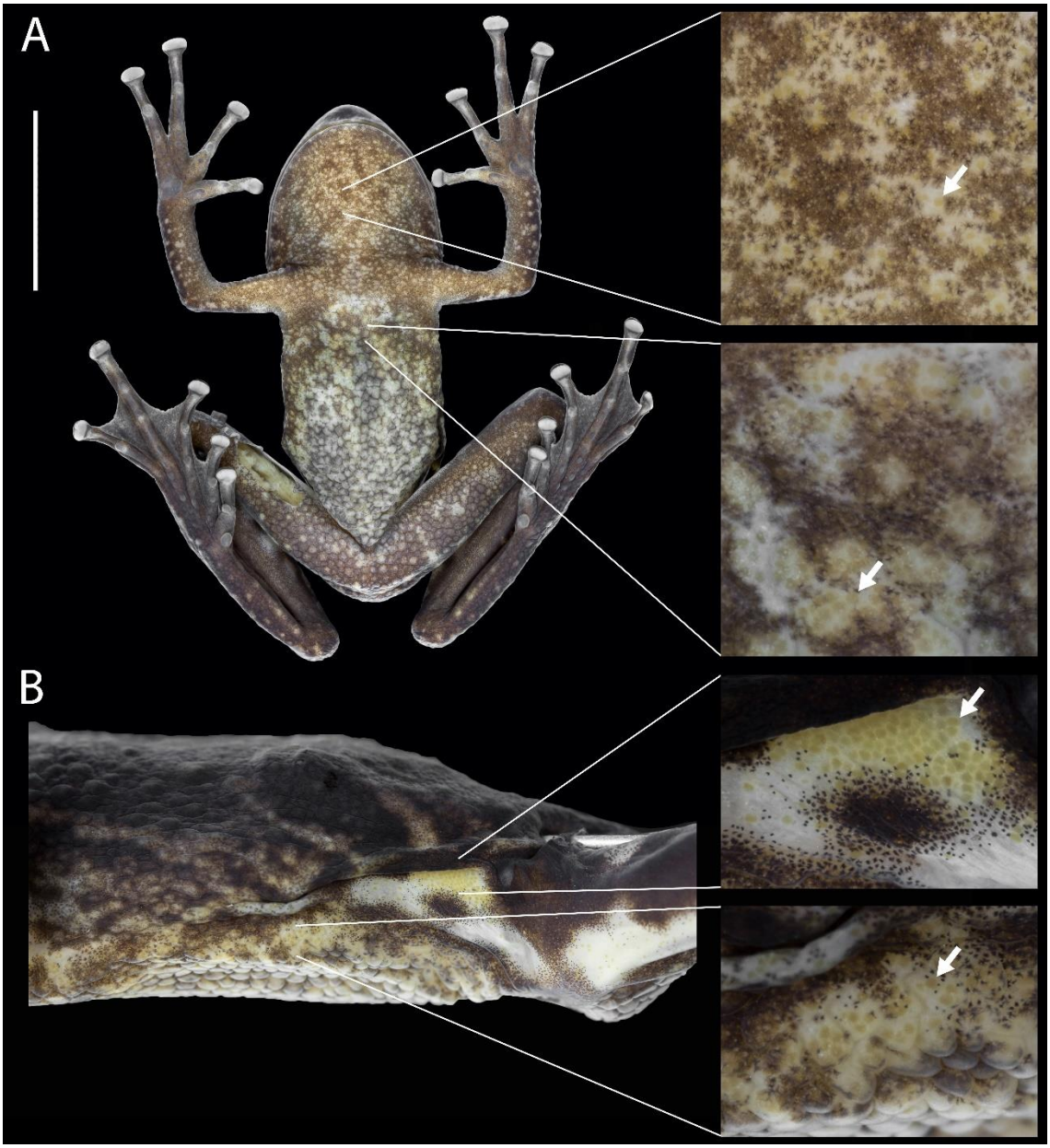


Fig. 5. *Ololygon machadoi* holotype (MZUSP 73669; immature female, SVL 20.6 mm): (A) dorsal and (B) ventral views. Scale bar = 10 mm.

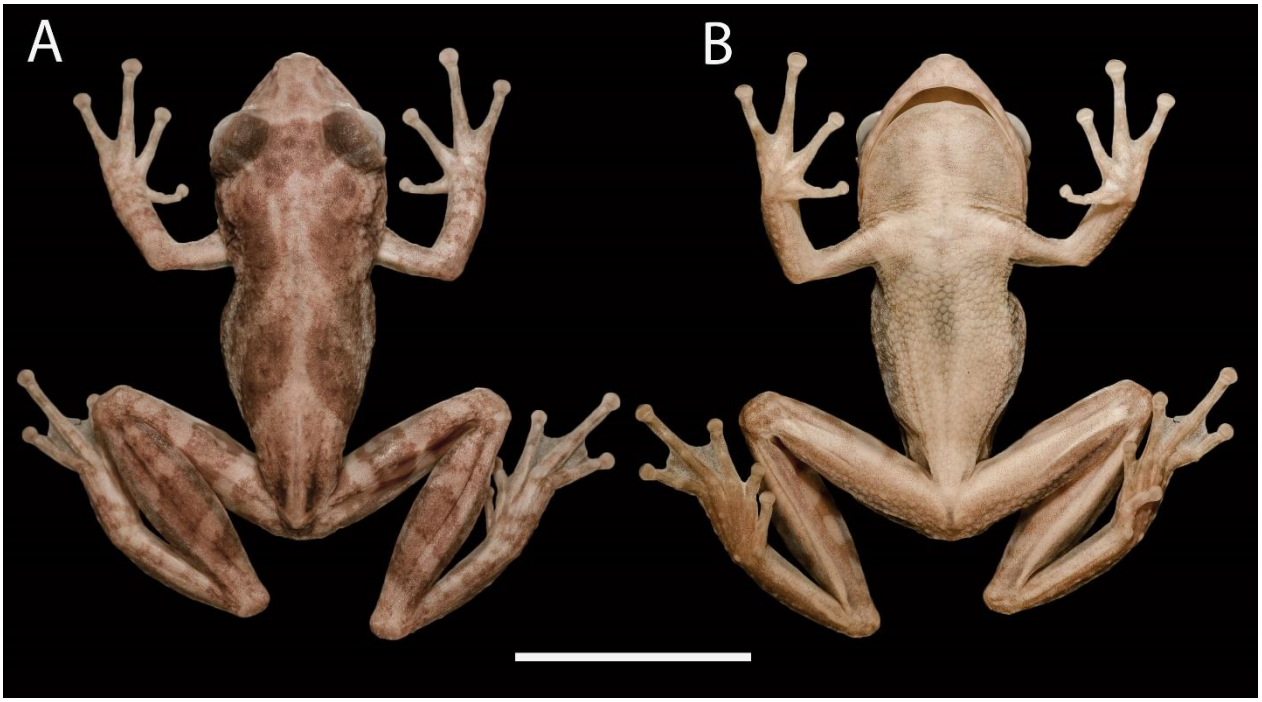


Fig. 6. *Ololygon machadoi* holotype (MZUSP 73669; immature female): (A) dorsal and (B) lateral views of head, (C) ventral views of right hand and (D) right foot. Scale bar = 5 mm.

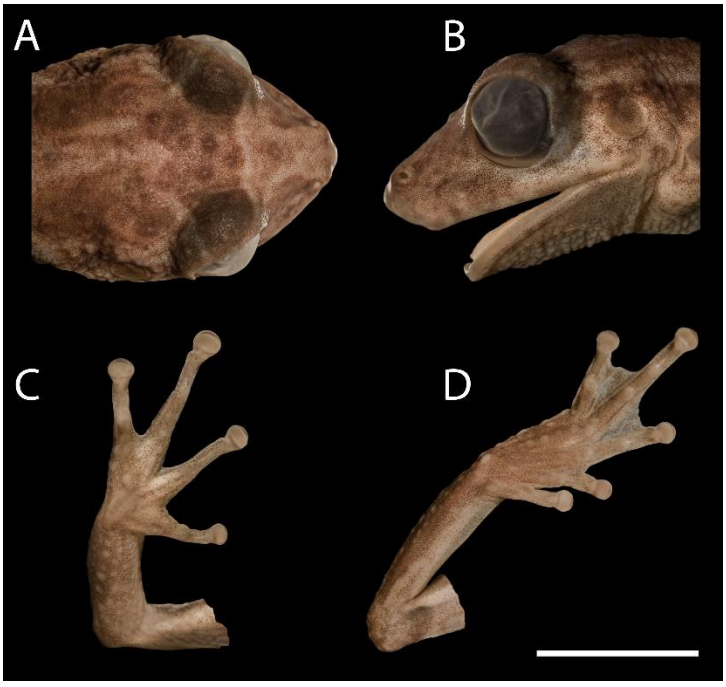


Fig. 7. Topotypes individuals of *Ololygon machadoi* in dorsal (right) and ventral (left) views: (A) adult female UFMG 19571; (B) adult male UFMG 19575; (C) adult male UFMG 19576.

Fig. 8. Geographic distribution of *the Ololygon machadoi* species complex**.** Numbers refer to populations delimited by STACEY analysis as shown in Figs. 1 and 8. Blue bar indicate the southern limit of the Espinhaço Range. Red bars indicate the main altitudinal breaks along the Espinhaço Range: (A) between the South (QF) and Mid-south regions, (B) between the Serra do Cabral and the Diamantina plateu; (C) between the Mid-south and Mid-north regions, (C) between Mid-north and North regions. Abbreviations: MG = Minas Gerais State and BA = Bahia State.

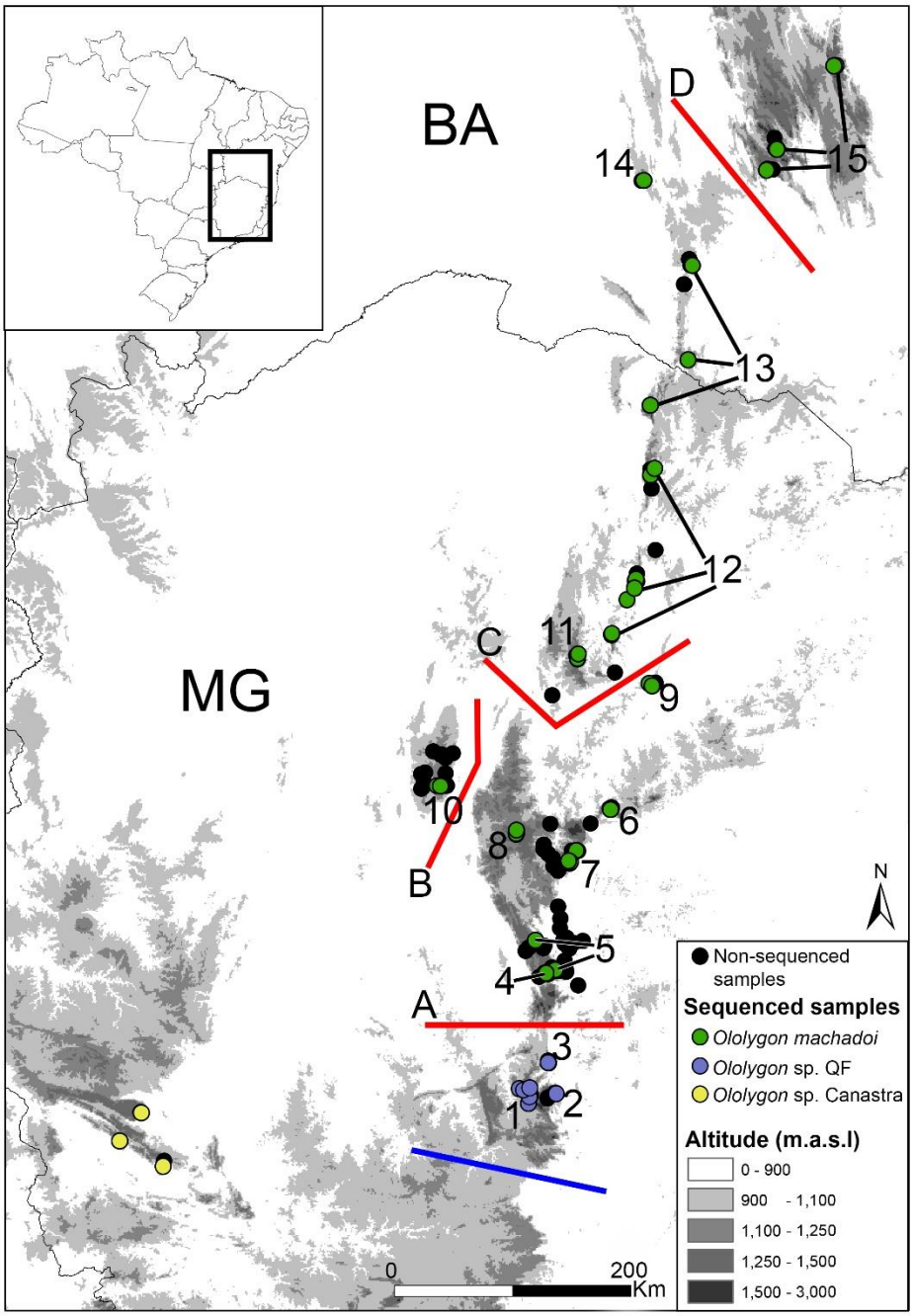
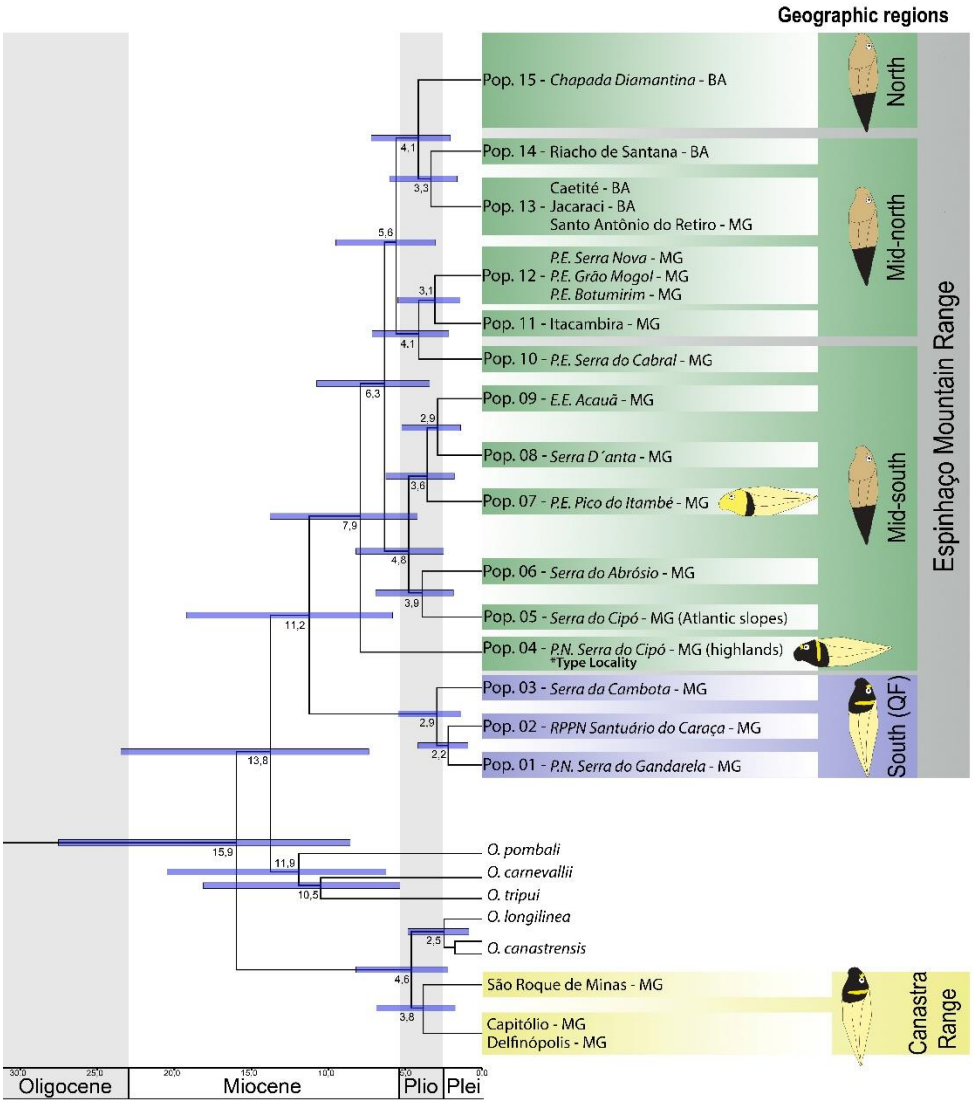


Fig. 9. Time-calibrated tree based on concatenated dataset of the mitochondrial gene COI and nuclear genes POMC, RAG1, RHOD and βFib7. Bars indicate the 95% highest probability density interval for divergence date. Numbers below branches indicate mean node ages in million years before present. Tadpoles vertically oriented represent the morphotype present on the region in which it is in front of. Tadpoles horizontally oriented represent populational exceptions of the morphotype present in that region, present in the populations that they are in front of. Localities in italic refer to region names, non-italic refer to municipality names. Abbreviations refer to conservation units: P.N (National Park), P.E. (State Park), E.E. (Ecological Station), RPPN (Private Reserve of Natural Patrimony).



8. TABLES

Table 1. Measurements (in mm) of *Ololygon machadoi* paratypes and of specimens from all localities with occurrence of the species. Values are given as range above and mean ± standard deviation in parentheses, below.

Measurements	Paratype males n=33	Paratype females n=3	Non-type males n=192	Non-type females n=53
SVL	15.2–17.5 (16.3 ± 0.6)	22.2–25.4 (23.4 ± 1.7)	14.3–26.3 (19.7 ± 1.9)	21.0–30.9 (26.1 ± 2.3)
HL	6.3–6.9 (6.6 ± 0.2)	8.7–9.5 (9.1 ± 0.4)	5.2–9.3 (7.4 ± 0.7)	8.0–11.6 (9.6 ± 0.9)
HW	5.4–6.0 (5.7 ± 0.2)	7.8–9.1 (8.3 ± 0.7)	4.4–8.6 (6.6 ± 0.7)	7.0–11.0 (8.9 ± 0.9)
ESD	2.7–3.1 (3.0 ± 0.1)	4.0–4.2 (4.1 ± 0.1)	2.5–4.2 (3.2 ± 0.3)	3.3–5.0 (4.1 ± 0.4)
TD	0.9–1.1 (1.0 ± 0)	1.3–1.4 (1.4 ± 0)	0.9–1.5 (1.2 ± 0.1)	1.0–1.9 (1.4 ± 0.2)
THL	8.0–8.9 (8.5 ± 0.2)	11.4–13.0 (12.0 ± 0.9)	6.4–13.7 (10.2 ± 1.1)	10.9–16.3 (13.5 ± 1.3)
TIL	8.2–9.7 (9.1 ± 0.3)	12.1–13.7 (12.8 ± 0.8)	7.7–15.1 (10.8 ± 1.2)	11.9–17.2 (14.2 ± 1.2)
FL	11.4–13.2 (12.4 ± 0.4)	16.0–18.9 (17.4 ± 1.4)	10.6–19.8 (14.5 ± 1.5)	16.0–23.1 (19.0 ± 1.6)

Table 2. Uncorrected pairwise distance of COI (lower diagonal) among the populations delimited by STACEY analysis for *Ololygon* sp. QF (pop. 1 to 3, underlined) and *Ololygon machadoi* (pop. 4 to 15). Upper diagonal shows standard deviation.

Populations	Pop.1	Pop.2	Pop.3	Pop.4	Pop.5	Pop.6	Pop.7	Pop.8	Pop.9	Pop.10	Pop.11	Pop.12	Pop.13	Pop.14	Pop.15
Pop.1		0.008	0.009	0.010	0.009	0.010	0.009	0.010	0.010	0.010	0.011	0.011	0.010	0.010	0.010
Pop.2	0.048		0.006	0.010	0.009	0.010	0.010	0.010	0.010	0.010	0.011	0.010	0.010	0.010	0.010
Pop.3	0.056	0.027		0.010	0.009	0.010	0.010	0.010	0.010	0.010	0.011	0.011	0.010	0.010	0.010
Pop.4	0.086	0.091	0.090		0.007	0.009	0.008	0.009	0.010	0.009	0.010	0.010	0.010	0.010	0.010
Pop.5	0.078	0.086	0.082	0.046		0.008	0.008	0.008	0.009	0.009	0.010	0.009	0.008	0.009	0.009
Pop.6	0.081	0.084	0.083	0.074	0.062		0.009	0.009	0.009	0.009	0.010	0.009	0.010	0.010	0.010
Pop.7	0.102	0.100	0.099	0.059	0.066	0.077		0.007	0.009	0.009	0.010	0.009	0.009	0.010	0.009
Pop.8	0.086	0.096	0.094	0.057	0.052	0.066	0.055		0.008	0.009	0.010	0.010	0.009	0.010	0.009
Pop.9	0.090	0.087	0.088	0.069	0.067	0.076	0.070	0.051		0.009	0.011	0.010	0.010	0.010	0.010
Pop.10	0.107	0.102	0.101	0.076	0.074	0.086	0.084	0.067	0.072		0.010	0.010	0.010	0.010	0.010
Pop.11	0.105	0.097	0.099	0.083	0.095	0.095	0.093	0.092	0.090	0.092		0.007	0.010	0.010	0.010
Pop.12	0.104	0.092	0.100	0.074	0.082	0.089	0.087	0.082	0.087	0.088	0.046		0.010	0.009	0.009
Pop.13	0.104	0.103	0.098	0.083	0.074	0.088	0.092	0.079	0.083	0.084	0.093	0.096		0.005	0.007
Pop.14	0.103	0.102	0.097	0.090	0.076	0.090	0.097	0.086	0.090	0.090	0.092	0.095	0.025		0.008
Pop.15	0.093	0.091	0.094	0.085	0.081	0.080	0.086	0.084	0.080	0.083	0.087	0.085	0.049	0.057	

Table 3. Uncorrected pairwise distance of COI (lower diagonal) among species of *Ololygon* included in the phylogenetic analyses. Upper diagonal shows standard deviation.

Species	*O.mach*	*O.*sp.QF	*O.*sp.Can	*O.cana*	*O.long*	*O.trip*	*O.car.*	*O.pom*
O. machadoi		0.007	0.010	0.010	0.010	0.012	0.011	0.010
O. sp. QF	0.095		0.010	0.011	0.011	0.013	0.012	0.012
O. sp. Canastra	0.149	0.138		0.010	0.010	0.013	0.012	0.011
O. canastrensis	0.149	0.143	0.100		0.007	0.014	0.013	0.012
O. longilinea	0.144	0.133	0.095	0.044		0.014	0.013	0.012
O. tripui	0.175	0.181	0.178	0.181	0.183		0.015	0.014
O. carnevallii	0.189	0.187	0.175	0.188	0.186	0.202		0.013
O. pombali	0.151	0.152	0.149	0.151	0.144	0.189	0.203	