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FILOGEOGRAFIA DO COMPLEXO *ISCHNOCNEMA LACTEA* E *ISCHNOCNEMA HOLTI* (ANURA, BRACHYCEPHALIDAE), SUDESTE DO BRASIL

LARYSSA SAKAYANAGI TEIXEIRA

Dissertação apresentada ao Instituto de Biociências do Campus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Mestre em Ciências Biológicas (Zoologia).

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Orientador: Célio Fernando Baptista Haddad

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Esta Dissertação é dedicada a *Daisaku Ikeda*,

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Resumo

Entre os diferentes ambientes da Mata Atlântica as áreas de maior altitude são o hábitat de algumas espécies endêmicas de anfíbios anuros. Tais espécies podem fornecer evidências para testar a hipótese de que os ambientes montanhosos e acidentados propiciam barreiras à dispersão, fazendo com que cada população, passando por processos independentes de evolução, sofra especiação. *Ischnocnema holti* encontra-se em áreas de altitude da Mata Atlântica e trabalhos anteriores revelaram que há divergência genética entre os espécimes de diferentes localidades, havendo confusão de identificação com *Ischnocnema lactea.* Os objetivos deste estudo foram caracterizar geograficamente a diversidade genética de *I. holti;* discutir a existência de possíveis novas espécies neste complexo e inferir os processos históricos envolvidos em sua diversificação. Para inferir as relações filogenéticas, utilizamos dados de sequências de dois genes de DNA mitocondrial e quatro genes nucleares juntamente com Inferência Bayesiana e Máxima Verossimilhança. Estimamos tempos de divergência (TMRCA) e também utilizamos a análise de agrupamento Bayesiano. Finalmente, nós modelamos o complexo sob condições atuais e passadas. Os resultados deste trabalho indicam que as populações de *I. holti* e *I. lactea* formam um complexo de espécies distribuídas nos topos das montanhas da Mata Atlântica do Sudeste do Brasil. Nós encontramos seis clados bem suportados pelos marcadores mitocondriais e nucleares (ainda que com pequenas variações) e geneticamente bem divergentes, porém não foi possível resolver a relação entre os mesmos. As modelagens sugerem que as populações deste complexo permaneceram restritas as regiões de altitude pelo menos desde o Último Interglacial (120 mil anos atrás), ainda que possam ter tido uma pequena expansão de habitat no último Máximo Glacial. A datação da separação das linhagens também sugere um isolamento mais antigo, sendo que a separação teria ocorrido entre 7 e 11 milhões de anos atrás. Portanto, os resultados sugerem um padrão diferente de expansão para as populações que vivem em altitudes muito elevadas.

Palavras-chave: Anfíbio, Diversidade genética, Filogeografia, Mata Atlântica, Modelagem paleoclimática.

Abstract

Among the different environments of the Atlantic forest, the high altitude areas are habitat for some endemic species of amphibians. Such species can provide evidence to test the hypothesis that mountainous environments provide barriers to the dispersion. Thus, each population can evolve independently, suffering speciation. *Ischnocnema holti* is found in high altitude areas of the Atlantic forest. Previous papers have shown that there is genetic divergence between samples from different locations and that there is misidentification with *Ischnocnema lactea*. The aims of this study were to characterize geographically the genetic diversity of *Ischnocnema holti*, to discuss the existence of possible new species in this complex, and to infer the historical processes involved in the diversification of *Ischnocnema holti*. We infer phylogenetic relationships using DNA sequence data from two mitochondrial and four nuclear genes coupled with Bayesian and Maximum Likelihood reconstructions.We estimated divergence times (tMRCA) and also used Bayesian clustering analysis. Finally, we modelled the location of suitable climate for the complex species under present-day conditions and paleoclimates (SDMs). These results indicate that populations of *I. holti* and *I. lactea* form a complex of species distributed in the tops of the mountains of the Atlantic Forest of southeastern Brazil. We found six clades supported by mitochondrial and nuclear markers (even with minor variations) and genetically divergent, however we could not resolve the relationship between them. The modeling suggests that the populations of this complex remained restricted regions of altitude at least since the Last Interglacial (LIG), although they may have had a small habitat expansion in the last Glacial Maximum. The dating of the separation of lineages also suggests an older isolation, and the separation would have occurred between 7 and 11 million years ago. Therefore, the results suggest a different expansion pattern for the populations living at very high altitudes.

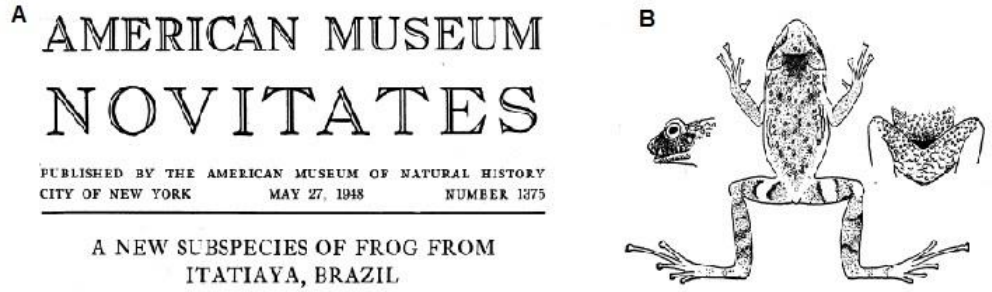
Keywords: Amphibian, Genetic diversity, Phylogeography, Atlantic forest, SDMs.

Sumário

Introdução geral

O gênero *Ischnocnema* Reinhardt e Lütken (1862) (Brachycephalidae) foi removido da sinonímia de *Eleutherodactylus* com base em evidências moleculares (Heinicke et al. 2007; Hedges et al. 2008). É composto por 33 espécies distribuídas na região leste do Brasil e ao norte da Argentina (Canedo et al. 2012; Canedo & Haddad 2012; Frost 2015). Tais espécies estão organizadas em quatro séries: *Ischnocnema guentheri*, *Ischnocnema lactea*, *Ischnocnema parva* e *Ischnocnema verrucosa*, além de poucas espécies não incluídas em nenhuma série de espécies. A série de *I. lactea* é composta por 10 espécies: *I. concolor, I. gehrti*, *I. holti, I. lactea, I. melanopygia, I. nigriventris, I. paranaenses*, *I. randorum, I. spanios* e *I. vizottoi* (Canedo & Haddad 2012).

Ischnocnema holti foi descrito por Cochran em 1948 (Figura 1) como *Eleutherodactylus holti*, subespécie de *Eleutherodactylus unistrigatus* (Günther, 1859), atualmente chamado *Pristimantis unistrigatus,* conhecido apenas no Equador e Bolívia. O exemplar único utilizado na descrição foi coletado na parte alta de Itatiaia, em 1921, durante uma excursão chefiada por Frank Chapman do American Museum of Natural History, onde o mesmo encontra-se depositado (Targino & Carvalho 2008).


A

AMERICAN MUSEUM
NOVITATES

PUBLISHED BY THE AMERICAN MUSEUM OF NATURAL HISTORY
CITY OF NEW YORK MAY 27, 1948 NUMBER 1375

A NEW SUBSPECIES OF FROG FROM
ITATIAYA, BRAZIL

B

Figura 1: (A) Jornal American Museum Novitates onde foi publicado pela primeira vez a descrição de *Ischnocnema holti*. (B) Vista lateral da cabeça, vista dorsal do corpo, e vista ventral da região do fêmur do tipo de *Eleutherodactylus unistrigatus holti*. Figura: Cochran, 1948.

Posteriormente, *Eleutherodactylus holti* foi sinonimizado por Bokermann (1966) com *Basanitia nigriventris* Lutz, 1925, cuja localidade-tipo é Itatiaia e Cubatão. Lynch (1968) sinonimizou *Basanitia* Miranda-Ribeiro, 1923 com *Eleutherodactylus* (então sob a combinação de *Eleutherodactylus nigriventris*, atualmente *Ischnocnema nigriventris*).

Heyer (1985) coletou espécimes no Brejo da Lapa, no Parque Nacional do Itatiaia, Itamonte, MG, e verificou sua similaridade com o holótipo de *E. holti*. Seus estudos apontaram que a sinonímia proposta por Bokermann (1966) não se sustentava, pois havia diferenças morfológicas com os tipos *E. nigriventris* em relação ao tamanho do corpo, rugosidade do dorso e tamanho do apêndice calcâneo. Também encontrou diferenças significativas com *Eleutherodactylus unistrigatus*, confirmando assim seu status de espécie plena, sob a combinação *Eleutherodactylus holti*. Heinicke et al. (2007), elaboraram uma nova classificação em que *E. holti*, assim como todos os *Eleutherodactylus* da Mata Atlântica foram denominados *Ischnocnema*.

Targino & Carvalho (2008) publicaram uma redescrição da espécie, restringindo sua ocorrência ao Parque Nacional do Itatiaia, entre 2000 e 2400m de altitude, embora seu canto possa ser ouvido a partir da localidade da Garganta do Registro a 1670 m de altitude. Costa et al. (2008) expandiram a distribuição geográfica desta espécie, registrando sua ocorrência para Teresópolis, no estado do Rio de Janeiro. Espécimes de identificação dúbia e morfologicamente similares a esta espécie podem ser encontrados também em outras áreas de altitude da Mata Atlântica do Sudeste brasileiro (C. Canedo, com. pess.).

Ischnocnema holti (Figura 2) é uma espécie de porte médio e aspecto rugoso, com cabeça mais larga que longa e vistas lateral e dorsal da cabeça arredondadas. Possui discos adesivos grandes, expandidos e emarginados, além de presença de apêndice calcâneo de tamanho variado e presença de tubérculos supranuméricos pouco pronunciados. Sua coloração apresenta grande variação. O olho possui íris esverdeada com pontuações escuras e há, na maioria, uma faixa supratimpânica marrom escuro. Encontra-se em sua maioria em matas fechadas embora possa ser encontrado em barrancos no meio de liquens ou nos campos de atitude no meio de formações de poáceas e musgos (Targino & Carvalho 2008).

Figura 2: (A – I) Exemplares de *Ischocnema holti* e suas variações dos padrões dorsais de cor em preservativo. Fotos: Targino & Carvalho 2008.

Canedo & Haddad (2012) fizeram o primeiro trabalho de filogenia molecular com várias espécies do gênero *Ischnocnema*, sendo que os espécimes identificados como *I. holti* não foram recuperados como monofiléticos (Figura 3), destacando a necessidade de estudos com foco em sua taxonomia, ecologia, citogenética e de variação molecular.

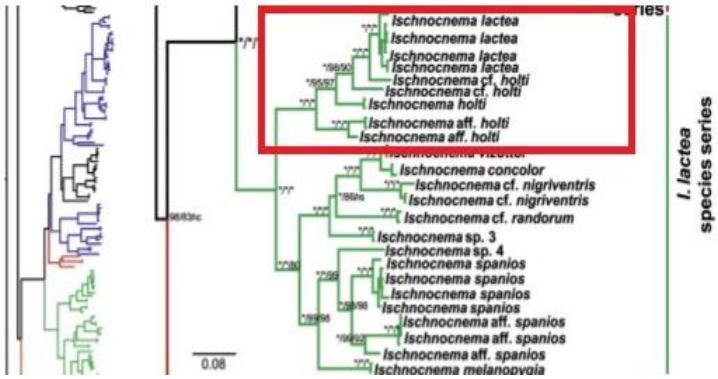


Figura 3: Árvore da série de espécies de *Ischnocnema lactea*, demonstrando as relações filogenéticas entre as espécies *I. holti* e *I. lactea*. Reprodução parcial da figura 1 de Canedo & Haddad (2012).

Portanto, os objetivos deste trabalho foram caracterizar geograficamente a

diversidade genética de *Ischnocnema holti*, discutir a existência de possíveis novas espécies neste complexo e inferir os processos históricos envolvidos em sua diversificação.

Genetic structure in the montane *Ischnocnema holti* (Anura, Brachycephalidae)

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Introduction

The Atlantic forest has a great diversity of organisms and therefore is considered a "hotspot" of global biodiversity (Myers et al. 2000). On the other hand, it is constantly being threatened by many human interference, primarily deforestation (Galindo- Leal & Hall 2005). Accordingly, it is considered one of the high priority areas for biodiversity conservation (Myers 1988).

Its complex evolutionary history, the unique climate conditions, and the large altitudinal range of the Atlantic forest have favored the high diversity and endemism of amphibians of this biome (Haddad & Prado 2005). More than 530 species occur in the region, which correspond about 20% of the species in South America (Haddad et al. 2013). Among the different environments of the Atlantic forest, areas of higher altitude are the habitat of many endemic amphibians (Duellman 1999).

Studies that focus in these environments have shown the existence of several new species, sometimes rare and endemic (Giaretta Jr & Aguiar 1998). Because of the mountainous and rugged environments that provide barriers to the dispersal, each population can evolve independently (Andrade 2010), suffering speciation. An example are the species of the genus *Brachycephalus* (Haddad et al. 2010), whose studies have helped to understand the speciation processes related to geological changes of the Serra do Mar and Serra da Mantiqueira.

Geological changes can form barriers to gene flow between populations (Funk et al. 2005). In Brazil there were notable erosion of crystalline shields the Devonian, Cretaceous, and Tertiary Periods, which today are transformed into

different mountain ranges (e.g., Serra do Mar and Serra da Mantiqueira - Ab'saber, 1975). These mountains were carved by various actions such as epeirogenic lifting, faults interference, and the action of regional climate processes that commanded the erosion during the geological periods (Sartori & Sartori 2013; Penha et al. 1998).

To better understand the processes that shaped the patterns of genetic diversity in these Brazilian mountains, with a broader goal to contribute to regional conservation, many studies have combined the tools of correlative distribution modelling under paleoclimatic scenarios with analyses of molecular data (Pie et al. 2012).

Carnaval et al. (2014) analyzed the effects of current and past climatic variation on the genetic diversity of 25 vertebrates in the Brazilian Atlantic forest. This study suggests that climatic variability through the last 120 kyr impacts the northern and southern forests differently, and that presently montane species had revealed distinct biological responses under cooler climates relative to lowland species (Carnaval et al. 2009). In particular, the data suggest that the southern montane regions of the Atlantic forest, as well as their associated taxa, have persisted under the Last Glacial Maximum, being probably more widespread than today (Rodrigues et al. 2009). During warmer interglacial periods, e.g. present time, these cold-adapted species and habitats have likely experienced fragmentation, genetic divergence, and potentially speciation (Hewitt 2000).

Ischnocnema holti is a montane anuran described by Cochran (1948). In 2008, Targino and Carvalho published a re-description of this species, restricting its occurrence to the Parque Nacional do Itatiaia, Itamonte, Minas Gerais, Brazil (2000 to 2400 m above sea level). Costa *et. al*. (2008) expanded the geographic distribution of this species, recording it in Teresópolis, Rio de Janeiro. Specimens of dubious identification and morphologically similar to this species can also be found in additional high elevation areas of the Atlantic forest in Southeastern Brazil, but is not common. Canedo & Haddad (2012) conducted the first study of molecular phylogeny with several species of the genus *Ischnocnema*, finding that *I. holti* is not monophyletic.

The aims of this study are to characterize geographically the genetic diversity of *Ischnocnema holti*, to discuss the existence of possible new species in this complex, and to infer the historical processes involved in the diversification.

Material and Methods

Population Sampling. Sixty-one tissue samples of individuals previously identified as *Ischnocnema holti, Ischnocnema* aff. *holti, Ischnocnema lactea*, and the closely related outgroups (*Ischnocnema oea, Ischnocnema manezinho, Ischnocnema venancioi, Ischnocnema parva, Ischnocnema* sp., *Ischnocnema juipoca, Ischnocnema spanios, Ischnocnema* cf. *randorum, Ischnocnema sambaqui, Ischnocnema* aff. *guentheri,* and *Ischnocnema nasuta)* were gathered from 23 localities of southeast of Brazil. The vouchers are deposited in Brazil (Appendix A, Figure 1), in the collections: Célio F.B. Haddad (CFBH; Departamento de Zoologia, I.B., Universidade Estadual Paulista Júlio de Mesquita Filho, Rio Claro, São Paulo), Museu Nacional, Rio de Janeiro (MNRJ; Universidade Federal do Rio de Janeiro, Rio de Janeiro), Museu de Zoologia da Universidade de São Paulo (MZUSP, São Paulo), and Miguel Trefaut Rodrigues (MTR; I.B., Universidade de São Paulo, São Paulo).

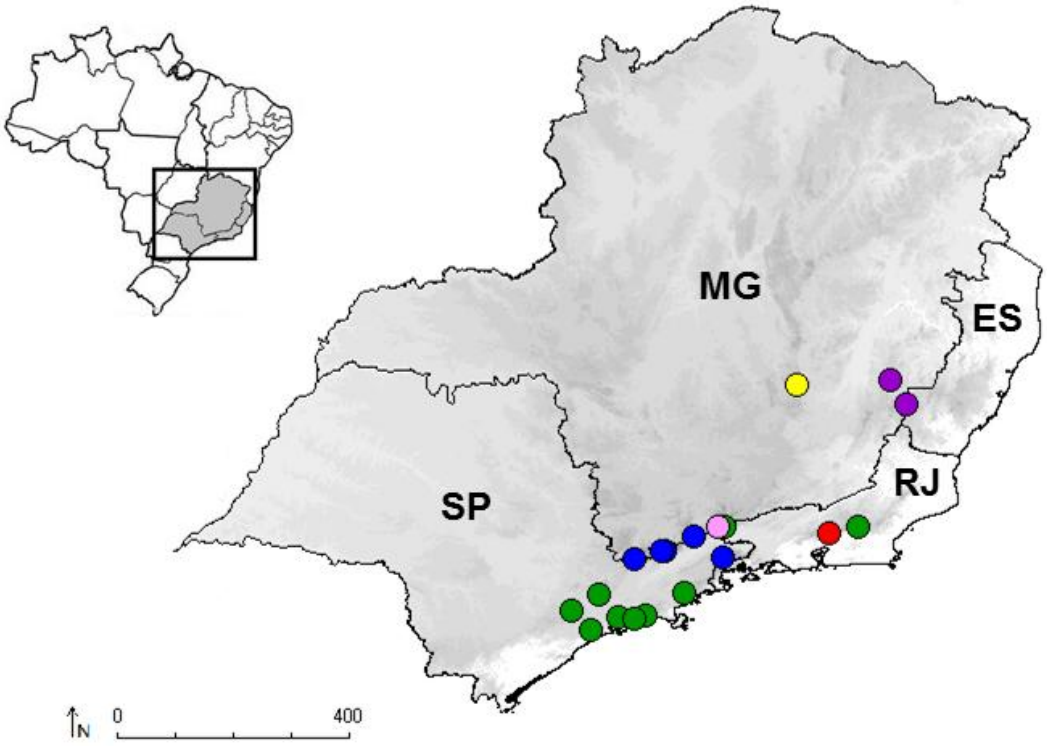

Figure 1: Map of the geographical distribution of the specimens used in this study. The abbreviations for the Brazilian states are: SP - São Paulo, MG - Minas Gerais, RJ – Rio de Janeiro, and ES – Espírito Santo. The colors indicate the mitochondrial clades of *I.*

holti/ I. lactea complex (green – *I. lactea*, pink – *I. holti*, blue – Clade I, purple – Clade II, red – Clade III, and yellow – Clade IV).

Data Collection. Two mitochondrial and four nuclear gene fragments were successfully sequenced from most of the 61 sampled individuals (Appendix B). The mitochondrial DNA consisted of a fragment ca. 630 bp of the gene Cytochrome c Oxidase subunit I (CO1) and a fragment ca. 600 bp of the gene 16S ribosomal RNA (16S). Nuclear fragments included recombination activating gene 1 (RAG1; ca. 521 bp), chemokine receptor 1 (CXCR1; ca. 650 bp), Tensin3 (TNS3; ca. 530 bp), and Tyrosinase (TYR; ca. 549 bp). We extracted genomic DNA from frozen or ethanol-preserved tissues using the DNeasy tissue extraction kit (Qiagen Inc.) or following a standard salt extraction protocol adapted from Maniatis et al. (1982). We performed Polymerase Chain Reaction (PCR) for the amplification of the selected fragments using PCR Master Mix (2X) Fermentas (0.05 u/ll Taq DNA Polymerase, reaction buffer, 4 mM MgCl2, 0.4 mM of each dNTP) and specific primers (Table 1). For mitochondrial and nuclear fragments (TYR and RAG-1), we amplified with the same conditions described in the paper Canedo & Haddad (2012). For CXCR1 and TNS3 fragments the standard reaction conditions were an initial hold for 60s at 94 ºC; 40 cycles of 94 ºC for 30 s, 52 ºC for 30 s, 54 ºC for 30 s and 72 ºC for 1 min, followed by a final hold of 72 ºC for 3 min, and terminating the reaction at 4 ºC. PCR products were visualized in 1% agarose gels. Purification and sequencing were done by Macrogen Inc., Seoul, South Korea (sequencing conducted under BigDye™ terminator cycling conditions, reacted products purified using ethanol precipitation, and runs performed in Automatic Sequencer 3730XL). Chromatograms were fully inspected, embedded primer sequences were deleted, and forward and reverse strands were compared before assembling consensus sequences with CodonCode Aligner 3.5 (Codon Code Corporation). Data also include sequences downloaded from GenBank (NCBI). We aligned the sequences in MEGA 6.06 (Molecular Evolutionary Genetics Analysis; Tamura et al. 2013) using CLUSTAL W (Thompson et al. 1994).

Nuclear sequences were phased with the algorithm PHASE (Stephens et al. 2001), than we built median-joining networks (Bandelt et al. 1999) with NETWORK 4.6.1.1 (www.fluxus-engineering.com). To describe levels of divergence within our data set, we measured the mean corrected *p*-distance between each

mitochondrial DNA (mtDNA) clade and the mean distance across individuals within clades using MEGA 6.06 (Tamura et al. 2013).

Table 1 - Primers used in this study.

Primer		Gene	Sequence	Reference
M13F-AnF1	F	COI	TGTAAAACGACGGCCAGTACHAAYCAYAAAGAYATYGG	M. L. Lyra (unpublished data)
M13R-puc-AnR1	R	COI	CAGGAAACAGCTATGACACTTCTGGGTGTCCGAAAAATC	M. L. Lyra (unpublished data)
16sAR	F	16S	CGCCTGTTTATCAAAAACAT	Palumbi et al. (1991)
16sWilk2	R	16S	GACCTGGATTACTCCGGTCTGA	Wilkinson et al. (1996)
Tyr1C	F	Tyrosinase	GGCAGAGGAWCRTGCCAAGATGT	Bossuyt and Milinkovitch (2000)
Tyr1G	R	Tyrosinase	TGCTGGGCRTCTCTCCARTCCCA	Bossuyt and Milinkovitch (2000)
R182	F	RAG1	GCCATAACTGCTGGAGCATYAT	Heinicke et al. (2007)
R270	R	RAG1	AGYAGATGTTGCCTGGGTCTTC	Heinicke et al. (2007)
WL423	F	TNS3	CAGCATAGGTACTTTATCATCATCAG	Smith et al. (2007)
WL421	R	TNS3	CAGTGTTGGAGAAGATGGTATGTC	Smith et al. (2007)
CXCF1	F	CXCR1	TCCAGAACCATGACTGATAAGTA	Castroviejo-Fisher et al. (2015)
			CAAGGCTTCTGTGATGGAGATCC	
CXCR1	R	CXCR1		Castroviejo-Fisher et al. (2015)

Molecular Data Analysis. Phylogenetic inferences were performed with the methods of Bayesian Inference (BI) and Maximum Likelihood (ML). We built Bayesian trees in MrBayes v 3.2.5 (Ronquist & Huelsenbeck 2011) first by using PartitionFinder (Lanfear et al. 2012) to select locus-specific models of nucleotide evolution that best fit the data. We ran four simultaneous Metropolis-coupled Markov chain Monte Carlo chains (MCMC), each lasting 10,000,000 generations; trees were sampled every 1,000 generations. Trees were built for the concatenated mitochondrial markers, as well as all individual nuclear fragments and all concatenated markers. We evaluated convergence to stationary in TRACER 1.5 (Rambaut & Drummond 2009) using log-likelihood values for all trees. All Bayesian Inference trees were visualized in FigTree v1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/). For Maximum Likelihood trees, we built in MEGA 6.06 (Tamura et al. 2013). The best evolutionary model for each gene was estimated in the same program and we used 1,000 replicas for the bootstrap test (Felsenstein 1985).

To investigate population structure within a Bayesian probabilistic genetic clustering framework, we implemented by Structure 2.3 (Pritchard et al. 2000) using a genotype matrix of all nuclear DNA (nuDNA) sequences. We explored a large range of values by running 20 replicated analyses over a range of *K* (2-10).

Each of these 180 independent runs implemented 500,000 generations and incorporated the possibility of mixed ancestry. The optimal K value was estimated based on the rate of change of the log probability of the data between successive K values, using ΔK (Evanno et al. 2005), as calculated by Structure Harvester. (http://taylor0.biology.ucla.edu/structureHarvester/).

Climatic Modelling. To evaluate if the environmental space occupied by the many lineages sampled through our study is similar across lineages, and hence evaluate whether we can confidently model them as a single entity (hence effectively increasing sample sizes for modeling purposes), we generated an environmental principal component analysis (PCA) using the dismo package in R (Venables & Smith 2005) by combining information from weather-station based bioclimatic variables freely available from the WorldClim database (Hijmans et al. 2005) and all occurrence data gathered for our species complex.

To predict the spatial distribution of suitable environments for *I. holti/ I. lactea* complex in the past and present, we developed species distribution model (SDMs) employing the maximum entropy algorithm in MAXENT v3.3.3 (Phillips et al. 2009) using georeferenced points. Given that the available occurrence data often show strong spatial bias in sampling efforts, we used SDMTOOLBOX v1.0b (Brown 2014) to reduce spatial autocorrelation in the occurrence data by selecting one record within a 5 km radius (Kramer-Schadt et al. 2013). We created a layer of the Gaussian kernel density of sampling locations (i.e., a bias layer) with a bandwidth of 50 km to control for background sampling effort. The area of modeling was set including a 10 km buffer. We obtained eight bioclimatic (BIO 1, 4, 10, 11, 12, 15, 16, and 17) layers using the Hadley Centre Climate model (HadCM3 [Fuchs et al. 2013]) from Carnaval et al. (2014) and used ARCGIS and SDMTOOLBOX to produce a base-map and bioclimatic layers with the same map projection and resolution. We used these layers for the modeling purpose because they are the only bioclimatic variables available at a large set of time slices for projection into the past. Using MAXENT, we first built SDMs based on current bioclimatic data then projected the current SDMs to paleoclimate scenarios (6 kya, 21 kya or Last Glacial Maximum – LGM, and 120 kya or Last Interglacial Maximum – LIG) to predict suitable climatic areas during the Pleistocene and Holocene.

Because optimizing model feature class and regularization parameters per species can result in higher-quality output than employing default settings (Radosavljevic & Anderson 2014), we compared models built with linear (L) features, linear (L) + quadratic (Q), only hinge (H) feature, linear (L) + quadratic (Q) + hinge (H), and linear (L) + quadratic (Q) + hinge (H) + threshold (T). For each of these sets of feature classes, we used a suite of regularization multipliers (1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, and 5.0) to explore the possibility of this parameter affecting model output. The performances of these models were then compared, and the best one selected for downstream analyses. The best model was evaluated choosing the highest area under the curve (AUC) and the lowest omission rate (OR).

Divergence Time Estimates. Using the 16S dataset, we estimated the time to the most recent common ancestor (TMRCA) for the complex using relaxed Bayesian molecular clock with uncorrelated lognormal rates. Due to lack of fossil calibrations for this group, we used the protein-coding mtDNA mutation rate proposed by Heinicke et al. (2007) for Terrarana (0.0075 mutations/site/Myr). Although divergence times inferred based on nucleotide substitution rates are susceptible to errors, we considered the use of this approach still valuable to infer a timeframe for hypotheses of historical biogeography and patterns of diversification within *I. holti/ lactea* complex (Fusinatto et al. 2013, Pulquério & Nichols, 2006). We used BEAST v.2.1.3 program (Bouckaert et al. 2014) and we adopted the Yule speciation process and followed default settings for other parameters. Data were analyzed by four independent runs of 10 million generations sampled each 5000 generations, and 25% removed as burn-in using TreeAnnotator v 2.1.3. Convergence to stationarity was evaluated in TRACER 1.5 (Rambaut & Drummond 2009) using log-likelihood values for the tree was visualized in FigTree v1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

Results

Molecular analysis. The mitochondrial Bayesian and Maximum Likelihood reconstructions of the phylogenetic relationships of *I. holti/ lactea* complex based

on concatenated mtDNA markers (COI and 16S; Figure 2), suggest six distinct mitochondrial lineages, which we here refer to as mitochondrial clades.

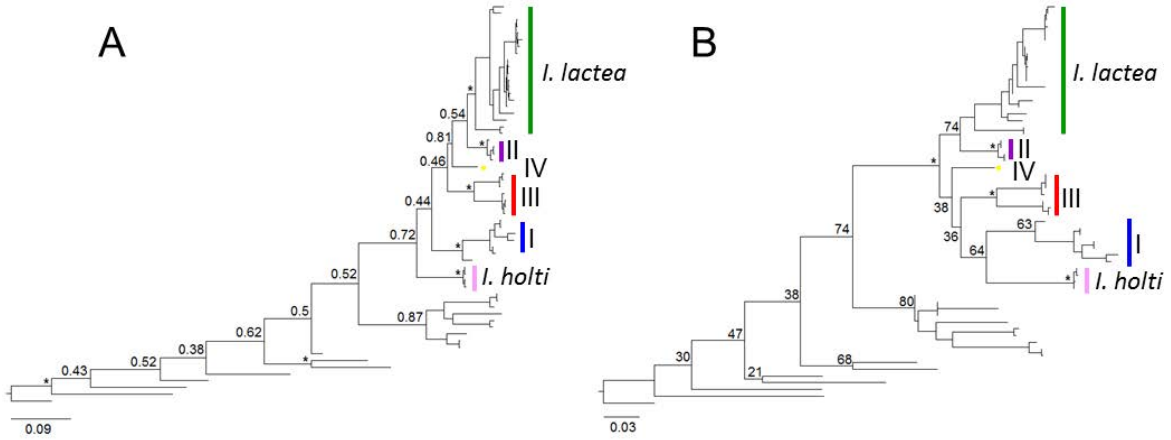

Figure 2: A) Bayesian phylogenetic hypothesis for *I. holti/ I. lactea* complex based on concatenated mtDNA markers COI and 16S. Posterior probabilities are indicated left to nodes (* ≥ 90). B) Maximum Likelihood phylogenetic hypothesis for *I. holti/ I. lactea* complex based on concatenated mtDNA markers COI and 16S. Bootstraps are indicated left to nodes (* ≥ 90). The colors indicate the mitochondrial clades of *I. holti/ I. lactea* complex (green – *I. lactea*, pink – *I. holti*, blue – Clade I, purple – Clade II, red – Clade III, and yellow – Clade IV).

The clade with the highest number of specimens is associated to *I. lactea strictus sensus* (Figure 2; in green), with 20 individuals distributed in Serra do Mar, SP, and RJ, and Serra da Mantiqueira. The clade in pink corresponds to *I. holti*; individuals match the species description and the clade includes specimens collected in the type locality of the species, in the high areas of the Itatiaia montains, near the border between RJ, SP, and MG. Clade I (in blue) includes individuals from Campos do Jordão and Pico dos Martins, São Paulo, and near the border between SP and MG, being relatively supported (> 95% posterior probability and 62 bootstrap). Clade II (in purple) appears divergent from *I. holti* and *I. lactea* clades in the phylogenetic trees with high support (> 95% posterior probability and > 95 bootstrap); its specimens occur in Serra do Caparaó, MG and surrounding area. Clade III (in red) includes individuals from Teresópolis, in Serra

do Órgãos, RJ, being well-supported (> 96% posterior probability and 94 bootstrap). Finally, Clade IV (in yellow) is distinct from the others and includes a single individual from Catas Altas, at Serra do Caraça, MG.

The divergence levels within clades (Table 2) indicated low differentiation; however, the divergence among clades (Table 2) indicated high differentiation among mitochondrial groups. Analyzing the COI fragment, there are from nearly 15% up to 20% of divergence between *I. holti* and the unnamed clades. Usually, with lower divergence of values, the 16S fragment also showed differentiation across groups, reaching 2.8% up to 5.5% between *I. holti* and the unnamed clades.

Table 2: Tamura-Nei corrected mean pairwise distance matrix within clades (the first two columns) and among clades of *Ischnocnema* based on mtDNA marker 16S (inferior diagonal) and mtDNA marker COI (superior diagonal).

	COI	16S	*I. holti*	*I. lactea*	Clade I	Clade II	Clade III	Clade IV	Outgroup
I. holti	0.2%	0		19.6%	17.8%	15.4%	19.3%	16.8%	27.5%
I. lactea	7.7%	2.3%	5.5%		17.3%	11.4%	14.9%	12.4%	26.6%
Clade I	7.0%	1.2%	4.7%	5.8%		18.2%	17.6%	16.3%	26.6%
Clade II	0.9%	0	3.6%	2.7%	4.4%		15.8%	12.3%	22.7%
Clade III	6.4%	1%	4.7%	5.3%	4.8%	3.3%		13.8%	26.4%
Clade IV	-	-	2.8%	4.1%	3.9%	2.1%	3.2%		26.4%
Outgroup	-	-	9.5%	8.2%	9.4%	8.3%	9.9%	8.3%	

Phylogenetic reconstructions based on independent nuclear genes showed similar structure relative to the mtDNA genealogy (Figure 3 and 4). The TNS3 gene suggests the same clades as mitochondrial clades, CXCR1 suggests five clades (Clade IV within *I. lactea*); TYR separates the Clade I from the others ingroup, and RAG-1 suggests four clades (*I. lactea* with Clade III, *I. holti*, Clade I and Clade IV). TYR and RAG-1 genes are more conserved and therefore we could expect a less resolved phylogeny.

Median joining networks of the nucDNA dataset showed many distinct haplotypes, suggesting high genetic diversity (Figure 5 – Appendix C). Twenty-three distinct haplotypes of RAG-1 were present; twenty- one distinct haplotypes of CXCR1 were present; thirteen distinct haplotypes of TNS3 were present; nineteen distinct haplotypes of TYR were present and only one was shared

between clades IV, *I. lactea* and *I. holti.* This happens, because TYR is more conserved and perhaps is an incomplete lineage sorting.

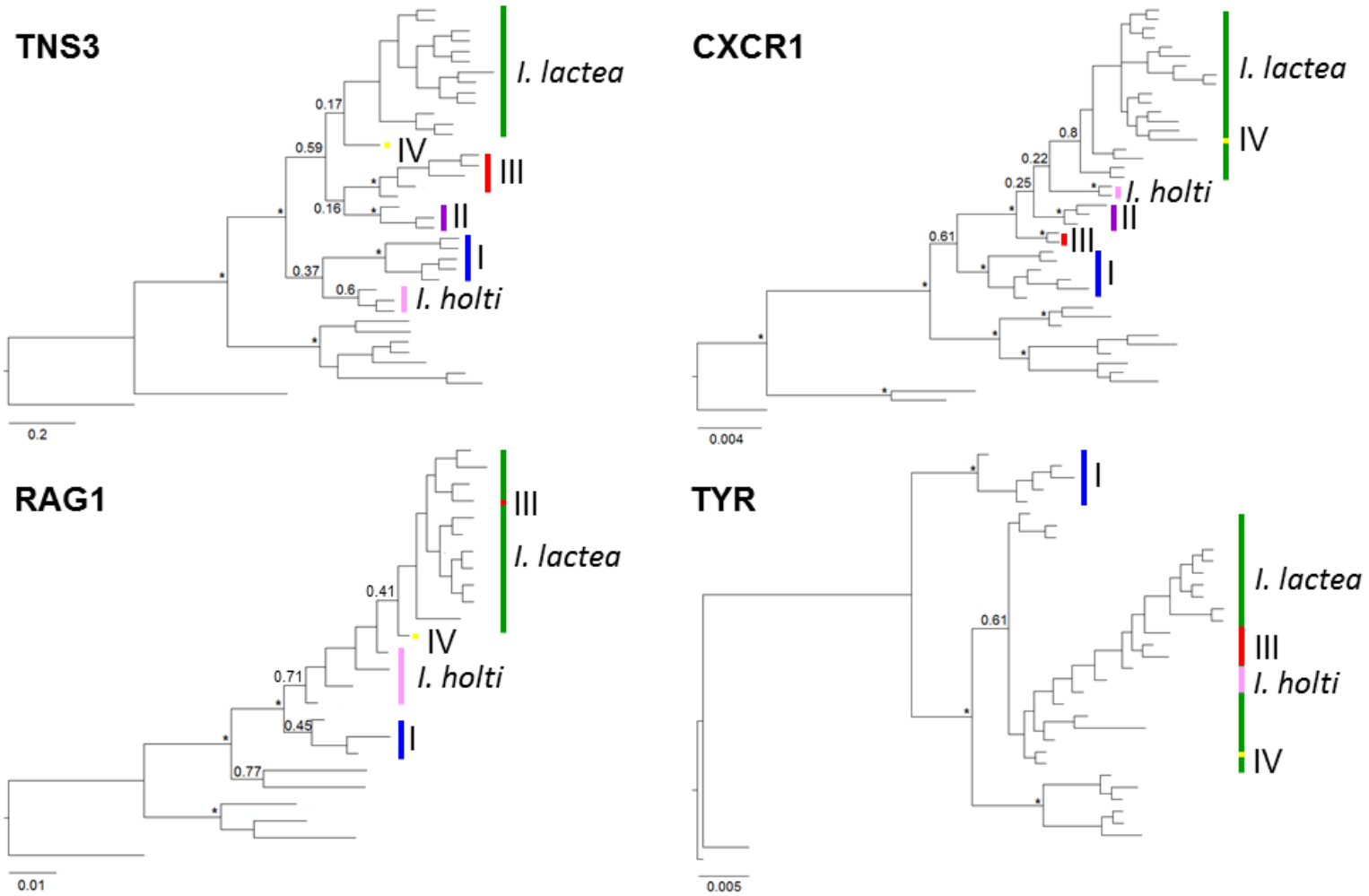

Figure 3: Bayesian phylogenetic hypothesis for *I. holti/ lactea* complex based on nucDNA markers (green – *I. lactea*; pink – *I. holti*; blue – Clade I; purple – Clade II; red – Clade; yellow- Clade IV). Posterior probabilities are indicated left to nodes. * > 0.90.

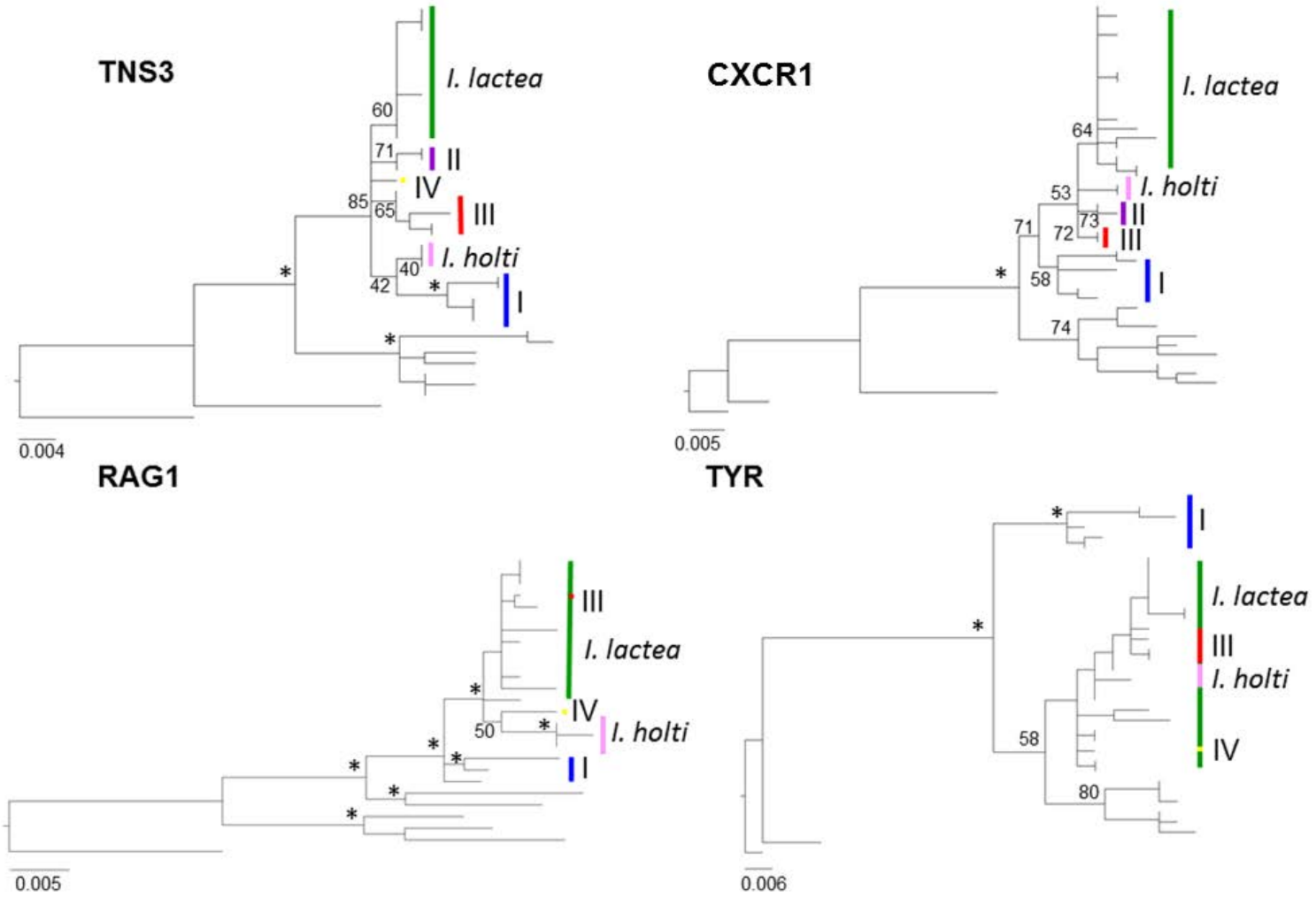

Figure 4: Maximum Likelihood phylogenetic hypothesis for *I. holti/ I. lactea* complex based on nucDNA markers (green – *I. lactea*; pink – *I. holti*; blue – Clade I; purple – Clade II; red – Clade; yellow- Clade IV). Bootstrap values are indicated left to nodes. * > 90.

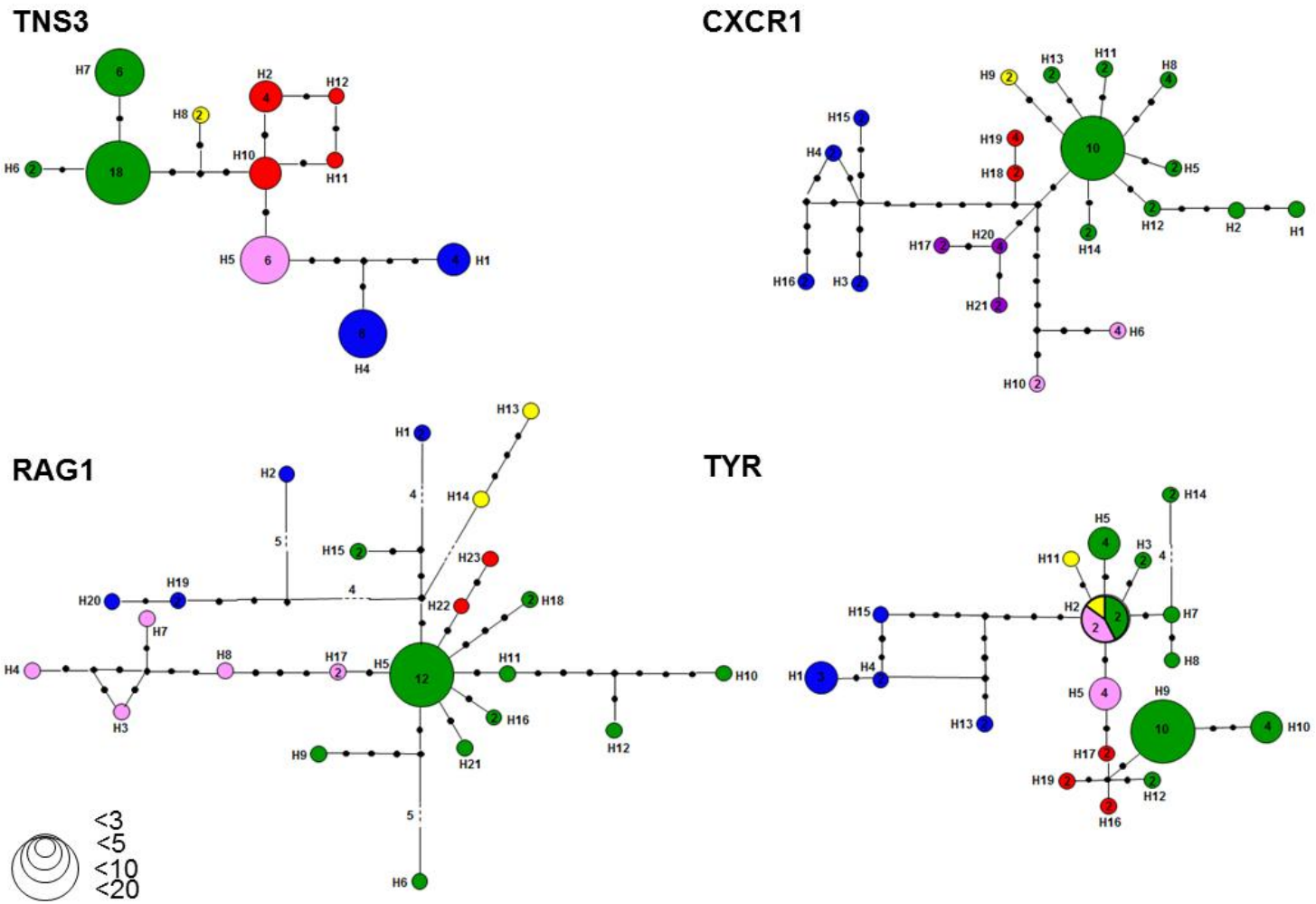

Figure 5: Median joining network showing detailed distribution of haplotypes in the complex. The colors indicate the mitochondrial clades of *I. holti/ I. lactea* complex (green – *I. lactea*, pink – *I. holti*, blue – Clade I, purple – Clade II, red – Clade III, and yellow – Clade IV). Numbers in the circles indicate the number of individuals who share a haplotype, except for single haplotypes.

A Bayesian and Maximum Likelihood reconstructions of the phylogenetic relationships of *I. holti/ I. lactea* complex, based on concatenated mtDNA markers (COI and 16S) and nucDNA (TYR, RAG1, CXCR1, and TNS3), suggest the same six distinct mitochondrial lineages for both methods.

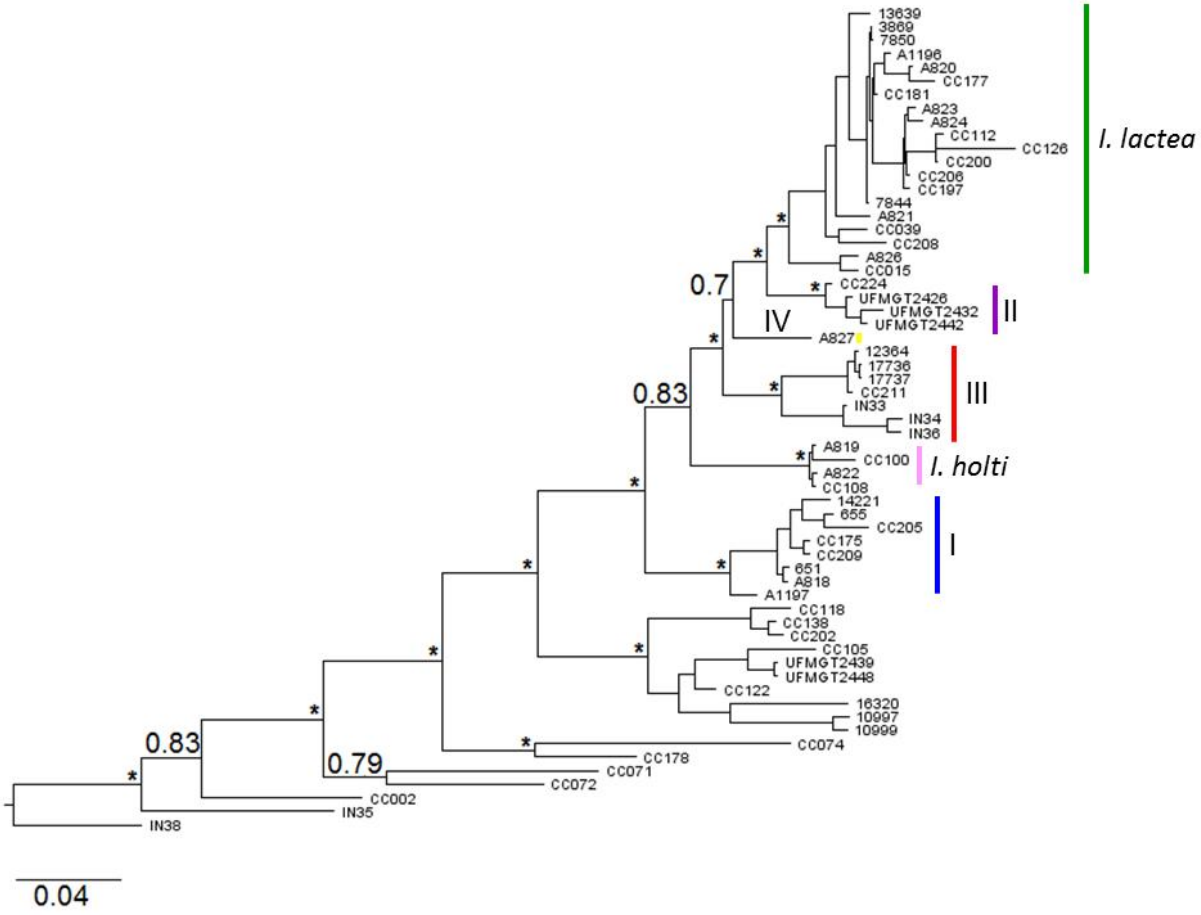

Figure 6: Bayesian phylogenetic hypothesis for *I. holti/ I. lactea* complex based on concatenated genes. Posterior probabilities are indicated left to nodes (* ≥ 0.90). The colors indicate the mitochondrial clades of *I. holti / I. lactea* complex (green – *I. lactea*, pink – *I. holti*, blue – Clade I, purple – Clade II, red – Clade III, and yellow – Clade IV).

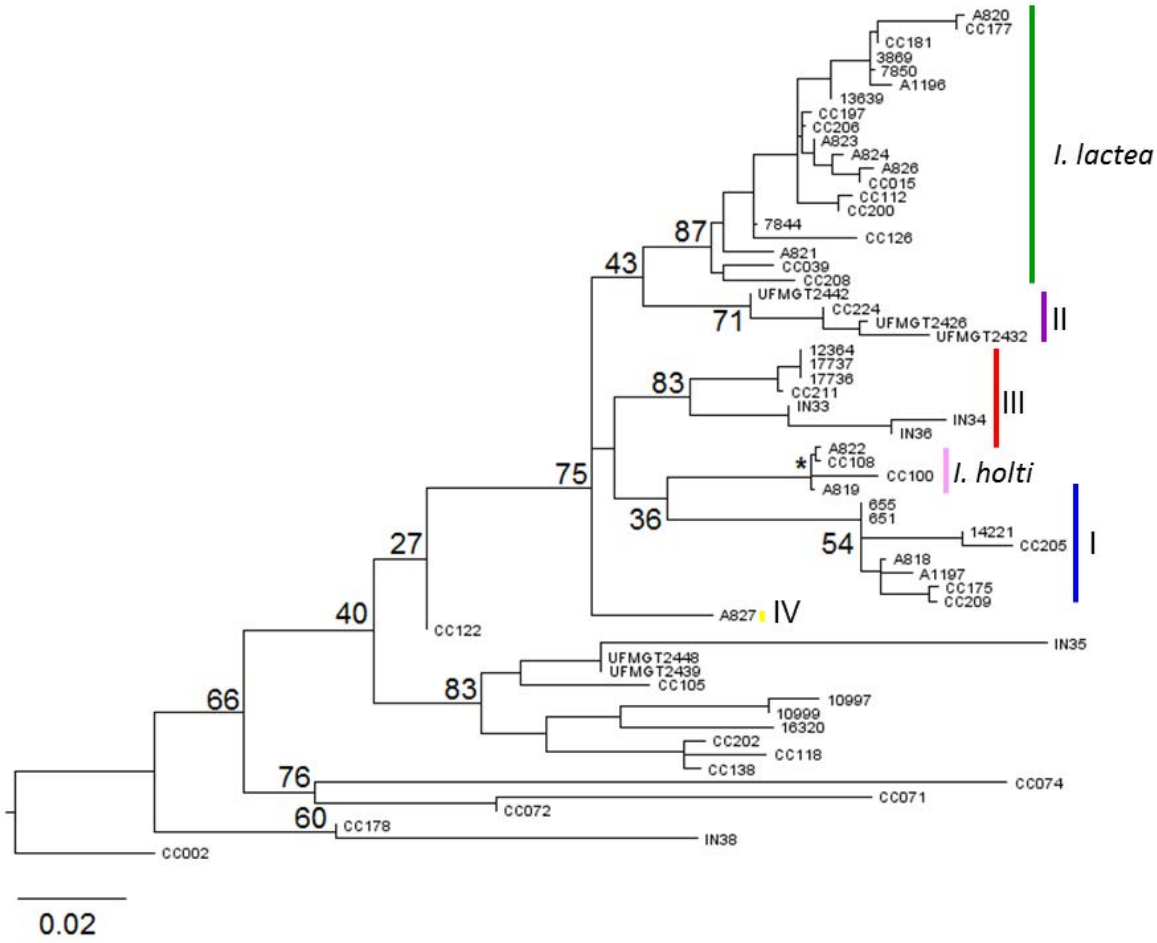

Figure 7: Maximum Likelihood phylogenetic hypothesis for *I. holti / I. lactea* complex based on based on concatenated genes. Bootstraps are indicated left to nodes (* ≥ 90). The colors indicate the mitochondrial clades of *Ischnocnema holti* complex (green – *I. lactea*, pink – *I. holti*, blue – Clade I, purple – Clade II, red – Clade III, and yellow – Clade IV).

Population Structure. Calculations detected a peak at K = 4, (Appendix D) representing a different nuclear marker structure relative to the phylogenetic trees (Figure 8) but, according to Evanno et al. (2005), estimates for the number of groups given by Structure often does not correspond to the number of mitochondrial lineages.

One of the groupings detected at K = 4 is congruent with Clade I (blue). The pink Structure cluster represents all specimens collected in Minas Gerais, including individuals falling in the mitochondrial Clades *I. holti*, II, and IV. This entire cluster is distributed along the Serra da Mantiqueira, MG. The red cluster

includes all specimens of the mitochondrial Clade III, and specimens of *I. lactea* from Nova Friburgo, RJ and Paranapiacaba, SP. The individuals of *I. lactea*, from Nova Friburgo, and individuals of Clade III, from Teresópolis, may have gene flow because they occur in the same formation (Serra dos Órgãos, RJ). This cluster and *I. lactea* cluster (green) are distributed in Serra do Mar.

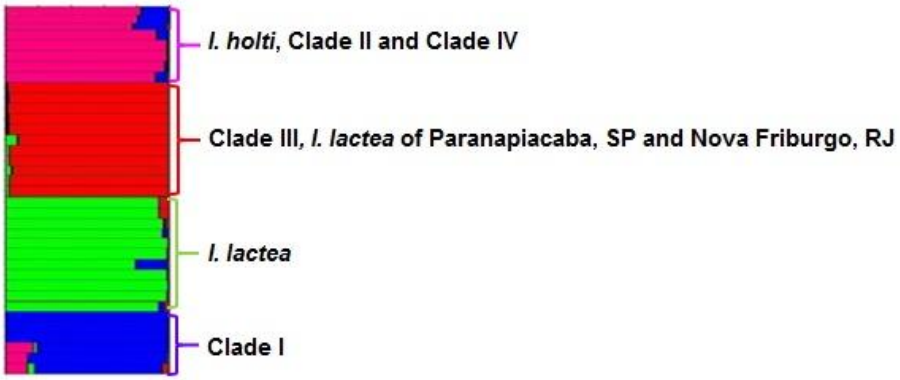

Figure 8: Structure clusters (K = 4). Each population of *Ischnocnema* cluster is represented by a different color.

Climatic Modelling. The environmental PCA showed that the individuals belonging to Clades I, II, III, *I. lactea*, and *I. holti*, occupy similar environmental spaces and Clade IV occupies a very distinct environmental space compared to the others. Given this observation, we decided to pool their localities to generate a distribution model of the whole group. Because the Clade IV occupies a more distinct environment and being a single individual, we opted not to merge it for the modeling procedure to reduce the error.

The best model parameter set showed an AUC of 0.947, corresponded to the linear feature class, and a regularization multiplier of 0.5. Precipitation of Wettest Quarter was the variable that contributed most significantly to this model.

The predicted distribution of *I. holti*/ *I. lactea* complex was also severely restricted during Last Interglacial (120Ky) period, for which only the highest peaks

of Serra do Mar, SP and Itatiaia, near the border between RJ, SP, and MG were predicted to be suitable. The cold climate of the Last Glacial Maximum (21Ky) and Mid-Holocene (6Ky) periods revealed a little expansion of suitable habitat, manly in São Paulo, Minas Gerais and Espírito Santo. Present day SDMs suggest that the complex is still restricted to small remnants of suitable climates (Figure 9).

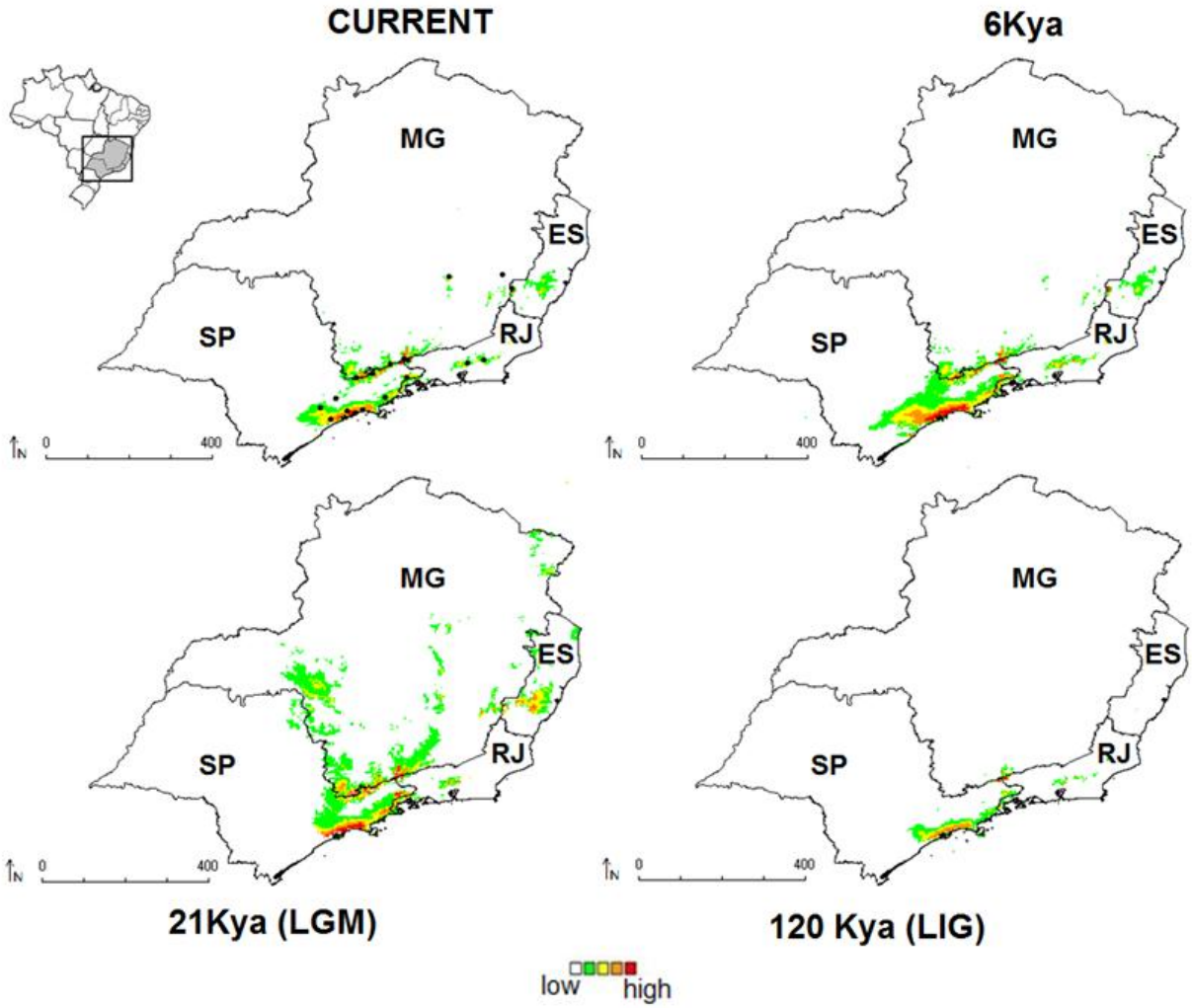

Figure 9: Modelled suitable climatic conditions for *I. holti/ I. lactea* complex across Quaternary climatic fluctuations and current climate. A – Current (with the geographical distribution of the specimens), B - 6Kya, C - LGM (21Kya), and D - LIG (120Kya). Green color indicates low predicted suitability, yellow to red colors indicate higher values, grey areas indicate those pixels with values below the Minimum Training Presence (MTP) threshold, as determined based on the calibration data. The abreviativos for the Brazilian states are: SP - São Paulo, MG - Minas Gerais, RJ – Rio de Janeiro, and ES – Espírito

Santo.

Divergence Time Estimates. The time of the most recent common ancestors estimated for the *I. holti / I. lactea* complex correspond to the Miocene and Pliocene (Figure 10). The most recent common ancestor of all lineages dates from about 10.64 million years.

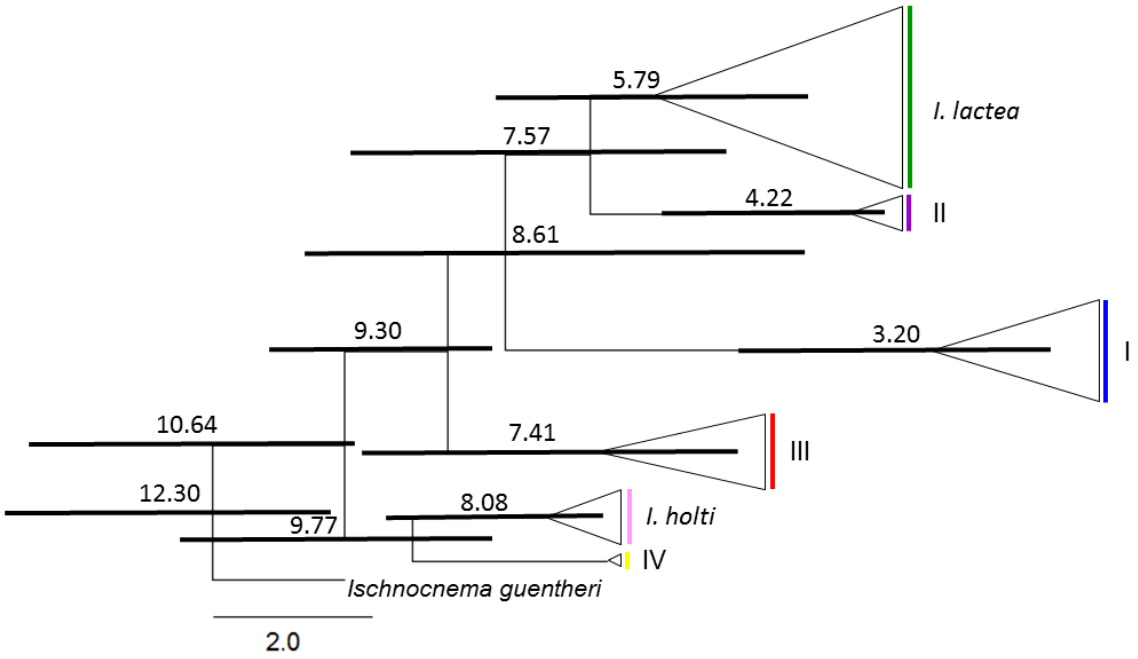

Figure 10: Topology found in tMRCA analysis for *I. holti/ I. lactea* complex. Numbers above branches indicate the estimated values for the tMRCA (in millions of years - Mya); black bars indicate pattern deviation.

Discussion

Phylogeography. The results suggest a high genetic diversity among populations, with six clades supported by mitochondrial and nuclear markers (even with minor variations), indicating several cryptic species and it has been observed for others *Ischnocnema* groups (e.g., Gehara et al. 2013). However, we could not resolve the relationship between them.

The Clade I includes species from Serra do Mar, SP and Serra da Mantiqueira, along the border between SP, RJ, and MG. It is the best supported clade from both the nuclear and mitochondrial analyses, generally recovered as

the most basal lineage within the complex. It is evident from phylogenetic analyses that the Clade I is distinct from the remaining populations, as well as in the clusters generated by Structure.

Ischnocnema lactea (Miranda-Ribeiro, 1923) inhabits the southeastern Brazil, specifically in coastal mountains, occurring between 800-2500 m altitude. Its type locality is Iguape, state of São Paulo (Cruz & Carvalho 2014). We identified the Clade *I. lactea* because of morphological similarity of the type specimens and the relative proximity of Iguape.

The Clade *I. holti* includes specimens collected in the type locality of the species, in Alto do Itatiaia, Parque Nacional do Itatiaia, Itamonte, MG. (Targino & Carvalho 2008). Costa et al. (2008) expanded the geographic distribution of *I. holti*, recording its occurrence in Teresópolis, Rio de Janeiro, but individuals collected in Teresópolis belonged to the mitochondrial Clade III and did not fall within the *I. holti* Clade according to the phylogenetic and structure analyses; these two lineages are also very divergent from each other.

The Clade II is well structured and is composed by individuals of Caparaó National Park and Simonésia, both in the state of Minas Gerais. Such locations are close to each other and connected through a single mountain range. These localities share other amphibian species in common as *Hylodes babax* (da Silva et al. 2012).

The Clade IV includes only an individual; however, the result of environmental PCA showed that this species inhabits a different environment compared to the rest, demonstrating the need for additional field efforts at this site to better understand the position of Clade IV within the phylogeny.

The divergence levels among clades suggested by genetic distance indicates high differentiation across mitochondrial groups. Vences et al. (2005) suggested limits for amphibian species around 5% divergence in 16S and 10% divergence in COI. Fouquet et al. (2007) argued in favor of the use of 3% of divergence in 16S to identify candidate Neotropical frog species. Crawford et al. (2010) suggested values higher than 6% divergence in COI or 1.2% divergence in 16S for identify candidate species. Smaller population sizes lead to fewer dispersing individuals, whose genetic variation would stand a slim chance of fixation within the bulk of the population existing in all habitats (Holt 1996). This could be being observed in this complex because it inhabits different and rugged

mountain environments in southeast of Brazil, facilitating the fixation of genetic variants in each population, and generating high divergence values.

In general, all values presented in this study are higher than values above, but we do not have other evidence to confirm the *status* of candidate species (e. g. bioacoustics, morphology - Vieites et al. 2009), and there are some critics and disagreements for the establishment of divergence values (Ferguson 2002), thus, we decided to called them as a complex species, while other evidences are missing.

Climatic modeling and Divergence times. Species distribution modeling is a relatively new method and it has been criticized for including abiotic factors only and not taking biotic parameters (Elith & Leathwick 2009). Nonetheless, the high explanatory power of our models (AUC = 0.95) represented high consistence between actual and predicted occurrences. We chose MAXENT algorithm to ran the analysis because it is considered the best model for low sample sizes (Wisz et al. 2008).

In this study, we assume that the *I. holti/ lactea* complex is restricted to higher montane habitats in the past to the current days. This distribution profile is different when compared with lowland and mid-altitude species of the southern Atlantic rainforest (Carnaval et al. 2009; Fitzpatrick et al. 2009) Under LGM conditions, these species appeared climatically unsuitable restricting itself to the refuges in the northern portion of the forest. Currently, these species live in milder climates and high temperatures, expanding their ranges (Carnaval et al. 2009).

Tropical montane regions harbors have high levels of taxa diversity because of their endurance to past climate change (Bell et al. 2010). These taxa that are restricted to high elevations were probably more widespread under cooler climates, and are currently restricted in their distribution (Rodrigues et al. 2009). Amaro et al. (2011) suggested that the *Proceratophrys boiei* persisted regions of southern Atlantic rainforest during the LGM, confirming the hypothesis that the altitude species profile distribution have a pattern opposite to that observed in lowland forms.

The *I. holti / I. lactea* complex revealed a little expansion of suitable habitat in LGM as expected for altitude species. However, the overall distribution profile of the complex is little changed during periods proposed by modeling (we observed

no significant changes of expansion and contraction of suitable habitat), suggesting a different pattern of expansion for population living at very high altitudes.

Given that current temperatures are warmer than to recent climate cycles, we expect that this complex will have its range further reduced under current climate change (Pounds et al. 1999).

The time of divergence estimated by the complex (about 11 Mya) is older than the time included in the species distribution modeling (21,000 years), confirming that this complex have been isolated a long time on the tops of mountains. These results are consistent with the results generally found to Terrarana, where the divergence times are generally estimated for the 50 Myr (Heinicke et al. 2007).

On the other hand, the dating of emergence of some mountain ranges, such as the Serra do Mar, Serra da Mantiqueira, and Serra do Espinhaço (it is estimated that the uplift of these mountains began in Cretaceous period - Petri & Fúlvaro 1983; Mello et al. 1985; Riccomini et al. 1989) and some geographical location of faults and neotectonic lineament (Figure 11 - Saadi et al. 2002) as a Caratinga Fault and Rio Paraíba do Sul crustal discontinuity (it is estimated that the uplift of these faults began in Neoproterozoic period) and Além Paraíba Fault Zone (it is estimated that the uplift of this fault began in Eocene period), are older than the time of divergence of the complex. All these faults and lineaments were identified as possible explanations for diversification of the *Rhinella crucifer* group (Thomé et al. 2010) and are nearly coincident with some clades limits for the *I. holti/ I. lactea* complex. However, we need more studies on the dispersion and vicariant process, to relate the emergence of these populations with the formation of these mountain ranges and faults.

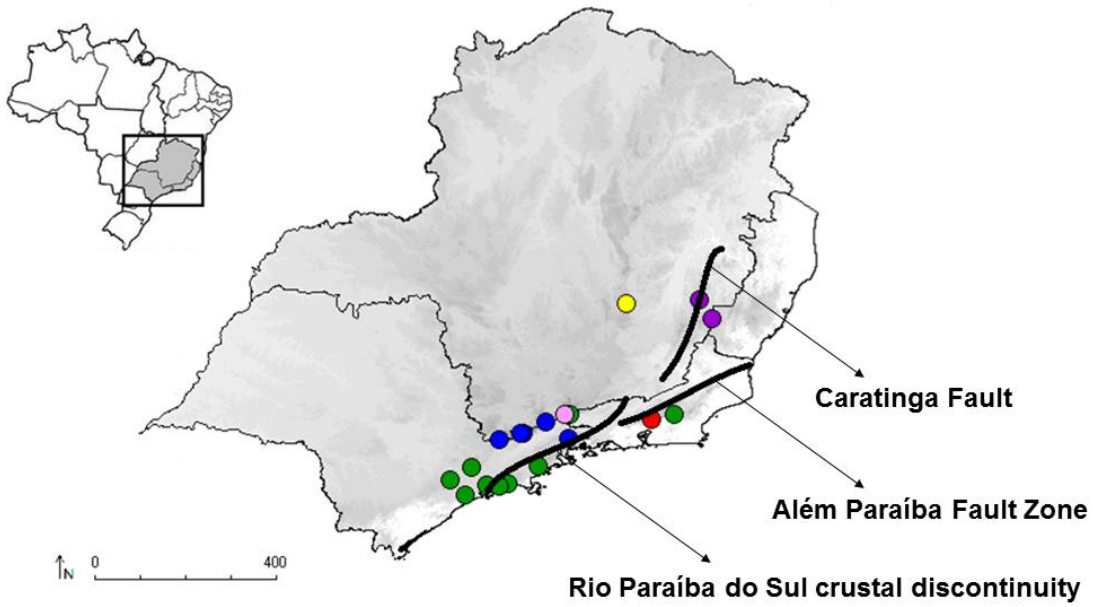

Figure 11: Distribution of the clades of the *I. holti/ I. lactea* complex, and approximate location of faults and lineaments quaternary (sensu Saadi *et al.* 2002). The colors indicate the mitochondrial clades of *I. holti / I. lactea* complex (green – *I. lactea*, pink – *I. holti*, blue – Clade I, purple – Clade I, red – Clade III, and yellow – Clade IV). The abbreviations for the Brazilian states are: SP - São Paulo, MG - Minas Gerais, RJ – Rio de Janeiro, and ES – Espírito Santo.

Conclusion

This study suggests that the *Ischnocnema holti* and *Ischnocnema lactea* form a complex of species distributed in the tops of mountains of the Atlantic Forest, southeastern Brazil. We found six clades supported by mitochondrial and nuclear markers (even with minor variations) and genetically divergent, however we could not resolve the relationship between them. The modeling suggests that the populations of this complex remained restricted regions of altitude at least since the Last Interglacial (LIG), although they may have had a small habitat expansion in the last Glacial Maximum. The dating of the separation of lineages also suggests an older isolation, and the separation would have occurred between 7 and 11 million years ago. Therefore, the results suggest a different expansion pattern for the populations living at very high altitudes. This study demonstrates the importance to expand research to the species that inhabit the Atlantic forest,

especially those who occupy areas of altitude, because of the high endemism of these areas and their importance for biodiversity conservation.

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Appendix A – Samples of specimens of Ischnocnema and collection sites. Collection abbreviations follow Sabaj Pérez (2010), except for MLP DB (Diego Baldo collection, at Museo de La Plata, Argentina), MTR (tissue collection and Miguel Trefaut Rodrigues collection, both at Instituto de Biociências, Universidade de São Paulo, Brazil), and CTMZ (tissue collection at Museu de Zoologia da Universidade de São Paulo, Brazil). The abbreviations for the Brazilian states are: SP - São Paulo, MG - Minas Gerais, RJ – Rio de Janeiro, and ES – Espírito Santo.

Species	Voucher	Nº extraction	Locality	Longitude	Latitude
Ischnocnema gr. *lactea*	CFBH6719	A818	Camanducaia, MG	-46.038	-22.863
Ischnocnema holti	CFBH10316	A819	Itamonte, MG	-44.628	-22.369
Ischnocnema sp. (gr. *lactea)*	CFBH11170	A820	PESM, SP	-46.743	-23.985
Ischnocnema holti	MNRJ57641	A821	PARNA, RJ	-44.734	-22.362
Ischnocnema holti	MNRJ57645	A822	Itamonte, MG	-44.628	-22.369
Ischnocnema sp. (gr. *lactea)*	CFBH28167	A823	Paranapiacaba, SP	-46.291	-23.770
Ischnocnema gr. *lactea*	MZUSPfield1574	A824	Paranapiacaba, SP	-46.291	-23.770
Ischnocnema sp.	MNRJ57308	A826	Nova Friburgo, RJ	-42.556	-22.368
Ischnocnema gr. *lactea*	MNRJ55003	A827	Catas Altas, MG	-43.496	-20.139
Ischnocnema cf. *lactea*	CFBH32293	A1196	PESM, SP	-46.743	-23.985
Ischnocnema gr. *lactea*	CFBH33877	A1197	Serra da Bocaina, SP	-44.686	-23.020
Ischnocnema oea	MNRJ34949	CC002	Santa Teresa, ES	-40.644	-19.977
Ischnocnema cf. *holti*	MNRJ51474	CC015	Nova Friburgo, RJ	-42.556	-22.368
Ischnocnema lactea	CFBH14066	CC039	Santa Virgínia, SP	-45.264	-23.405
Ischnocnema venancioi	MNRJ44564	CC071	Teresópolis, RJ	-43.000	-22.466
Ischnocnema parva	MNRJ 44559	CC072	Teresópolis, RJ	-43.000	-22.466
Ischnocnema sp. (gr. *lactea*)	CFBH12177	CC074	Caraguatatuba, SP	-45.492	-23.619
Ischnocnema holti	MNRJ57639	CC100	Itamonte, MG	-44.628	-22.369
Ischnocnema sp.	MNRJ57644	CC105	PARNA, RJ	-44.734	-22.362
Ischnocnema holti	MNRJ57646	CC108	Itamonte, MG	-44.628	-22.369
Ischnocnema lactea	MTR 10435	CC112	Paranapiacaba, SP	-46.291	-23.770
Ischnocnema spanios	CA field# 148	CC118	Mogi das Cruzes, SP	-46.163	-23.748
Ischnocnema cf. *randorum*	MCL field# 0117	CC122	PESM, SP	-46.743	-23.985
Ischnocnema cf. *holti*	USP-T CX80ST41	CC126	Boracéia, SP	-45.869	-23.745
Ischnocnema sp.	USP-T CX128ST80	CC138	Paranapiacaba, SP	-46.291	-23.770
Ischnocnema aff. *holti*	MTR 11068	CC175	Campos do Jordão, SP	-45.583	-22.733
Ischnocnema lactea	AF lab# 1576	CC177	Caucaia do Alto, SP	-47.021	-23.684

Ischnocnema juipoca	MCL field# 0069	CC178	Boracéia, SP	-45.869	-23.630
Ischnocnema lactea	CFBH16772	CC181	PESM, SP	-46.743	-23.986
Ischnocnema lactea	CFBH23477	CC197	Bertioga, SP	-46.058	-23.806
Ischnocnema cf. *lactea*	CFBH29054	CC200	Paranapiacaba, SP	-46.291	-23.770
Ischnocnema cf. *spanios*	MZUSPfield741	CC202	Paranapiacaba, SP	-46.291	-23.770
Ischnocnema aff. *holti*	CFBH24163	CC205	Pico dos Martins, SP	-45.121	-22.502
Ischnocnema cf. lactea	CFBH29107	CC206	Paranapiacaba, SP	-46.291	-23.770
Ischnocnema cf. *lactea*	MZUSPfied933	CC208	Cantareira, SP	-46.600	-23.414
Ischnocnema aff. *holti*	MTR10965	CC209	Campos do Jordão, SP	-45.613	-22.742
Ischnocnema cf. *holti*	CFBH24762	CC211	Teresópolis, RJ	-43.000	-22.466
Ischnocnema cf. *holti*	USP-T CX71ST33	CC224	Caparaó, MG	-41.792	-20.448
Ischnocnema cf. *holti*	ZUFRJ13M106	IN33	Teresópolis, RJ	-43.000	-22.466
Ischnocnema cf. *holti*	ZUFRJ13M101	IN34	Teresópolis, RJ	-43.000	-22.466
Ischnocnema aff. *guentheri*	ZUFRJ13M102	IN35	Teresópolis, RJ	-43.000	-22.466
Ischnocnema cf. *holti*	ZUFRJ13M103	IN36	Teresópolis, RJ	-43.000	-22.466
Ischnocnema nasuta	UFMG13905	IN38	PESM, SP	-46.743	-23.985
Ischnocnema lactea	UFMG13329	UFMG2426	Simonésia, MG	-42.038	-20.061
Ischnocnema lactea	UFMG13337	UFMG2432	Simonésia, MG	-42.038	-20.061
Ischnocnema sp.	UFMG13347	UFMG2439	Simonésia, MG	-42.038	-20.061
Ischnocnema lactea	UFMG13344	UFMG2442	Simonésia, MG	-42.038	-200.61
Ischnocnema sp.	UFMG10961	UFMG2448	Simonésia, MG	-42.038	-20.061
Ischnocnema gr. *lactea*	CFBH6720	651	Camanducaia, MG	-46.038	-22.863
Ischnocnema gr. *lactea*	CFBH6721	655	Camanducaia, MG	-46.038	-22.863
Ischnocnema sp. (gr. *lactea*)	CFBH12212	3869	PESM, SP	-46.743	-23.985
Ischnocnema sp.	CFBH19342	7844	PESM, SP	-46.743	-23.985
Ischnocnema sp.	CFBH19368	7850	PESM, SP	-46.743	-23.985
Ischnocnema sp. (aff. *lactea)*	CFBH23297	10997	Tapirai, SP	-47.507	-23.963
Ischnocnema sp. (aff. *lactea)*	CFBH23310	10999	Piedade, SP	-47.426	-23.714
Ischnocnema sp. (gr. *lactea)*	CFBH24762	12364	Teresópolis, RJ	-43.000	-22.466
Ischnocnema sp. (gr. *lactea*)	CFBH28167	13639	Paranapiacaba, SP	-46.291	-23.770
Ischnocnema aff. *holti*	CFBH29127	14221	Pico dos Martins, SP	-45.121	-22.502
Ischnocnema sp.	CFBH32302	16320	PESM, SP	-46.743	-23.985
Ischnocnema holti	CFBH30945	17736	Teresópolis, RJ	-43.000	-22.466
Ischnocnema holti	CFBH30946	17737	Teresópolis, RJ	-43.000	-22.466

Appendix B - Samples included in molecular phylogenetic analysis.

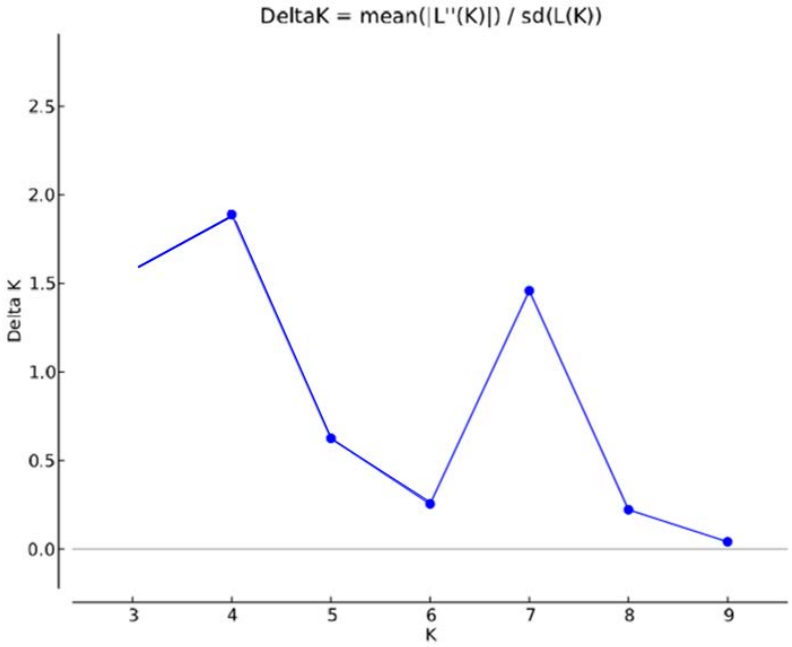
Nº extraction	Voucher	COI	16S	RAG	CXC	WL	TYR
A818	CFBH6719	X	X	X	X	X	X
A819	CFBH10316	X	X	X		X	X
A820	CFBH11170	X	X	X	X	X	X
A821	MNRJ57641	X	X	X			X
A822	MNRJ57645	X	X	X	X	X	X
A823	CFBH28167	X	X	X	X	X	X
A824	MSUSPfield1574	X	X	X	X	X	X
A826	MNRJ57308		X		X		
A827	MNRJ55003	X	X	X	X	X	X
A1196	CFBH32293	X	X		X		X
A1197	CFBH33877		X		X		
CC002	MNRJ34949	X	X	X			X
CC015	MNRJ51474	X	X	X	X		X
CC039	CFBH14066	X	X	X	X	X	X
CC071	MNRJ44564	X	X	X			
CC072	MNRJ 44559		X	X			
CC074	CFBH12177	X	X			X	
CC100	MNRJ57639	X	X				
CC105	MNRJ57644	X	X	X	X	X	
CC108	MNRJ57646	X	X	X	X	X	X
CC112	MTR 10435	X	X		X	X	X
CC118	CA field# 148	X	X	X	X	X	X
CC122	MCL field# 0117					X	X
CC126	USPTCX80S4	X	X	X	X	X	X
CC138	USPTCX128ST80	X	X		X	X	X
CC175	MTR 11068	X		X		X	X
CC177	AF lab# 1576	X	X	X	X	X	X
CC178	MCL field# 0069		X	X	X	X	
CC181	CFBH16772	X	X	X	X	X	X
CC197	CFBH23477	X	X	X	X	X	
CC200	CFBH29054	X	X	X	X	X	X
CC202	MZUSPfield741	X	X		X	X	X
CC205	CFBH24163	X	X		X	X	
CC206	CFBH29107	X	X	X	X	X	X
CC208	MZUSPfied933	X	X		X		X
CC209	MTR10965	X	X	X	X	X	X
CC211	CFBH24762	X	X	X		X	X
CC224	USPTCX71ST33	X	X		X	X	
IN33	ZUFRJ13M106	X				X	X
IN34	ZUFRJ13M101	X			X	X	
IN35	ZUFRJ13M102	X			X		X
IN36	ZUFRJ13M103	X	X		X	X	X
IN38	UFMG13905	X	X		X		
UFMG2426	UFMG13329	X	X		X	X	
UFMG2432	UFMG13337	X				X	
UFMG2439	UFMG13347		X		X	X	
UFMG2442	UFMG13344		X		X		
UFMG2448	UFMG10961		X		X		
651	CFBH6720		X		X		X
655	CFBH6721						X
3869	CFBH12212	X	X		X		
7844	CFBH19342		X		X	X	
7850	CFBH19368	X	X		X	X	
10997	CFBH23297	X	X		X	X	X

10999	CFBH23310		X	X		X
12364	CFBH24762		X			
13639	CFBH28167		X			
14221	CFBH29127		X		X	
16320	CFBH32302	X		X		
17736	CFBH30945	X	X		X	
17737	CFBH30946	X	X			

Appendix C – Samples of *I. holti/ I. lactea* complex used in this study (without outgroup), with their respective genes and haplotype cod.

Nº extraction	Voucher	RAG	CXC	WL	TYR
A818	CFBH6719	H1, H2	H4	H4	H1
A819	CFBH10316	H3, H4		H5	H5
A820	CFBH11170	H5, H6	H5	H3	H6
A821	MNRJ57641	H5			H7,H8
A822	MNRJ57645	H7,H8	H6	H5	H2
A823	CFBH28167	H9,H10	H7	H6	H9
A824	MSUSPfield1574	H11,H12	H7	H7	H10
A826	MNRJ57308		H8		
A827	MNRJ55003	H13,H14	H9	H8	H2,H11
A1196	CFBH32293		H1, H2		H2,H3
A1197	CFBH33877		H3		
CC015	MNRJ51474	H15	H8		H15
CC039	CFBH14066	XH16	H7	H3	H9
CC100	MNRJ57639		H10		
CC108	MNRJ57646	H7,H17	H6	H5	H2
CC112	MTR 10435			H7	H10
CC126	USPTCX80S41	H18		H3	H9
CC175	MTR 11068	H19,H20		H4	H13
CC177	AF lab# 1576	H5	H7	H3	H6
CC181	CFBH16772	H5	H12	H3	H1,H3
CC197	CFBH23477	H5	H13	H3	
CC200	CFBH29054	H5	H14	H3	H9
CC205	CFBH24163	H21	H15	H1	
CC206	CFBH29107	H5	H16	H7	H9
CC208	MZUSPfied933				H14
CC209	MTR10965	H1,		H4	H15
CC211	CFBH24762	H22, H23		H2	H16,H17
CC224	USPTCX71ST33		H17	H9	
IN33	ZUFRJ13M106		H18	H10	H16,H17
IN34	ZUFRJ13M101		H19	H11,H12	
IN36	ZUFRJ13M103		H20	H10	H19
UFMG2426	UFMG13329		H20	H13	
UFMG2439	UFMG13347			H13	
UFMG2442	UFMG13344		H20		
651	CFBH6720				H18
655	CFBH6721				H1
3869	CFBH12212				
7844	CFBH19342			H3	
7850	CFBH19368			H3	
12364	CFBH24762				
13639	CFBH28167				
14221	CFBH29127			H1	
17736	CFBH30945			H2	
17737	CFBH30946				

Appendix D – Graphic generated by Structure Harvester demonstrating the best K. Calculations detected a peak at K = 4.



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CONCLUSÕES FINAIS E PERSPECTIVAS

Os resultados deste trabalho indicam que as populações de *I. holti* e *I. lactea* formam um complexo de espécies distribuídas nos topos das montanhas da Mata Atlântica do Sudeste do Brasil. Nós encontramos seis clados bem suportados pelos marcadores mitocondriais e nucleares (ainda que com pequenas variações) e geneticamente bem divergentes, porém não foi possível resolver a relação entre os mesmos. As modelagens sugerem que as populações deste complexo permaneceram restritas as regiões de altitude pelo menos desde o Último Interglacial (120 mil anos atrás), ainda que possam ter tido uma pequena expansão de habitat no último Máximo Glacial. A datação da separação das linhagens também sugere um isolamento mais antigo, sendo que a separação teria ocorrido entre 7 e 11 milhões de anos atrás. Portanto, os resultados sugerem um padrão diferente de expansão para as populações que vivem em altitudes muito elevadas.

Como perspectivas futuras, visamos aumentar nossos dados geográficos através de parcerias com coleções e colaboradores o que nos dará maior confiabilidade em nossos modelos de distribuição de espécies, haja vista, que neste trabalho modelamos o complexo.

Ruane et al. (2015) afirma que o pequeno número de marcadores moleculares utilizados nas análises filogenéticas, pode resultar em topologias equivocadas, por isso visamos aumentar o número de loci para compreender melhor a estrutura genética deste complexo, já que são espécies difíceis de serem coletadas.

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