Outstanding diversity and microendemism in a clade of rare Atlantic Forest montane frogs

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ABSTRACT

Distributed across topographically complex landscapes that vary from lowland to high elevation, the Atlantic Forest harbors one of the richest biotas worldwide. Atlantic Forest amphibians are particularly speciose, taxonomic accounts are rising and the group is used as model for biogeographic inference. Past climate-related habitat fragmentation is often invoked to explain diversification, with montane taxa expected to become more widespread during glacial times and restrained at interglacials. In this study we investigate diversification in *Ischnocnema lactea* and *I. holti* (Anura: Brachycephalidae), two rare frog species inhabiting Atlantic Forest montane regions in Southeastern Brazil. Previous phylogenetic accounts have suggested uncertain limits between these two sister species. We assembled a multilocus DNA dataset, delimited lineages in this clade, and used ecological niche modeling to explore past and future putative ranges. Assignment analyses and traditional and coalescent phylogenetic methods confirmed the existence of a species complex of Miocene origin comprising nine lineages, most of which show very narrow ranges. Lineages were fully supported as species based in coalescent species delimitation, but the phylogenetic relationships among lineages in higher elevation were unresolved. Models of past ranges suggest extensive suitable areas at the last glacial maximum which, along with phylogenetic uncertainty, are consistent with a hypothesis that climate driven vicariance at higher elevation areas resulted in hard polytomies. Species distribution models under future climates suggest narrower ranges of the lineages relative to now, but no species are currently considered endangered. Overall, our results argue in favor for the reassessment of the taxonomic and conservation status of the *I. holti – I. lactea* species complex.

1. Introduction

Mountains affect the origin and maintenance of biodiversity directly and indirectly, and are often high biodiverse areas themselves (Hoorn et al., 2013). Montane systems in the tropics sustain even larger number of species and striking degrees of endemism at higher elevations (Cadena et al., 2012; Merckx et al., 2015). Mountain taxa diversification is often explained by neutral and allopatric models: populations become isolated in mountaintops and differentiate after enduring intense genetic drift (e.g. Firkowski et al., 2016), albeit non-neutral and sympatric diversification across elevation gradients have also been explored (e.g. Patton and Smith, 1992).

The Brazilian Atlantic Forest is a hyperdiverse biome distributed across the tropical and subtropical Atlantic margin of the South American continent. In eastern Brazil, topography is especially complex and landscapes vary in elevation from sea level to nearly 3,000 m above sea level (Pico da Bandeira, 2892 m a.s.l.). Diversification hypotheses invoke isolation of populations following Plio-Pleistocene climate-related habitat fragmentation (Carnaval et al., 2009) or populations being bounded by geographic barriers (Thome et al., 2014). In this context, whereas the complex relief is permeated by neotectonic faults and lineaments (Saadi et al., 2002), a preference for the cold would cause montane taxa to respond to past climate change in a particular way, becoming more widespread during glacial times and restrained to mountaintops at interglacials (Amaro et al., 2012; Carnaval et al., 2014; Rodrigues et al., 2009).

Atlantic Forest amphibians constitute one of the most diverse vertebrate groups globally; a recent account listing an impressive number of 625 species likely represents an underestimation as new taxa are frequently described (Rossa-Feres et al., 2017). Because of particular life history traits that enhance phylogeographic signal retention, such as low vagility and habitat specificity (Avise, 2000), Atlantic Forest amphibians have been used as models to elucidate the origins of the biome's biodiversity, attributed to a combination of distinct evolutionary mechanisms and deep histories (Turchetto-Zolet et al., 2013). Particularly for amphibians, diversity is sustained by a combination of large altitudinal and latitudinal ranges with a complex relief creating moisture gradients and climatic conditions that vary at local scale (da Silva et al., 2012; Haddad and Prado, 2005).

One of the most emblematic anuran families in the Atlantic Forest is the Brachycephalidae, comprising ground-dwelling, direct-developing frogs in the genera *Brachycephalus* and *Ischnocnema* (Hedges et al., 2008). Both show high species diversity and extreme endemism levels. About half of the Brachycephalidae species were described in the last decade (Frost, 2019), and the availability of molecular data has played a fundamental role in solving phylogenetic uncertainty within complexes of cryptic species. A relevant aspect of the ecology of the family is the preference for montane habitats and very restricted ranges; often, its species span a single mountaintop. This trait is prevalent in *Brachycephalus* but less frequently reported for *Ischnocnema*, although recent molecular studies revealed that several *Ischnocnema* species correspond, in fact, to complexes of multiple, highly divergent lineages with restricted distributions (e.g. Gehara et al., 2017; Taucce et al., 2018a), including cases of microendemism (*I. guenteri*, Gehara et al., 2013).

Ischnocnema currently comprises 38 described species distributed in five species series, as supported by recent phylogenetic studies; they are the *I. guentheri, I. lactea, I. parva, I. venancioi,* and *I. verrucosa* species series (Canedo and Haddad, 2012; Taucce et al., 2018b). The *Ischnocnema lactea* species series is at the base of the *Ischnocnema* tree and comprises 11 species (Canedo and Haddad, 2012; Taucce et al., 2019). Within this series, a particular clade containing *I. lactea* (Miranda-Ribeiro, 1923) and *I. holti* (Cochran, 1948) was highlighted as a possible species complex, given that *I. holti* was recovered paraphyletic in relation to *I. lactea* (Canedo and Haddad, 2012; Taucce et al., 2018a, 2018b). The two species are associated to montane Atlantic Forest physiognomies in Southeastern Brazil, being notably rare in the field and poorly represented in museum collections. *Ischnocnema lactea* inhabits the Serra do Mar mountain range at moderate elevations (700–1120 m a.s.l., Silva-Soares et al., 2018), and was recently redescribed by Silva-Soares et al. (2018). *Ischnocnema holti* was redescribed by Targino and de Carvalho-e-Silva (2008), who restricted its occurrence to a single high elevation site in the Serra da Mantiqueira mountain range (Itamonte, 2000–2400 m a.s.l.). Costa et al. (2008) expanded this geographic distribution to Serra dos Órgãos, and specimens of dubious identification (morphologically similar to *I. holti*) were also found in other high elevation areas of southeastern Brazil (see Table 1, Fig. 1). Little is known about natural history of the populations assigned to the two species names, with individuals of *I. lactea* being reported to reproduce during cold fronts in the winter season, under relatively low temperatures (Silva-Soares et al., 2018).

In this study, we prospect tissue samples from major museum collections in southeastern Brazil to access the genetic diversity of the rare *I. holti – I. lactea* species complex. We delimit lineages by combining traditional frequency and tree-based methods with coalescent methods under a multilocus approach. We use a molecular clock to define a time window for diversification in the complex, and explore aspects of its evolutionary history by contrasting lineage distributions to maps of geographic barriers and past putative ranges obtained with ecological niche modeling (ENM). Finally, we project the *I. holti – I. lactea* species complex climatic niche to the future and discuss its conservation under a global climate change scenario.

2. Material and methods

2.1. Taxon and gene sampling

We gathered tissue samples from vouchers previously identified as *Ischnocnema holti, I.* cf. *holti, I.* aff. *holti, I. lactea, I.* cf. *lactea, I.* aff. *lactea, I.* gr. *lactea, Ischnocnema* sp. (gr. *lactea*), and *Ischnocnema* sp., deposited in six main tissue collections in Brazil. This strategy resulted in 37 tissue samples from 18 localities (Fig. 1, Table S1). We also sampled five species to be used as outgroups in phylogenetic analyses, following results in Canedo and Haddad (2012) and Taucce et al. (2018a, 2018b): *I. randorum* and *I. spanios* from the same species series, *I. juipoca* from the *I. verrucosa* species series*, I. oea* from the *I. guenteri* species series, and *I. venancioi* from the *I. venancioi* species series.

We produced a multilocus dataset including two mitochondrial and four nuclear gene fragments: the mitochondrial fragments consisted of 501 bp of the cytochrome *c* oxidase subunit I gene (CO1) and 542 bp of the 16S ribosomal RNA gene (16S). The nuclear dataset consisted of 581 bp of the recombination-activating 1 gene (RAG1), 632 bp of the solute carrier family 8 member 1 gene (SLC8A1), 385 bp of the tensin 3 gene (TNS3), and 431 bp of the Tyrosinase gene (TYR).

2.2. Laboratory procedures and sequence alignment

We extracted total 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) to amplify selected fragments using the PCR Master Mix (2X) Fermentas (Waltham, USA) (0.05 u/ll Taq DNA Polymerase, reaction buffer, 4 mM MgCl2, 0.4 mM of each dNTP) and specific primers (Table S1). For the two mitochondrial fragments and the nuclear fragments TYR and RAG-1, we used the PCR conditions described in Canedo and Haddad (2012). For the nuclear SLC8A1 and TNS3, PCR conditions were as follows: an initial hold for 60 s at 94 °C; 40 cycles of 94 °C for 30 s, 52 °C for 30 s or 54 °C for 30 s and 72 °C for 1 min, followed by a final hold of 72 °C for 3 min, and terminating the reaction at 10 °C. We sent purified PCR products to Macrogen Inc. (Seoul, South Korea) to sequence the forward and reverse strands under BigDye™ terminator chemistry, in an Automatic Sequencer 3730XL.

We inspected the chromatograms, deleted primer sequences and assembled consensus sequences in Geneious v.6 (Biomatter Ltd.). Obtained sequences were submitted to GenBank and aligned with previously available sequences (Supplementary Table S1) using the MAFFT online service (Katoh and Standley, 2013). We used the default strategy (Auto) for all fragments except the 16S, for which we used the Q-INS-i strategy considering RNA secondary structure (Katoh and Toh, 2008).

2.3. Genetic structure and phylogenetic inferences

We employed two approaches to identify possibly independently evolving lineages in the *I. holti – I. lactea* species complex. First we took advantage of our multilocus dataset to estimate the number of populations and population boundaries with Bayesian assignment analyses. Second, we recovered phylogenetic relationships with traditional phylogenetic reconstruction methods under Maximum Likelihood (ML) and Bayesian inference (BI). For the Bayesian assignment analyses we used Structure 2.3 (Pritchard et al., 2000) with nuclear fragment sequences previously phased in PHASE v.2.1.1 (Stephens et al., 2001; Stephens and Scheet, 2005). For each nuclear alignment we performed 10 separate runs using 100 iterations for burnin followed by the default number of iterations of 100, and a thinning interval of 1. We then coded those sequences phased with a probability equal or superior to 0.9 in all analyses into alleles, considering all variable positions conjunctly (i.e, each fragment as a single and independent locus, Table S1). Because of

Table 1

Locality, elevation, population, clade, and lineage for samples of the *Ischnocnema holti – I. lactea* species complex. Coordinates for localities and voucher information are available in Supplementary Table S1. Elevation group refers to results of Bayesian assignment analyses for K = 2, whereas population refers to K = 7. Clades refer to results from phylogenetic analyses. Lineages correspond to categories combining information on populations and clades, and were used for species tree inference.

Sample	Locality	Locality	Elevation	Population	Clade	Lineage
S1	L01	Piquete, SP	high	1	1	1
S2	L02	Campos do Jordão, SP	high	1	1	1
S3	L02	Campos do Jordão, SP	high	1	1	1
S4	L03	Serra da Bocaina, Silveiras, SP	high	1	1	1
S5	L04	Camanducaia, MG	high	1	1	1
S6	L04	Camanducaia, MG	high	1	1	1
S7	L05	Itamonte, MG	high	2	2	2
S8	L05	Itamonte, MG	high	2	2	2
S9	L05	Itamonte, MG	high	2	2	2
S10	L06	Teresópolis, RJ	high	3	3	3
S11	L06	Teresópolis, RJ	high	3	3	3
S12	L06	Teresópolis, RJ	high	3	3	3
S13	L06	Teresópolis, RJ	high	3	3	3
S14	L06	Teresópolis, RJ	high	3	3	3
S15	L07	Simonésia, MG	high	4	4	4
S16	L07	Simonésia, MG	high	4	4	4
S17	L07	Simonésia, MG	high	4	4	4
S18	L08	Caparaó, MG	high	4	4	4
S19	L09	Catas Altas, MG	high	5	5A	5A
S20	L10	Nova Friburgo, RJ	high	5	5B	5B
S21	L10	Nova Friburgo, RJ	high	5	5B	5B
S22	L11	Itatiaia, RJ	Moderate	–	8	8
S23	L12	Cantareira, São Paulo, SP	Moderate	–	8	8
S24	L13	Santa Virgínia, São Luis do Paraitinga, SP	Moderate	6	6	6
S25	L14	Caucaia do Alto, Cotia, SP	Moderate	6	6	6
S26	L15	Mogi das Cruzes, SP	Moderate	6	6	6
S27	L16	Boracéia, Salesópolis, SP	Moderate	6	6	6
S28	L17	Curucutu, São Paulo, SP	Moderate	6	6	6
S29	L17	Curucutu, São Paulo, SP	Moderate	6	6	6
S30	L17	Curucutu, São Paulo, SP	Moderate	6	6	6
S31	L17	Curucutu, São Paulo, SP	Moderate	6	6	6
S32	L17	Curucutu, São Paulo, SP	Moderate	6	6	6
S33	L18	Paranapiacaba, Santo André, SP	Moderate	6	6	6
S34	L18	Paranapiacaba, Santo André, SP	Moderate	7	6 / 7	7
S35	L18	Paranapiacaba, Santo André, SP	Moderate	7	6 / 7	7
S36	L18	Paranapiacaba, Santo André, SP	Moderate	7	6 / 7	7
S37	L18	Paranapiacaba, Santo André, SP	Moderate	7	6 / 7	7

the restricted number of nuclear fragments, we included mitochondrial information by coding supported clades of a mitochondrial phylogenetic tree as alleles (Fig. S1), a strategy that proved useful under similar conditions (Thomé et al., 2012). We obtained the tree for the two concatenated mitochondrial fragments in RAXML v. 8.2.12 (Stamatakis, 2014), with methods described below. We ran analyses with the "no admixture" model with correlated frequencies and using sampling locations as prior. We performed a first run to estimate the lambda parameter under K = 1, and subsequently ran ten replicates for each K value, ranging from one to 15. In each replicate we conducted one million iterations as burnin, followed by one million iterations. We estimated optimal K based on the mean LnP(K) per K, and the ΔK criterion (Evanno et al., 2005) as calculated by Structure Harvester (Earl, 2012).

Prior to the phylogenetic analyses, we used PartitionFinder 2.1.1 (Lanfear et al., 2017) to optimize partitions in our concatenated alignment, which were then implemented in both ML and BI. For ML reconstruction we used RAXML v. 8.2.10 (Stamatakis, 2014), searching for the best tree with 1,000 replicates and the GTRCAT substitution model. We then estimated node support with 1,000 non-parametric bootstraps under the same model. For BI we used PartitionFinder to select the best nucleotide substitution model for each partition used in BI. For coding fragments (all fragments except 16S) we considered each codon position as a possible partition. Then we ran BI analyses in MrBayes 3.2.6 (Ronquist et al., 2012), running two independent analyses of four chains for 15 million generations, sampling each 1,000 generations and discarding 25% as burnin. We assumed convergence by

checking that the standard deviation of split frequencies was bellow 0.01 and that estimated sample sizes of parameters (ESS, examined in Tracer v1.6 (Rambaut et al., 2014)) were higher than 200. We considered node posterior probabilities as support values. We re-rooted all trees in the clade *I. venancioi + I. oea*, following Canedo and Haddad (2012) and Taucce et al. (2018a, 2018b). We ran analyses in CIPRES (Miller et al., 2010).

2.4. Lineage delimitation

Following the results of genetic diversity, we used the multispecies coalescent model as implemented in BPP v.4.0 (Bayesian Phylogenetics and Phylogeography, Yang and Rannala, 2010) for joint estimation of number of species and species tree (analysis A11). Following program instructions, we ran the analyses with distinct prior combinations of population size (theta) and divergence time (tau), using algorithms 1 and 2. The theta parameter varied from small (IG = 3, 0.004) to large (IG = 3, 0.04), and the tau parameter varied from deep (IG = 3, 0.02) to shallow (IG = 3, 0.002). For each prior combination (four in total) we ran three replicates under algorithms 1 and 2, totaling 24 analyses. We ran each analysis with automatic fine-tune for 1,000,000 generations, with 100,000 generations discarded as burn-in, checking swapping rates were between 0.3 and 0.7. We also estimated, using the software MEGA v.6 (Tamura et al., 2013), the average genetic distances (uncorrected p-distances) within and between the lineages recovered by these strategies for the two mitochondrial genes (COI and 16S).
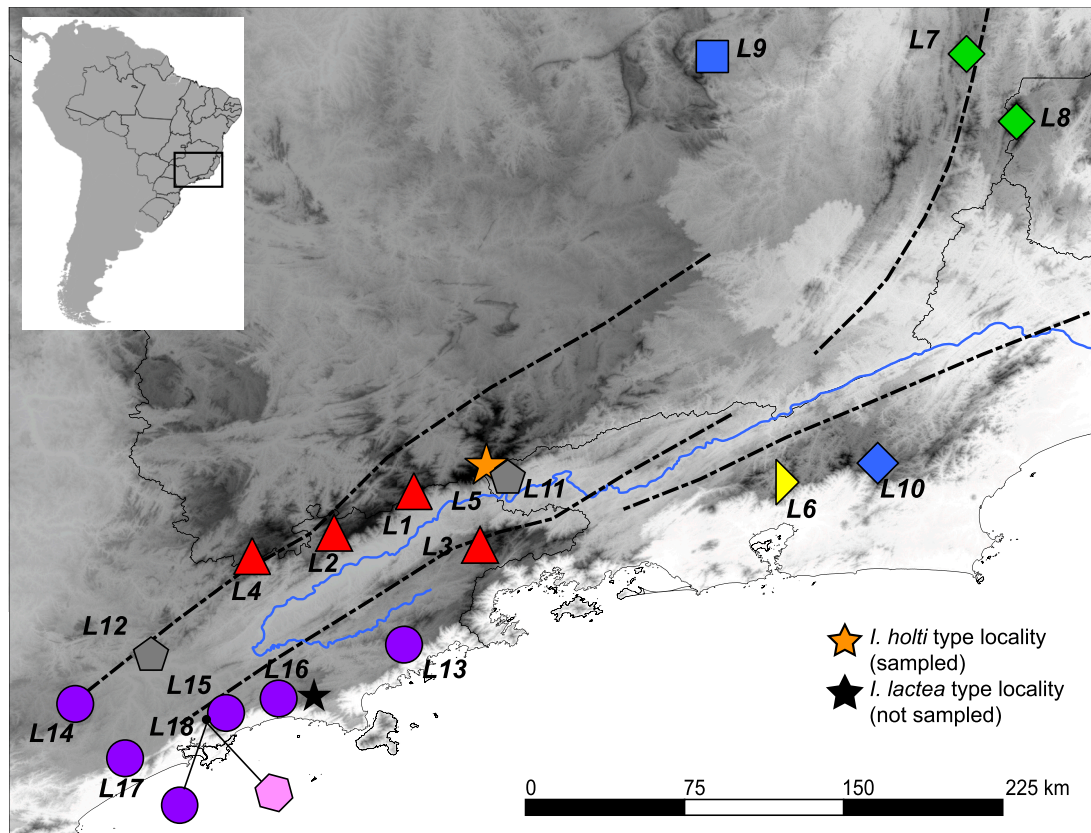
Fig. 1. Map showing localities sampled for the *Ischnocnema holti – I. lactea* species complex, and the location of the type localities for *I. holti* and *I. lactea*. Locality codes follow Table 1, and colours follow Figs. 2 and 3. The blue line represents the Paraíba do Sul river, and dashed lines represent neotectonic barriers (see text for details). For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

2.5. Molecular clock

To set a timeframe for lineage divergence in the system, we used the multispecies coalescent method StarBeast2 (Ogilvie et al., 2017) to reconstruct a dated species tree in the software package BEAST2 v.2.5.0 (Bouckaert et al., 2014), considering as species the lineages identified with BPP. We used a substitution rate obtained in a previous *Ischnocnema* study (Gehara et al., 2017) for the mitochondrial fragment 16S, and excluded other mitochondrial fragment to avoid over-parameterization. We used the bModelTest option (Bouckaert and Drummond, 2017) to estimate nucleotide substitution models for all fragments using nucleotide empiric frequencies, assumed a strict clock for all, and a Yule model for the species tree. We used default priors for most parameters, except for the clock rates of nuclear fragments for which we used a $1/X$ prior. We ran the analysis for 100 million generations, storing trees at each 10,000 and discarding 10% as burnin. We checked convergence by examining ESS values for each parameter as described above.

2.6. Ecological niche modeling

To investigate putative shifts in the distribution of the *I. holti – I. lactea* species complex, we used bioclimatic data at a 2.5-min resolution to model the climatic niche across a selected region in southeastern Brazil, projecting potential distributions for both past and future times. To avoid spatial autocorrelation, we considered occurrences at least 10 km distant (Aiello-Lammens et al., 2015). We obtained the climate layers from Hijmans et al. (2005) and selected five variables after conducting a factor analysis with varimax rotation (Revelle, 2018; Supplementary table S2). They were mean diurnal range, isothermality, mean temperature of warmest, precipitation of the wettest quarter, and

precipitation of the driest quarter. Present-day niche models were calibrated and projected onto reconstructed climatic conditions for the last glacial maximum (LGM, 21,000 ya), mid-Holocene (6,000 ya), and future (Representative Concentration Pathway 4.5, predicted for 2061–2080). Two general circulation models (GCM) for projected climates were used, the Community Climate System Model (CCSM4; Gent and Danabasoglu, 2011) and the Model for Interdisciplinarity Research on Climate (MIROC-ESM; Watanabe et al., 2011). We used four methods: Bioclim (Busby 1991), Gower distance (Carpenter et al., 1993), Support Vector Machine (SVM; Guo et al., 2005), and Maximum Entropy (MaxEnt; Phillips et al., 2006), combined into the ensemble forecasting approach (Araújo and New, 2007). We ran all models in the R v. 3.3.3 environment (R Core Team, 2017) with the package "dismo" (Hijmans et al., 2017) accounting for specific function for each ENM method, except SVM, for which we used the function ksvm from "kernlab" R package (Karatzoglou et al., 2004). To evaluate models, we used the 'leave-one-out test' because of the small number of occurrence records. This method was described as a variation to the k-fold partitioning method, in which a jackknife sampling is imposed by excluding one record every time (see details in Pearson et al., 2007). For predictive consensual maps, we selected the best models ($p < 0.05$) and then weighted by their suitability maps (Hijmans, 2019) by respective D statistic. While the current scenario represents the ensemble prediction from four ENMs, the eight models (4 ENMs * 2 GCMs) were combined for each climate period. Also, we applied a two-way ANOVA using the predicted suitability from all models (4 ENMs × 2 AOGCMs × 3 times) as the response variable to disentangle and map the uncertainties in the potential distribution due to ENMs, AOGCMs over time (Diniz-Filho et al., 2009). The algorithms accounted for most of the variability around the consensus projections (Appendix A), as observed in past studies (e.g. Diniz-Filho et al., 2009).
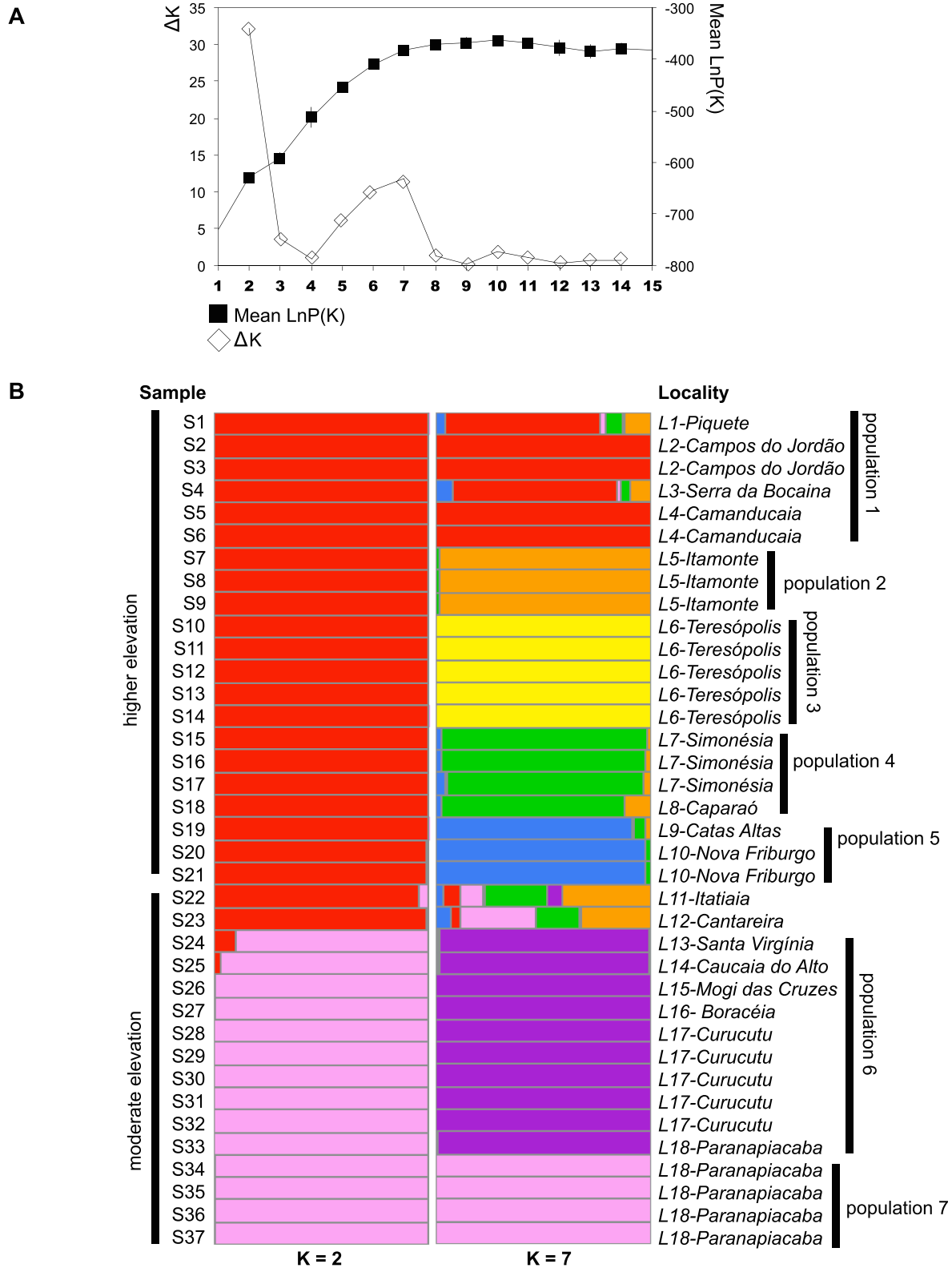
Fig. 2. Results of Bayesian assignment analyses of the *Ischnocnema holti – I. lactea* species complex based in four nuclear and one mitochondrial fragments. A, plot of mean values for LnP(K) at each K value and Evanno's delta K (ΔK); B, barplots at K = 2 and K = 7 with each sample represented as a bar and colors representing the probability of assignment to each inferred population. Sample and locality codes follow Table 1, colours follow Figs. 1 and 3.

3. Results

3.1. Genetic structure and phylogenetic inferences

Structure analyses suggest the existence of seven unique genetic clusters within the *I. holti* – *I. lactea* species clade. The plot with mean LnP(K) across K values shows an inflexion point at K = 7, with similar mean LnP(K) values above that. The Evanno criterion ΔK peaks twice, at K = 2 and K = 7 (Fig. 2A), indicating hierarchical genetic structure: population limits at K = 2 are also recovered at K = 7 (Fig. 2, Table 1). At K = 2 all samples are attributed with high q values, being clustered into a group that ranges through 12 sampled localities (L01–12), spanning most of the geographic distribution of the species complex and the populations that occurs in higher elevation, and another group with a smaller distribution that includes samples from six localities in the Serra do Mar mountain range that are at moderate elevations (L13–18) (Figs. 1 and 2A). At K = 7 most samples are attributed with high q values with two exceptions, one in the southern Serra da Mantiqueira mountain range (L11), and another in the Serra da Cantareira mountain range (L12). One group ranges through most of the southern Serra da Mantiqueira mountain range (L01-02, and L4), also comprising the Serra da Bocaina mountain range (L03) (hereafter population 1), a second group is restricted to a single locality in the northern Serra da Mantiqueira mountain range (L05, hereafter population 2), a third group is restricted to a single locality in the Serra dos Órgãos mountain range (L06, hereafter population 3), a fourth group occurs in two nearby localities in Serra do Caparaó and Simonesia (L07-08, hereafter population 4), a fifth clusters samples from two relatively distant sites, one in the southern Serra do Espinhaço mountain range (L09) and other in the northern Serra dos Órgãos mountain range (L10) (hereafter population 5), a sixth group clusters samples from the Serra do Mar mountain range (L13-18) (hereafter population 6), and a seventh group is restricted to one locality in the Serra do Mar mountain range (L18, hereafter population 7). Only the last two groups are found to co-exist at a given locality (Paranapiacaba, L18) (Figs. 1 and 2B).

The phylogenetic inferences were mostly concordant, and broadly concordant with the Structure analyses. PartitionFinder supported ten partitions for the concatenated dataset, including three for fragment CO1, one for the 16S, one for the 1st codon positions of SLC and TYR, one for the 2nd codon positions of the same fragments, one for the 3rd codon positions of SLC and TNS3, one for 1st and 2nd codon positions of RAG1 and TNS3, one for the 3rd codon position of RAG1, and the last for the 3rd codon position of TYR. Selected nucleotide substitution models were as follows: K80 + G, HKY + I, GTR + G, GTR + I + G, GTR + I + G, HKY + I + G, HKY + G, F81, K80 + G, and GTR + G. The ML and BI trees showed mostly concordant topologies, diverging only in poorly supported clades (Fig. 3A, Supplementary Fig. S2). Both recovered the ingroup with maximum support, and samples clustered in well-supported clades that recover most populations identified in the Structure analyses (see Table 1): clade 1 corresponds to population 1, clade 2 corresponds to population 2, clade 3 corresponds to population 3, clade 4 corresponds to population 4, and clade 7 corresponds to population 7. Samples from population 5 clustered in two different clades (clade 5A with the sample from L09 – Catas Altas, and clade 5B with samples from L10 – Nova Friburgo). Populations 6 and 7 are not reciprocally monophyletic, with population 6 containing population 7 (thus, clade 6 includes clade 7 and corresponds to the Serra do Mar group in the Structure analyses). The two samples with low assignment coefficients (q values) clustered in a separate clade (clade 8). More inclusive well-supported clades include: clade 8 + clade 6, clade 5B (clade 8 + clade 6), and clade 4 [clade 5B (clade 8 + clade 6)] (Fig. 3A).

3.2. Lineage delimitation

We considered the results from both assignment analyses and phylogenetic trees complementarily to define nine putative independently

evolving lineages: lineage 1 corresponding to population/clade 1, lineage 2 corresponding to population/clade 2, lineage 3 corresponding to population/clade 3, lineage 4 corresponding to population/clade 4, lineage 5A corresponding to clade 5A, lineage 5B corresponding to clade 5B, lineage 6 corresponding to population 6, lineage 7 corresponding to population/clade 7, and lineage 8 corresponding to clade 8 (Table 1).

All BPP analyses recovered those same nine lineages as species in the *I. holti* – *I. lactea* species complex with maximum posterior probability (1.0) after values are rounded beyond the fourth decimal case. Each one of the nine lineages was supported as a distinct species with maximum posterior probabilities after values are rounded beyond the sixth decimal case. However, the maximum posterior probability for the best species tree was very low, not surpassing 0.026 considering all analyses. Thus, we do not report on BPP species trees topologies.

Genetic distances between lineages in the COI fragment varied from 3.7% to 18.1% and in 16S varied from 0.8% to 11.5% (Table 2). Lineage 1, with samples in Serra da Mantiqueira and Serra do Mar, had the highest intra-clade divergences (COI: 6.3% and 16S: 3.9%).

3.3. Molecular clock

The most recent common ancestor for the *I. holti* – *I. lactea* species complex was dated back to the Miocene, with a median value around 11 Mya. Most of the divergences in the complex fell within the same geologic period, although the divergence between lineages 6 and 7 was dated back to the middle to late Pleistocene, whereas the divergence between lineage 8 and lineage 6+ lineage 7 was dated to the Pleistocene, with the lower confidence interval reaching the Pliocene.

The dated tree had weakly supported nodes (Fig. 3B), and the topology slightly differed from that of previous analysis (Fig. 3A, Supplementary Fig. S2). Lineage 3 (= clade 3) clustered with lineage 5B, and a clade containing lineages 8, 6, and 7 (Fig. 3B), whereas in previous analyses clade 3 attained a more basal position (Fig. 3A). Other relationships were in full agreement with the previous analyses, as is the case of lineage 8 (= clade 8) clustering with lineage 6+ lineage 7 (= clade 6) with maximum support. In other cases the relationships are similar but support differed among trees, which is the case for lineage 1 (= clade 1) being the first to diverge with maximum support in the species tree, and low or no support in the previous phylogenetic analyses. The StarBeast analysis of the dated tree nonetheless converged with most ESS values above 2,000.

3.4. Ecological niche modeling

ENM suggests that the entire region of southeastern Brazil, and also montane regions of the central and northeastern Brazil, were suitable for this complex in the LGM (Fig. 4). They also suggest that this potentially wide distribution decreased over time, as a result of decreased suitability. Projecting into the future, it is expected that a smaller portion of the Serra do Mar will be climatically suitable for the species complex (Fig. 4). Our models showed that areas further north of Minas Gerais are climatically suitable for *I. holti* – *I. lactea* species complex, in the south of Serra do Espinhaço. The analysis of variance (ANOVA; Table 3) indicated that the ENM component had the highest median proportion (96%) and amplitude (37–100%) of the total sum of squares (SS), in comparison with another components (i.e., atmosphere-ocean general circulation models – AOGCMs, ENM * AOGCM interaction).

4. Discussion

In this study we combined assignment analyses with phylogenetic inference to prospect lineages within the *I. holti* – *I. lactea* species complex, validated those lineages under the multispecies coalescent model, and used ENM to explore its past and future putative range. We
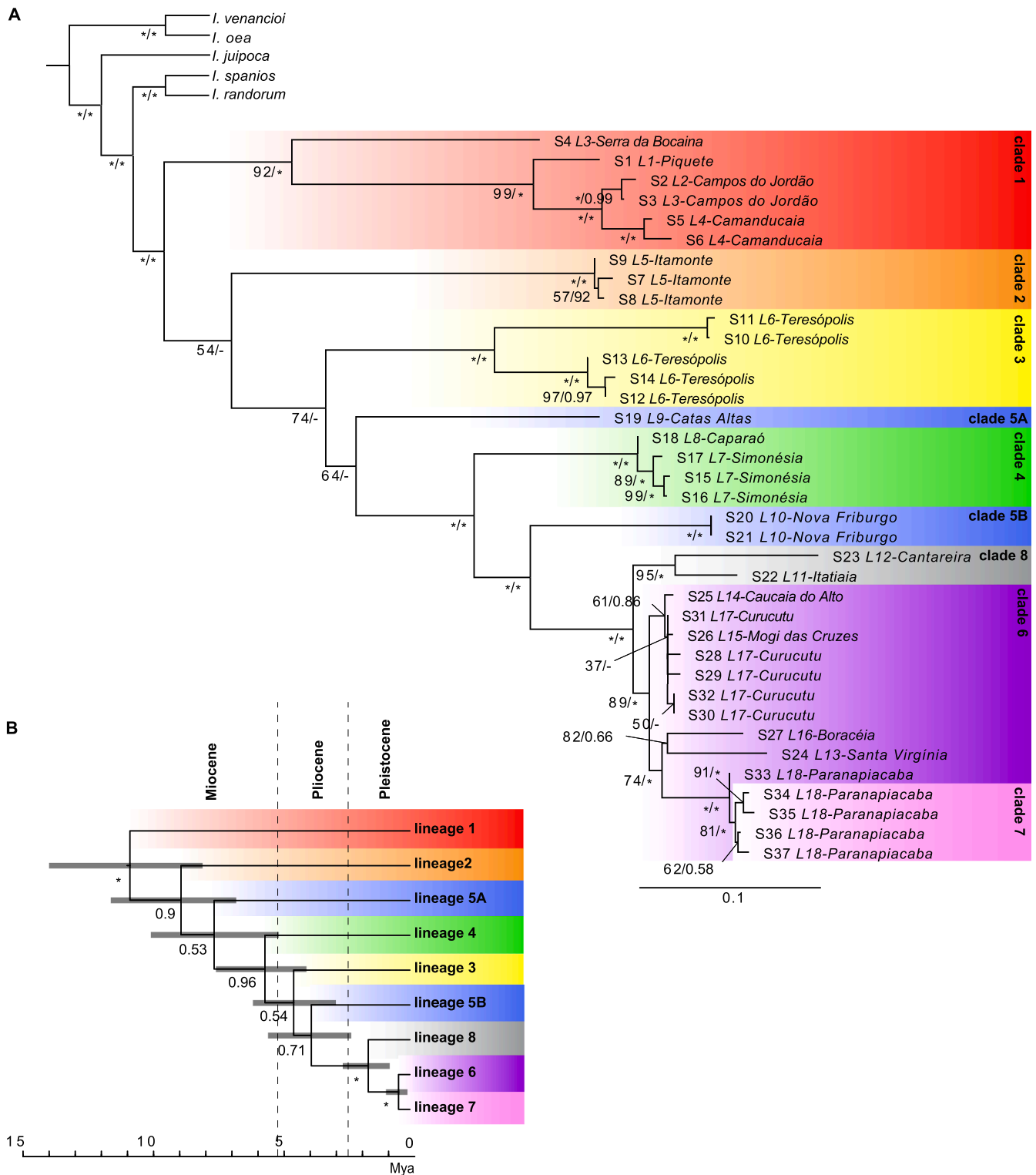
Fig. 3. Phylogenetic relationships among samples of the *Ischnocnema holti – I. lactea* species complex. A, Maximum Likelihood tree obtained with RAXML, with Maximum Likelihood bootstrap values and Bayesian posterior probabilities next to nodes indicating support. Asterisks indicate full support whereas dashes indicate nodes not recovered in the Bayesian analysis. Branch lengths of outgroups were edited for convenience. B, dated coalescent species tree from StarBeast. Grey bars indicate highest posterior density interval of node ages. Numbers indicate posterior probabilities as clade support. Sample and locality codes follow Table 1, colours follow Figs. 1 and 2.

detected nine lineages that were fully supported as species, but their phylogenetic relationships were not completely resolved. Extensive suitable areas at the LGM, along with phylogenetic uncertainty, are consistent with Miocene climate driven vicariance at higher elevation.

Projection for future climate suggests narrower ranges relative to now, but neither species are currently considered endangered. Our results highlight the need for reassessing the taxonomic and conservation statuses in this species complex.

Table 2
Estimates of average P-distances between and within clades (numbered according to Table 1) according to mitochondrial fragments (Average | standard error).
Upper-right: COI distances; lower-left: 16S.

	COI	16S	Gr1	Gr2	Gr3	Gr4	Gr5	Gr5b	Gr6	Gr7	Gr8
Clade1	0.063|0.007	0.039|0.005		0.154	0.153	0.157	0.142	0.162	0.155	0.157	0.155
Clade2	0.004|0.002	0|0	0.105		0.160	0.142	0.142	0.170	0.169	0.181	0.173
Clade3	0.055|0.007	0.040|0.007	0.109	0.086		0.146	0.118	0.147	0.136	0.145	0.136
Clade4	0.000|0.002	0|0	0.089	0.082	0.073		0.120	0.104	0.100	0.114	0.109
Clade5a	n/c|n/c	n/c|n/c	0.11	0.074	0.085	0.073		0.130	0.114	0.127	0.112
Clade5b	0|0	0|0	0.095	0.078	0.074	0.042	0.079		0.093	0.101	0.099
Clade6	0.002|0.001	0.008|0.002	0.104	0.093	0.083	0.048	0.093	0.039		0.037	0.059
Clade7	0.027|0.004	0.001|0.001	0.104	0.092	0.081	0.047	0.092	0.041	0.008		0.069
Clade8	0.056|0.008	n/c|n/c	0.115	0.094	0.091	0.048	0.094	0.043	0.011	0.014	

4.1. Lineage diversity and microendemism

Genetic structure in the *I. holti – I. lactea* species complex revealed an unexpected number of lineages. These were then confirmed unambiguously as nine well-supported species after species delimitation based on the multispecies coalescent (Yang and Rannala, 2010). Genetic distances among lineages obtained for the mitochondrial loci are compatible or superior to that between other *Ischnocnema* species (Gehara et al., 2013). The two methods used to prospect the putative lineages did show differences in their diversity accounts, with

Table 3
Median proportions of the total sum of squares from the two-way anova performed for each grid cell for evaluating the relative contributions of methods for niche models and Global Circulation Models (GCM) to the variability in forecasting the *Ischnocnema holti – I. lactea* species complex.

Source	SS Median	Min-Max
ENM	0.96	0.37–1.00
GCM	0.02	0–0.43
ENM * GCM	0.02	0–0.34

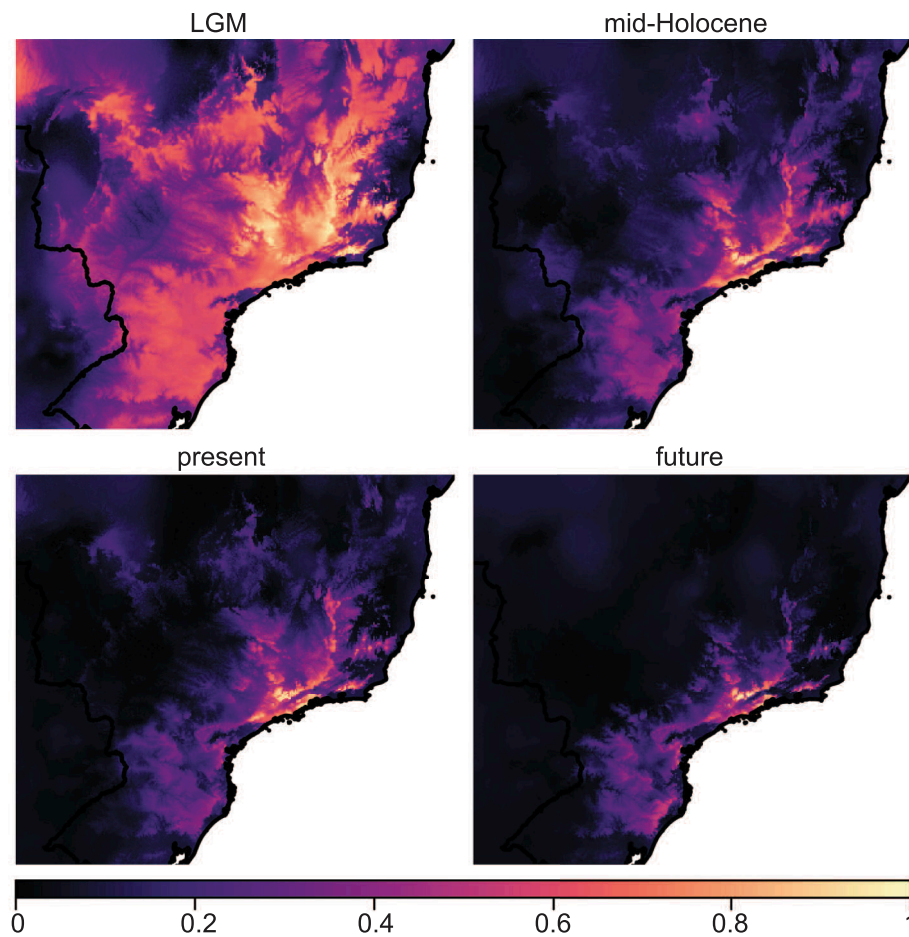### *I. holti - I. lactea* species complex

Fig. 4. Climatic suitability for the *Ischnocnema holti – I. lactea* species complex from consensual models. Models were projected to climatic conditions of the last glacial maximum (LGM), mid-Holocene, and for the future (2061–2080). Models were obtained for two general circulation models under four methods; the current scenario represents the ensemble prediction from four models and each climate period represents the eight models combined.

assignment analyses identifying seven populations and traditional phylogenetic analyses clustering samples into eight independent clades, plus a nested clade (clade 7 within clade 6, see Table 1). The results of assignment analyses must be interpreted with caution; for instance, the low genome and taxon sampling may have combined samples into a spurious group in population 5. Clustering of samples from distant localities seems rather unlikely in these low vagility frogs, and inspection of the input matrix suggests their clustering may have been caused by a combination of missing data due to both unamplified fragments and unsolved phasing, and allele sharing at the SLC8 nuclear fragment (Supplementary table S1). Also, two samples could not be attributed to any of the seven delimited populations, showing instead extreme low q values at K = 7. These samples clustered into a separate clade (clade 8) in traditional phylogenetic analyses. Considering geographic distance, the relatively long branches, and the fact that these are unique representatives of their localities (Fig. 3A, Supplementary Fig. S2), we argue that conclusive results about this lineage pend improved sampling. Finally, an additional lineage was revealed in assignment analyses (population 7) that appeared nested within clade 6 in phylogenetic analyses (Figs. 2 and 3). Samples of both populations were attributed with high q values by Structure and lineages diverged recently (Fig. 3), suggesting paraphyly likely results from incomplete lineage sorting rather than gene flow. Both are found at Paranapiacaba, but whether geographic ranges overlap remains unclear because this location comprises a large, continuous Atlantic Forest remnant and we lack more precise information on collection sites.

Elevation areas of southeastern Brazil can be roughly resumed by three main mountain systems: the Serra do Mar and the Serra da Mantiqueira mountain massifs show parallel SW-NE orientations and are separated by the Paraíba do Sul river valley, whereas the Serra do Espinhaço shows a more inland distribution that extends up to northeastern Brazil. Each system comprises minor units (mountain ranges); the Serra do Mar, Serra dos Órgãos, and Serra da Bocaina mountain ranges are all within the Serra do Mar massif. Likewise, the Serra da Mantiqueira massif also includes the Serra da Cantareira and Serra do Caparaó mountain ranges (see IBGE, 2013 for a detailed map). Of the nine delimited lineages, only two show somewhat large distributions: lineage 1 occurring along the southern Serra da Mantiqueira and Serra da Bocaina and lineage 6 spanning part of the Serra do Mar mountain range. In turn, lineage 4 occurs at Serra do Caparaó mountain range and a nearby mountainous area in Simonésia, and lineage 8 occurs at Serra da Cantareira and at Itatiaia (Serra da Mantiqueira). All remaining lineages are possible microendemics. Narrow, microendemic ranges characterize most of the species in the montane *Brachycephalus* (e.g. *B. brunneus* and *B. pitanga*, Frost, 2019), being sometimes reported in *Ischnocnema* (e.g. *I. nahallux*, Brusquetti et al., 2013; *I. guentheri*, Gehara et al., 2013). Our results add in reporting a preference for mountainous habitats associated to these narrow ranges for several putative species, further extending this trait within the Brachycephalidae.

Our data do not resolve the phylogenetic relationships among lineages in the *I. holti – I. lactea* species complex, with the topologies of the ML, BI, and the species tree differing in some degree. Differences between ML and BI are minor and restricted to poorly supported nodes, but substantial differences in supported nodes exist between traditional methods and the species tree. Also, the BPP analyses failed to infer a model for species diversification with an appreciable posterior probability, indicating that data contains more information about species delimitation than phylogeny (Yang, 2015). Aside from the inherent differences in method's philosophies, a possible explanation for tree discordance includes poorly supported nodes being 'soft' polytomies caused by insufficient genome sampling. This explanation seems rather unlikely because studies including smaller matrices yielded resolved relationships at deeper nodes for *Ischnocnema* (Canedo and Haddad 2012; Taucce et al., 2018b) and also for shallow nodes in this study. A second explanation is that poorly supported nodes constitute 'soft' polytomies caused by insufficient taxon sampling instead, either due to

underrepresentation or past extinction events. This explanation seems plausible, at least to some extent, because the relationships that are not conflicting (among samples from lineages 6, 7, and 8) refer to samples that are geographically not very distant, even though relationships among other lineages at similar distances (lineages 1 and 2) remained unresolved. A third explanation implies that poorly supported nodes are 'hard' polytomies resulting from simultaneous or temporally close speciation events. Events of this nature have been attributed to non-neutral diversification phenomena involved in adaptive radiations (e.g. Losos et al., 1998). In the case of organisms with patchy distributions, such as those inhabiting mountain systems and archipelagos, explanations often invoke large ancestral populations and neutral mechanisms of habitat fragmentation that result in reduced populations evolving under isolation and intense genetic drift (Firkowski et al., 2016). This explanation seems especially appealing because lineages 6, 7, and 8 whose relationships are resolved occupy moderate elevation compared to all other high elevation lineages, including lineages 1 and 2 at similar distances (Fig. 1). Thus, possible mechanisms causing the concomitant isolation of lineages at higher elevation deserve further investigation (discussed bellow).

4.2. Diversification framework

Our molecular clock indicates a Miocene origin for the *I. holti – I. lactea* species complex, which is in phase with a recent dated *Ischnocnema* phylogeny (Taucce et al., 2018a). Divergence times follow through the Miocene, Pliocene, and Pleistocene, rendering an older diversification time window in the *I. holti – I. lactea* species complex compared to that of the congeners in the *I. parva* species complex (Gehara et al., 2017). Comparison with diversification times in *Brachycephalus* reveals a striking contrast, as divergences in this group are bounded to the late Pleistocene (Firkowski et al., 2016). Similar diversification times have, however, been reported for other Atlantic Forest amphibians, such as in the *Thoropa miliaris – T. taophora* species complex (Sabbag et al., 2018), which share with the *I. holti – I. lactea* species complex a striking similar diversification timeframe as well as poor node support in the species tree.

The most elevated parts of the Atlantic Forest biome show intermediate elevation compared to other mountainous regions in the continent. In the Andes, intermediate elevation areas have shown particularly species rich because of higher diversification rates (the 'species pump' hypothesis) combined with older colonization (the 'museum' hypothesis, Smith et al., 2007). Previous studies in the Atlantic Forest provide support for the two hypotheses; regarding an 'Atlantic Forest's species pump', two main allopatric models are often invoked to explain diversification, the first being isolation due to habitat fragmentation related to climate change, and the second being isolation due to geographic barriers (see review in Turchetto-Zolet et al., 2013). Both models overlap in time at least to some extent: climate related habitat fragmentation was first invoked to explain Pleistocene diversification (Haffer, 1969) and latter extended to comprise the Tertiary (Haffer, 1997). Similarly, main geomorphologic units are old, resulting from uplift and the erosion of crystalline shields in the Devonian, Cretaceous, and Tertiary Periods (Ab'saber, 1975; Mello et al., 1985; Penha et al., 1998; Petri and Fúlvaro 1983; Riccomini et al., 1989), but Quaternary tectonics is also present and associated to many faults, lineaments, and river valleys lying within the Atlantic Forest domain (Saadi 1993; Saadi et al., 2002; Saadi et al., 2005). The two models do differ in their expectations regarding demographic trends, with variations in population sizes being expected only under the climate related habitat fragmentation model. Unfortunately, our dataset is inappropriate for demographic inference because of the low number of samples per lineage, and combining lineages into a single analyses have proven inappropriate as genetic structuring may result in false signals of population contraction (Heller et al., 2013).

Regarding an 'Atlantic Forest museum', Carnaval et al. (2009) first

detected extreme habitat instability for the southern region of the biome by coupling ENM and genetic data for three amphibians in the genus *Boana* (former *Hypsiboas*), and hypothesized that the southern AF was recently colonized by taxa at climatically stable areas located at the northern part of the biome. Later, Amaro et al. (2012) found glacial conditions to be much less stringent on the southern Atlantic Forest, and attributed LGM persistence of the amphibian *Proceratophrys boiei* to a particular physiological profile that would shield montane taxa from Pleistocene climatic changes. By gathering genetic and occurrence data of several taxa, Carnaval et al. (2014) finally confirm that montane Atlantic Forest species show distinct biological responses for cooler climates compared to lowland species. Thus, it is possible that these organisms were more widespread during glacial times than at interglacials (Rodrigues et al., 2009), and experienced population fragmentation during warmer periods (Hewitt, 2000), following colder climates through altitudinal migration (Firkowski et al., 2016). Diversification would then mostly result from non-adaptive radiations. Past projections of the climate niche for the *I. holti – I. lactea* species complex meet the large glacial range prediction, with a strikingly enlarged distribution inferred for the LGM. Two biogeographic scenarios can be drawn from this; first, habitat contraction from the LGM to the present time suggests preterit warmer climates, possibly in the Tertiary, might indeed constitute the mechanism behind concomitant isolation of higher elevation lineages as hypothesized above. Second, the moderate elevation lineages might result from later colonization from a high elevation 'museum' source during subsequent cooler periods (Smith et al., 2007). Nevertheless, the geographical coincidence among lineage limits in the *I. holti – I. lactea* species complex and some neotectonic faults and lineaments (such as the Caratinga Fault, the Rio Paraíba do Sul crustal discontinuity, and the Além Paraíba Fault Zone, Fig. 1) (Saadi et al., 2002) also raises the possibility for a role of geographic barriers in the diversification of lineages in this complex. Further population level sampling is required for formally testing these hypotheses in this species complex.

4.3. Taxonomic implications

Considering the concordant coalescence of gene genealogies (Baum and Shaw, 1995) as a secondary property of the general lineage concept of species (de Queiroz, 1998), it is clear from our results that the *I. holti – I. lactea* species complex harbors more diversity than that recognized by the current taxonomy. Although more objective than other approaches (Fujita et al., 2012), the use of strictly molecular methods for species delimitation has been criticized in the literature for many reasons, one being that results may vary among available methods (Carstens et al., 2013). A second source of criticism comes from the possibility that the multispecies coalescent model implemented in molecular species delimitation methods is not suitable for delimitation of true species (*sensu* Sukumaran and Knowles, 2017), delimiting instead population structure. Although valid, both issues seem of less importance for the taxonomy of Atlantic Forest amphibians because of a tradition in basing taxonomic decisions in multiple sources of evidence, with species diagnoses supported by morphological and/or acoustic evidence prior to genetic evidence (e.g. Taucce et al., 2018a). For instance, Gehara et al. (2013) combined genetic and acoustic data to delimit six distinct species in the *I. guenteri* species complex, four of which were undescribed and are in the process of having their taxonomic statuses formally reassessed (P. Taucce, personal communication).

The present work provides a first hypothesis accounting for species diversity in the *Ischnocnema holti – I. lactea* species complex, and lineages can be formally described as species following taxonomic scrutiny. Because of their narrow ranges, and with both *I. holti* and *I. lactea* being recently redescribed (Targino and de Carvalho-e-Silva,

2008; Silva-Soares et al., 2018), the two valid names can be associated to lineages delimited in the present study with some confidence based in their type localities (Fig. 1). In the case of *I. holti*, sampling of topotypes allow us to safely attribute this name to lineage 2 and in fact, Targino and de Carvalho-e-Silva (2008) redescribed the species based in topotypes, with M. Targino having personally verified the identification of the vouchers included here. *Ischnocnema lactea* can be attributed to clade 6 based in the geographic distribution (Silva-Soares et al., 2018), but a definitive association to lineages 6 and/or 7 pends on morphological inspection. We are not aware of other vacant names that can be attributed to any of the remaining lineages. However, a preliminary inspection of vouchers shows that some lineages do have morphological variation, which is the case of the specimens from the clade 5B, a species in process of description (T. Silva-Soares, pers. comm.).

4.4. Conservation

Concentration of the Brazilian human population in coastal areas resulted in intense fragmentation of the Atlantic Forest over the last 500 years (Morellato and Haddad, 2000). The high number of endemics combined with habitat loss rendered the biome the title of hotspot of global biodiversity (Myers et al., 2000). Despite the efforts of the scientific community, many amphibian species from the Atlantic Forest are still currently assigned as Data Deficient or Least Concern (IUCN, 2019) due to geographical distribution knowledge gaps or poorly representative taxonomy (the Wallacean and Linnean shortfalls, sensu Hortal et al., 2015). In 2004 the International Union for Conservation of Nature listed *Ischnocnema holti* as data deficient, possibly due to poor sampling. Likewise, *I. lactea* was listed as least concern due to a presumably larger occurrence area, regardless of an observation of population decline (IUCN, 2019). Neither *I. holti* nor *I. lactea* are listed in the latest assessment for the conservation status of Brazilian amphibian species.

Our results reduce both the Wallacean and Linnean shortfalls for these two species, and put in doubt their current conservation statuses. We show that current taxonomy fails to describe cryptic species diversity within the *I. holti – I. lactea* species complex, which possibly comprises nine species instead of two. Furthermore, most of these species are narrow endemics restricted to specific conditions at higher elevation locations. Finally, even though many Atlantic Forest remnants are located at higher elevation where human access is difficult (Ribeiro et al., 2009), new threats of habitat reduction under increasing carbon dioxide emissions challenge their future integrity (Loyola et al., 2014). ENM suggests that the ranges of suitable habitat are contracting since the LGM for these organisms, and will continue to contract alarmingly under global climate change, possibly leading to extinction. Thus, we propose not only taxonomy but that the conservation statuses of species in this complex should be carefully reassessed.

The 38 samples that we were able to gather represent the results of many decades of sampling by several institutions. This reduced number also highlights the low abundances in which these animals occur. The Atlantic Forest is one of the most studied biomes in Brazil (Turchetto-Zolet et al., 2013) and probably the best sampled one due to the geographic proximity with several universities and research institutes. In southeastern Brazil this sampling bias is intensified because science budgets benefit from local tax incomes derived from highly industrialized economies, intense mining, and oil prospection. Despite of such effort, we reached a maximum sample size of five samples for a single locality (Curucutu), a protection area that was systematically and intensively sampled for nearly 15 years (L. Malagoli, pers. comm.). Thus, we argue such low abundances are not a sampling artifact and should also be considered in the conservation reassessment of the species in the *I. holti – I. lactea* species complex.

CRediT authorship contribution statement

Maria Tereza C. Thomé: Writing - original draft, Formal analysis. **Mariana L. Lyra:** Data curation, Writing - review & editing. **Priscila Lemes:** Formal analysis, Writing - review & editing. **Laryssa S. Teixeira:** Data curation. **Ana Carolina Carnaval:** Writing - review & editing. **Célio F.B. Haddad:** Resources, Funding acquisition, Data curation, Writing - review & editing. **Clarissa Canedo:** Conceptualization, Supervision, Writing - review & editing.

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Appendix A. Number of records (N) and D-statistic of distribution models for *Ischnocnema holti – I. Lactea* species complex. Consensual models were built from the best models (p-value > 0.05) for each period. The greater the magnitude of D-statistic, the greater the ability to predict the excluded locality considering jackknife test. *p-value > 0.05.

Period	GCM	ENM	D-statistic (N = 18)
LGM	CCSM	Bioclim	5.9584
		Gower	4.3787
		SVM	14.1047
		Maxent	15.5031
	MIROC	Bioclim	2.9453
		Gower	3.9136
		SVM	11.5353
		Maxent	15.5701
Mid-Holocene	CCSM	Bioclim	9.9277
		Gower	9.2515
		SVM	16.1001
		Maxent	15.6907
	MIROC	Bioclim	9.9423
		Gower	10.6126
		SVM	16.6042
		Maxent	16.7723
Current		Bioclim	11.9412
		Gower	15.6367
		SVM	15.7099
		Maxent	15.6667
Future (2061–2080)	CCSM	Bioclim	3.9965
		Gower	15.7122
		SVM	15.3245
		Maxent	15.7132
	MIROC	Bioclim	4.9946
		Gower	14.7490
		SVM	5.8739
		Maxent	15.8339

Appendix B. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2020.106813.

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