



## Phylogenomic species delimitation in microendemic frogs of the Brazilian Atlantic Forest

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### ABSTRACT

The advent of next-generation sequencing allows researchers to use large-scale datasets for species delimitation analyses, yet one can envision an inflection point where the added accuracy of including more loci does not offset the increased computational burden. One alternative to including all loci could be to prioritize the analysis of loci for which there is an expectation of high informativeness. Here, we explore the issue of species delimitation and locus selection with montane species from two anuran genera that have been isolated in sky islands across the southern Brazilian Atlantic Forest: *Melanophryniscus* (Bufonidae) and *Brachycephalus* (Brachycephalidae). To delimit species, we obtained genetic data using target enrichment of ultraconserved elements from 32 populations (13 for *Melanophryniscus* and 19 for *Brachycephalus*), and we were able to create datasets that included over 800 loci with no missing data. We ranked loci according to their number of parsimony-informative sites, and we performed species delimitation analyses using BPP with the most informative 10, 20, 40, 80, 160, 320, and 640 loci. We identified three types of phylogenetic node: nodes with either consistently high or low support regardless of the number of loci or their informativeness and nodes that were initially poorly supported where support became stronger as we included more data. When viewed across all sensitivity analyses, our results suggest that the current species richness in both genera is likely underestimated. In addition, our results show the effects of different sampling strategies on species delimitation using phylogenomic datasets.

### 1. Introduction

Given that species are the main focus of the study of biological diversification, as well as the main focus for conservation efforts, accurate species delimitation forms the basis of much biodiversity research (Sites and Marshall, 2004; Adams et al., 2014). Two important advances in this area have occurred in recent years. The first is the development of species delimitation methods (e.g. Yang, 2002; Rannala and Yang, 2003; Knowles and Carstens, 2007; Yang and Rannala, 2010; Ence and Carstens, 2011; see Rannala, 2015 for a recent review) that are based on the multispecies coalescent (MSC) model (Takahata et al., 1995; Rannala and Yang, 2003). These methods provide an objective and operational way to infer species limits that is explicitly based on a rigorous population genetic framework (Fujita et al., 2011; Rannala,

2015; but see Sukumaran and Knowles, 2017). The second involves advances in sequencing technologies that allow for the generation of large-scale datasets (Bi et al., 2012; Faircloth et al., 2012; Lemmon et al., 2012; Lemmon and Lemmon, 2013; McCormack et al., 2013; Smith et al., 2013). Unfortunately, the computational demands of MSC species delimitation methods when dealing with large datasets means that the brute-force approach of including as many loci as possible might not be the most computationally efficient or cost-effective approach. The ideal approach might, instead, be to reduce the total number of loci by focusing analyses on those that are more informative while excluding those with low information content because this latter class of loci increases the computational burden but contributes little information to the analysis. In the analogous case of species-tree inference under the MSC, some methods perform worse with the addition

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of low-information loci (e.g. Manthey et al., 2016; Meiklejohn et al., 2016; Xu and Yang, 2016; but see Xi et al., 2015). Conversely, some recent speciation events might need a large number of loci to be properly detected, suggesting that researchers should use the largest number of loci possible (e.g. Hime et al., 2016; Meiklejohn et al., 2016). To address this problem, we need to understand how varying the number and the informativeness of loci affects the performance of species delimitation methods. While some studies have looked at this problem (e.g. Hime et al., 2016), they have not scaled delimitation analyses beyond 100 loci due to computational demands.

Here, we investigate the performance of MSC species delimitation with different numbers of loci and different degrees of locus informativeness when applied to two co-distributed, montane, anuran genera: *Melanophryniscus* (Bufonidae) and *Brachycephalus* (Brachycephalidae). The genus *Melanophryniscus* is broadly distributed throughout southeastern South America (Frost, 2018). *Melanophryniscus* of the southern Brazilian Atlantic Forests are characterized by montane endemic species with restricted and isolated distributions in cloud forests, *campos de altitude*, and grasslands (Langone et al., 2008; Steinbach-Padilha, 2008; Bornschein et al., 2015). These sky-island endemics include five of the 29 currently described *Melanophryniscus* species (Frost, 2018): *M. alipioi*, *M. biancae*, *M. milanoi*, *M. vilavelhensis*, and *M. xanthostomus*. Of these species, *M. biancae* and *M. vilavelhensis* represent a distinct lineage within montane *Melanophryniscus*, given their phylogenetic distance from the remaining species (Firkowski et al., 2016) and the unique type of vegetation in which they are found (Bornschein et al., 2015). The remaining three species (*M. alipioi*, *M. milanoi*, and *M. xanthostomus*) could be species complexes (Firkowski et al., 2016), but the considerable morphological variability found within species (see Bornschein et al., 2015), even within a given location, is a major hurdle for species delimitation using phenotypic data alone.

Members of the genus *Brachycephalus* are endemic to the Brazilian Atlantic Forest, with a distribution extending nearly 1700 km along the

biome although most species occur on isolated mountaintops from the Brazilian states of Bahia in northeastern Brazil to Santa Catarina in southern Brazil (Pie et al., 2013; Bornschein et al., 2016a). *Brachycephalus* includes both cryptic and aposematic toadlets which live in the forest leaf litter and are active during the day (e.g. Ribeiro et al., 2015). The most striking morphological feature of this genus is their extreme level of miniaturization (snout-vent length  $\approx$  1–1.5 cm), which has led to extreme modifications of their life histories (Hanken and Wake, 1993; Yeh, 2002). The genus was recently divided into three species groups (the *pernix*, *ephippium*, and *didactylus* groups - see Ribeiro et al. (2015)), with the *pernix* group including 19 described species distributed across the southern Atlantic Forest (Bornschein et al., 2016a).

Species of *Brachycephalus* and *Melanophryniscus* have traditionally been described using phenotypic data (Bornschein et al., 2015, 2016b; Pie and Ribeiro, 2015; Ribeiro et al., 2015), which can lead to underestimates of species diversity (Bickford et al., 2007). A recent delimitation study using 4–6 loci suggested that there could be several *Brachycephalus* and *Melanophryniscus* species that remain undescribed, but more extensive data were needed to firmly establish species boundaries (Firkowski et al., 2016). We focus on the lineages that occur where the two genera are co-distributed – the montane areas of the southern Brazilian Atlantic Forest, and our main goal is to delimit species of *Brachycephalus* and *Melanophryniscus* under the MSC using ultra-conserved elements (UCEs, Faircloth et al., 2012). Specifically, we used a Bayesian Markov chain Monte Carlo program for species delimitation (BPP 3.3; Yang, 2015) that relies on the MSC to compare different models of species delimitation while accounting for incomplete lineage sorting due to ancestral polymorphism and gene-tree conflicts (Yang and Rannala, 2010, 2014; Rannala and Yang, 2013). The lineages investigated in this study are particularly suitable for use with BPP because the candidate species are almost always allopatric, such that gene flow among populations is low to nonexistent, and BPP is robust to low levels of gene flow (Zhang et al., 2011). Our study, therefore, sheds

**Table 1**

Samples used in the present study, with the corresponding localities and geographical coordinates. Species sampled in more than one locality had their name associated only to the type locality, whereas the remaining specimens were not explicitly identified and were named according to their collection locality.

Species	Coordinates	Locality
<i>Melanophryniscus</i> sp.	25°36'24"S, 48°43'33"W	Serra da Prata, boundary of the municipalities of Morretes, Paranaguá, and Guaratuba, Paraná
<i>M. sp.</i>	25°36'40"S, 48°51'22"W	Morro dos Padres, Serra da Igreja, municipality of Morretes, Paraná
<i>M. alipioi</i>	25°07'49"S, 48°49'15"W	Capivari Grande, Serra do Capivari, municipality of Campina Grande do Sul, Paraná
<i>M. sp.</i>	25°13'30"S, 48°51'18"W	Itapiroca, Serra dos Órgãos, boundary of the municipalities of Campina Grande do Sul and Antonina, Paraná
<i>M. sp.</i>	24°51'17"S, 48°43'43"W	Caratutu, near the Parque Estadual das Lauráceas, municipality of Adrianópolis, Paraná
<i>M. sp.</i>	24°28'52"S, 48°47'12"W	Base of the Serra Água Limpa, municipality of Apiaí, São Paulo
<i>M. xanthostomus</i>	26°01'17"S, 48°59'47"W	Serra do Quiriri, municipality of Campo Alegre, Santa Catarina
<i>M. sp.</i>	26°08'48"S, 49°10'43"W	Condomínio Vale dos Lagos, municipality of Joinville, Santa Catarina
<i>M. sp.</i>	26°24'53"S, 49°13'08"W	Morro do Boi, municipality of Corupá, Santa Catarina
<i>M. sp.</i>	26°30'58"S, 49°03'14"W	Morro Boa Vista, on the border between the municipalities of Jaraguá do Sul and Massaranduba, Santa Catarina
<i>M. sp.</i>	26°46'42"S, 49°01'57"W	Morro do Cachorro, on the border between the municipalities of Blumenau, Gaspar, and Luiz Alves, Santa Catarina
<i>M. milanoi</i>	26°47'55"S, 48°55'55"W	Morro do Baú, municipality of Ilhota, Santa Catarina
<i>M. sp.</i>	26°45'49"S, 49°12'23"W	Morro Azul, on the border between the municipalities of Pomerode and Rio dos Cedros, Santa Catarina
<i>Brachycephalus auroguttatus</i>	26°00'21"S, 48°55'25"W	Pedra da Tartaruga, municipality of Garuva, Santa Catarina
<i>B. boticario</i>	26°46'42"S, 49°01'57"W	Morro do Cachorro, boundary of the municipalities of Blumenau, Gaspar, and Luiz Alves, Santa Catarina
<i>B. brunneus</i>	25°14'33"S, 48°50'04"W	Caratutu, Serra dos Órgãos, municipality of Campina Grande do Sul, Paraná
<i>B. sp.</i>	25°15'59"S, 48°50'16"W	Camapuã, Serra dos Órgãos, boundary of the municipalities of Campina Grande do Sul and Antonina, Paraná
<i>B. sp.</i>	25°36'58"S, 48°46'59"W	Serra Canasvieiras, boundary of the municipalities of Guaratuba and Morretes, Paraná
<i>B. ferruginus</i>	25°27'03"S, 48°54'59"W	Olimpo, Serra do Marumbi, municipality of Morretes, Paraná
<i>B. fuscolineatus</i>	26°47'58"S, 48°55'47"W	Morro do Baú, municipality of Ilhota, Santa Catarina
<i>B. iizecksohni</i>	25°37'25"S, 48°41'31"W	Serra da Prata, boundary of the municipalities of Morretes, Paranaguá, and Guaratuba, Paraná
<i>B. curupira</i>	25°42'07"S, 49°03'44"W	Serra do Salto, Malhada District, municipality of São José dos Pinhais, Paraná
<i>B. mariaetezeae</i>	26°06'51"S, 49°03'45"W	Reserva Particular do Patrimônio Natural Caetetal, top of the Serra Queimada, municipality of Joinville, Santa Catarina
<i>B. olivaceus</i>	26°24'42"S, 49°12'59"W	Morro do Boi, municipality of Corupá, Santa Catarina
<i>B. sp.</i>	25°13'29"S, 48°51'17"W	Abrigo 1, Serra dos Órgãos, municipality of Campina Grandedo Sul, Paraná
<i>B. pernix</i>	25°23'19"S, 49°00'15"W	Anhangava, Serra da Baitaca, municipality of Quatro Barras, Paraná
<i>B. pombali</i>	25°36'40"S, 48°51'22"W	Morro dos Padres, Serra da Igreja, municipality of Morretes, Paraná
<i>B. quiririensis</i>	26°01'17"S, 48°59'47"W	Serra do Quiriri, municipality of Campo Alegre, Santa Catarina
<i>B. sulfuratus</i>	25°20'17"S, 48°54'56"W	Corvo, municipality of Quatro Barras, Paraná
<i>B. sp.</i>	25°14'31"S, 48°47'47"W	Morro Tupiipiá, Serra dos Órgãos, municipality of Antonina, Paraná
<i>B. verrucosus</i>	26°12'44"S, 48°57'29"W	Morro da Tromba, municipality of Joinville, Santa Catarina
<i>B. sp.</i>	25°30'33"S, 48°58'58"W	Morro do Vigia, municipality of Piraquara, Paraná

light on species delimitation for a common and difficult scenario of judging reproductive isolation—that where lineages are allopatric—and does so with one of the largest genomic data sets applied to species delimitation, which allows for robust sensitivity analyses of locus number and locus informativeness.

## 2. Materials and methods

We obtained tissue samples from field-collected specimens of 13 populations of *Melanophryniscus* and 19 populations of *Brachycephalus* (Table 1), and we only included one specimen from each population. Although including multiple specimens per population could provide more information about intraspecific variation in putative species, BPP has been shown to correctly delimit species with single terminal nodes (Zhang et al., 2011). We deposited voucher specimens in the herpetological collection of the Department of Zoology of the Universidade Federal do Paraná (DZUP) in Curitiba, Brazil (more information on specimen collection methods and localities can be found in Firkowski et al. (2016)). These samples include most described species of *Brachycephalus* of the *pernix* group, except for *B. actaeus*, *B. albolineatus*, *B. coloratus*, *B. leopardus*, *B. mirissimus* and *B. tridactylus*. *Brachycephalus sulfuratus* does not belong to the *pernix* species group, but we included this species in the analyses to improve the rooting of the guide tree. Similarly, except for populations ascribed to *M. biancae* and *M. vilavelhensis*, we sampled all known records of montane *Melanophryniscus*, including several new records reported in Bornschein et al. (2015).

We extracted genomic DNA using the PureLink Genomic DNA kit (Invitrogen, USA), and we fragmented the extracted DNA using a BioRuptor NGS (Diagenode) to a size range of 300–500 bp. We prepared Illumina libraries using KAPA library preparation kits (Kapa Biosystems) and custom sequence tags unique to each sample (Faircloth and Glenn, 2012). To enrich targeted UCE loci, we followed an established workflow (Gnrke et al., 2009; Blumenstiel et al., 2010) while incorporating several modifications to the protocol detailed in Faircloth et al. (2012). Specifically, we pooled eight samples at equimolar ratios, prior to enrichment, and we blocked the Illumina TruSeq adapter sequence using custom blocking oligos. We enriched each pool using a set of 2560 custom-designed probes (MYcroarray, Inc.) targeting 2386 UCE loci (see Faircloth et al. (2012) and <http://ultraconserved.org> [last accessed May 23, 2017] for details on probe design). Prior to sequencing, we qPCR-quantified enriched pools, combined pools at equimolar ratios, and sequenced the combined libraries using two runs of a MiSeq PE250 (Cofactor Genomics).

We filtered reads for adapter contamination, low-quality ends, and ambiguous bases using an automated pipeline (<https://github.com/faircloth-lab/illumiprocessor>) that incorporates TRIMMOMATIC (Bolger et al., 2014). We assembled reads for each individual using Trinity (Grabherr et al., 2011). We used the PHYLUCE software package (Faircloth, 2015) to align assembled contigs back to their associated UCE loci, remove duplicate matches, and create a taxon-specific database of contig-to-UCE matches. We then generated two alignments: all *Brachycephalus* samples using *Melanophryniscus alipioi* as their outgroup, and all *Melanophryniscus* species using *B. sulfuratus* as their outgroup. We selected loci to create 100% complete data sets for both genera, leading to 820 loci in the *Brachycephalus* data set and 1227 loci in the *Melanophryniscus* data set. We aligned data for each individual in each data set using MAFFT (Katoh, 2013), and we trimmed resulting alignments using GBLOCKS (Castresana, 2000) with default parameters. Sequence reads for this project are available from NCBI BioProject PRJNA391191.

Ideally one would determine the informativeness of a given locus based on how much information it contributes to a given analysis (e.g. Townsend, 2007; Gilbert et al., 2015). However, this approach requires researchers to run entire analyses followed by the determination of locus informativeness *a posteriori*. A simple alternative is to calculate the absolute number of parsimony-informative sites (PIS – a single

nucleotide polymorphism that is present in more than one individual) for each locus and to use this measure as a proxy for informativeness. This approach was used for UCE loci in the context of phylogenetic inference by Hosner et al. (2016) and Meiklejohn et al. (2016) (see Edwards, 2016 for a general review on phylogenetic subsampling) and extended to species delimitation more recently by Hime et al. (2016). We calculated the number of PIS for each locus using  $IPS$  0.0–7 (Heibl, 2014) and compared them between datasets using a Spearman's test with the `COR.TEST` function in R 3.3.2 (R Core Team, 2017). We then ranked loci in each dataset according to their corresponding number of PIS, and we created subsets of the top 10, 20, 40, 80, 160, 320, and 640 most informative loci for subsequent analyses.

We used BPP 3.3 (Yang, 2015) in all species delimitation analyses. Important assumptions of the MSC model implemented in BPP include no recombination within a locus, free recombination between loci, no migration (gene flow) between species, and neutral evolution. UCEs are likely to be largely independently sorting (Derti et al., 2006) and recover topologies similar to those observed other classes of loci that are not under strong selection (Suh, 2016; Suh et al., 2015). It is also important to note that, although the core of UCEs tend to be highly conserved due to strong selection, flanking regions tend to have a similar level of informativeness as neutral DNA from introns (see Jarvis et al., 2014).

As a conservative starting point, we initially considered every population as a potential species (see Olave et al., 2014), allowing for the possibility that these populations would be lumped together by BPP depending on the analyzed data. We analyzed the data in BPP under three different sets of gamma priors for population size ( $\theta$ ) and divergence time at the root of the species tree ( $\tau_0$ ): (1) small ancestral population sizes and shallow interspecific divergences:  $\theta \sim \Gamma(2, 1000)$ ,  $\tau_0 \sim \Gamma(2, 1000)$ ; (2) large ancestral population sizes and shallow interspecific divergences:  $\theta \sim \Gamma(1, 10)$ ,  $\tau_0 \sim \Gamma(2, 1000)$ ; and (3) large ancestral population sizes and deep interspecific divergences:  $\theta \sim \Gamma(1, 10)$ ,  $\tau_0 \sim \Gamma(1, 10)$ . We used these three sets of priors to test their effect on the chosen species delimitation scheme, although given what is known about the microendemic distribution and recent divergence times of the species under study, the first set of priors fits the biology of each system and was given priority when conflicting results were detected.

We assigned other divergence time parameters a Dirichlet prior (Yang and Rannala, 2010: equation 2). We used the A10 model (speciesdelimitation = 1, speciestree = 0) for species delimitation using a user-specified guide tree (Yang and Rannala, 2010; Rannala and Yang, 2013). To reduce the computational burden imposed by modeling locus-specific mutation rates, we assumed that all loci have the same mutation rate (“locusrate = 0”). Estimating separate mutation rates would likely affect the  $\tau$  and  $\theta$  estimates for the root population but would likely not affect Bayesian comparison of different models of delimitation (Z. Yang, pers. comm.), as shown in the simulations presented in Zhang et al. (2011). We used the uniform rooted tree prior (speciesmodelprior = 1), and we assumed the same  $\theta$ s across loci (heredity = 0). To achieve adequate acceptance proportions (i.e., between 20 and 80%), we used the option of automatic fine-tuning of the MCMC. We estimated the guide tree by concatenating all loci in each dataset and carrying out maximum likelihood analyses in RAxML 8.2.8 (Stamatakis, 2014) using a single GTRGAMMA model. Although BPP has been shown to be robust to errors in guide trees (Zhang et al., 2014; Caviedes-Solis et al., 2015), we carried out a variety of species-tree analyses (e.g. STAR (Liu et al., 2009), ASTRAL-II (Mirarab and Warnow, 2015)) to ensure the guide tree we used was stable. The topologies we recovered were consistent across methods (see Pie et al., 2018).

We repeated analyses using the rjMCMC algorithm 0 ( $\epsilon = 2$ ) and algorithm 1 ( $\alpha = 2$ ,  $m = 1$ ), as well as including/excluding sites with gaps (cleandata = 0, 1), given that omitting gaps has been recently shown to potentially affect species delimitation using phylogenomic data (Domingos et al., 2017). To check the reliability of our results

(Yang, 2015), we ran each analysis at least twice. Given the number of tips on the guide tree, there were 768 potential species delimitation models in each analysis for *Brachycephalus* and 145 for *Melanophryniscus* and, in total, we ran 336 analyses (2 genera  $\times$  7 datasets with varying numbers of loci  $\times$  2 algorithms  $\times$  3 sets of priors  $\times$  2 treatments of gaps  $\times$  2 replicates). The parameters that we altered between analyses were those we assumed could have the most evident impacts on the obtained results. We ran each analysis for 10,000 generations, sampling every 10th generation, and we discarded the first 10% of posterior samples as burn-in. Although we are aware that BPP can carry out simultaneous species-tree and species delimitation analyses (i.e., the A11 model), preliminary analyses using a variety of phylogenetic inference methods produced consistent topologies, and we decided the substantial increases in run time that would result from the computational demands of co-estimating species trees (e.g. Caviedes-Solis et al., 2015) were not justified.

Finally, to discriminate the relative contribution of increasing the number of loci from prioritizing those with higher informativeness, we analyzed 10 randomly-sampled datasets including the same numbers of loci (10, 20, 40, 80, 160, 320, and 640) under model A10, algorithm 0, clean-data = 0, and priors  $\theta \sim \Gamma(2, 1000)$ ,  $\tau_0 \sim \Gamma(2, 1000)$  and compared the results we obtained to the analyses using ranked loci. To facilitate this comparison, we present only the mean PPs between replicates for the ranked loci and the mean PPs among the 10 randomly selected loci datasets.

### 3. Results

We found considerable variation among UCE loci in their informativeness, as indicated by the distribution of the number of PIS for each locus (Fig. S1). Generally, many loci have relatively few PIS and thus contain little phylogenetic information. To determine whether UCE loci were consistently informative between the two genera, we used a Spearman correlation of their PIS. We found a significant association ( $\rho = 0.31$ ,  $p = 1.88e-15$ ), meaning that information content for a given locus was correlated between genera. However, this relationship was driven largely by loci with low information content in both genera, whereas the loci with high information content in one genus were not consistently high information content in the other (Fig. 1). Nevertheless, these results suggest that one could, in principle, select UCE loci that are most informative based on similar datasets analyzing lineages that are not very closely related (e.g. within Anura), although this strategy might potentially lead to bias in analyses of unrelated taxa.

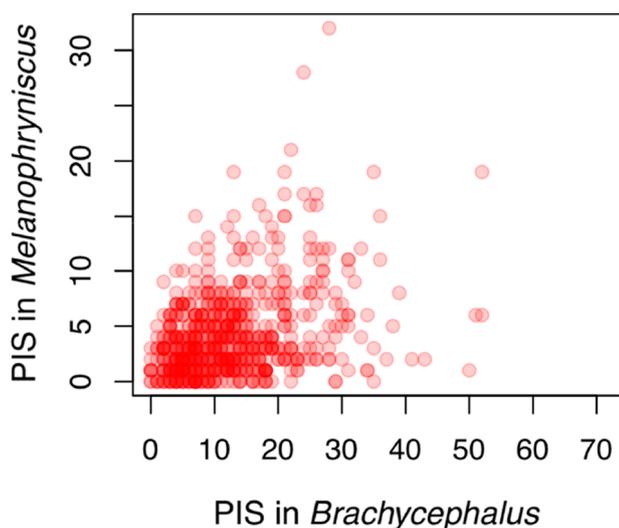


Fig. 1. Relationship between the number of parsimony-informative sites (PIS) for the same loci in the *Brachycephalus* and *Melanophryniscus* datasets (N = 610 loci).

One should also keep in mind that preferentially using variable loci might affect estimates of demographic parameters, such as divergence times and effective population sizes.

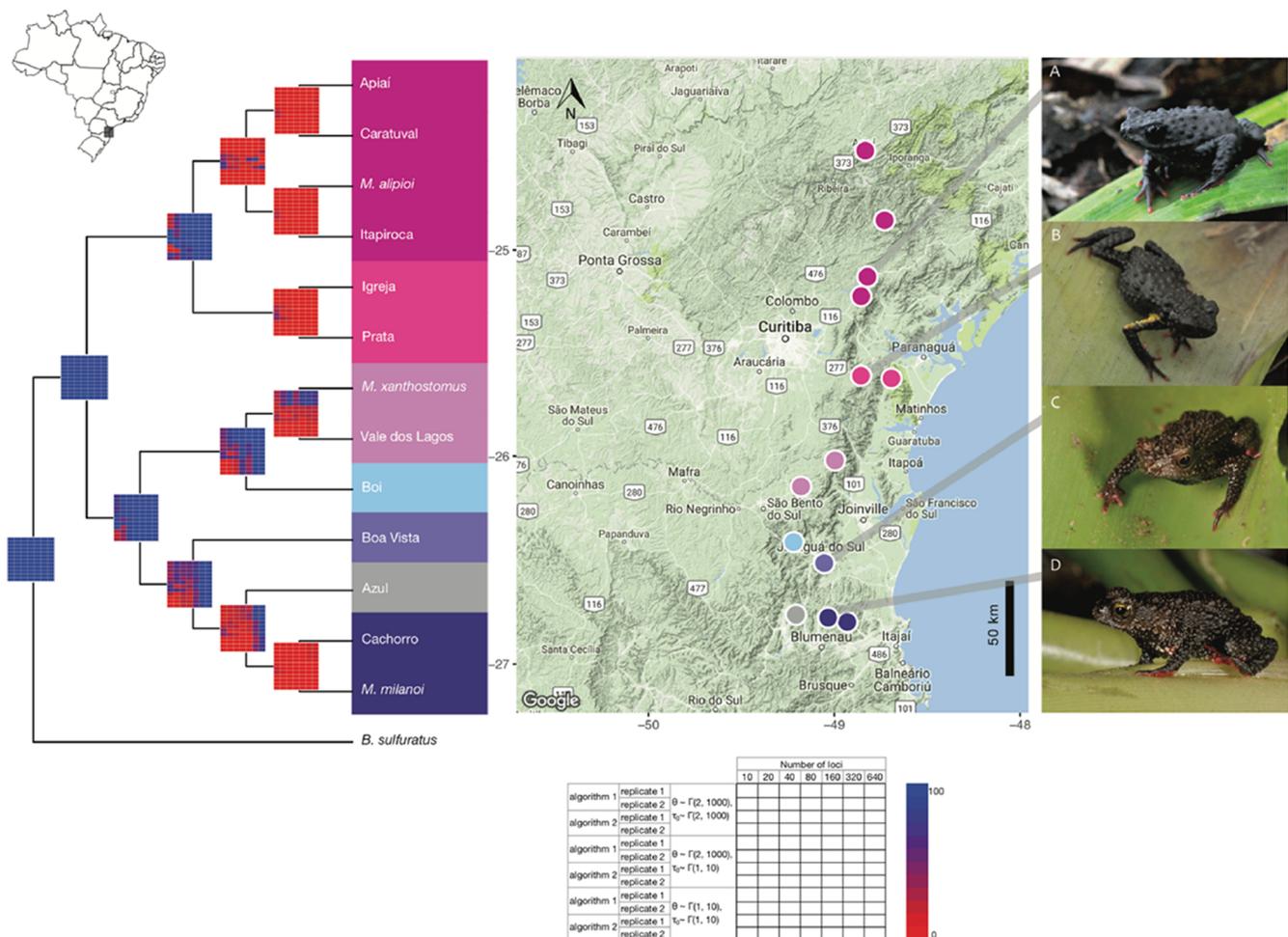
Summaries of the species delimitation analyses for *Melanophryniscus* and *Brachycephalus* are shown in Figs. 2 and 3. There were three main types of support for splits among populations: (1) nodes showing 1.0 posterior probabilities (PPs) in all analyses, which were usually those closest to the base of the guide trees, (2) nodes with low (or at least inconsistent) support across analyses, which were more often found near the tips of the guide trees; and finally, (3) nodes ranging from low to very high support. In the third case, a clear pattern emerged of a monotonic increase in support when a larger number of loci were included in the analyses (Fig. S2). This behavior is expected in statistically consistent methods, although we observed a slight decrease in the posterior probabilities between 20 and 40 loci for some analyses (e.g. Fig. S2E, F, L). We noticed that excluding sites with gaps (clear = 1) led to more unstable posterior probability estimates (Fig. S2), potentially because problematic sites had already been removed using Gblocks prior to the species delimitation. After taking into account variation in the obtained PPs across analyses, our results corroborate the distinctiveness of species that were originally described using phenotypic data alone, and these analyses suggest we have identified six putatively undescribed species (four species of *Melanophryniscus* and two species of *Brachycephalus*).

In general, analyzing loci ranked according to their informativeness seems to have a positive effect on the performance of BPP. PPs for ranked loci were modestly, but consistently, higher than randomly selected loci. In a few cases, random loci performed slightly better, and the effect of higher PPs for ranked loci appears to dissipate after 80 to 160 loci (Fig. S3). This is not unexpected, given that both ranked and random loci begin to converge on the size of the total data set at higher locus numbers.

### 4. Discussion

Phylogenomic data are still under-utilized for species delimitation (Pyron, 2015; but see Herrera and Shank, 2016; de Oca et al., 2017), yet we anticipate that these large data sets will soon become a valuable part of the toolkit for species discovery, delimitation, and diagnosis. The development of several new molecular methods allows us to collect phylogenomic data from a huge number of organisms (e.g. Bi et al., 2012; Faircloth et al., 2012; Lemmon et al., 2012; Lemmon and Lemmon, 2013; McCormack et al., 2013; Smith et al., 2013; Branstetter et al., 2017; Edwards et al., 2017; Faircloth, 2017), effectively removing the data-collection barrier. Yet, our general understanding of how to analyze these large datasets lags behind our ability to generate them, and unanswered questions range from the suitability of particular marker classes (e.g., UCE, exon, RAD-seq) for delimitation analyses to the various filtering steps that should or should not be used with loci of a given class.

A first and very general result of our study is demonstrating the effective use of UCE data to delimit species in two anuran genera. To our knowledge, only three other studies in different taxonomic groups have used UCES for a similar purpose beginning with Smith et al. (2013), who demonstrated that UCE loci, while highly conserved, contain sufficient genetic information to differentiate a suite of avian species using MSC programs like BPP. Subsequently, Oswald et al. (2016) used single SNPs extracted from UCE loci to perform Bayes factor species delimitation with the BFD\* method (Grummer et al., 2014; Leaché et al., 2014). Although the approach used by Oswald et al. (2016) differs substantially from the workflow we used, their analyses were able to identify strongly divergent lineages within a bird species (*Tringa semipalmata*; Scolopaciidae), with no evidence of admixture between them. Finally, Newman and Austin (2016) extended the use of UCES to amphibian taxa to establish the recognition of seven salamander species within *Plethodon serratus* sensu lato, although

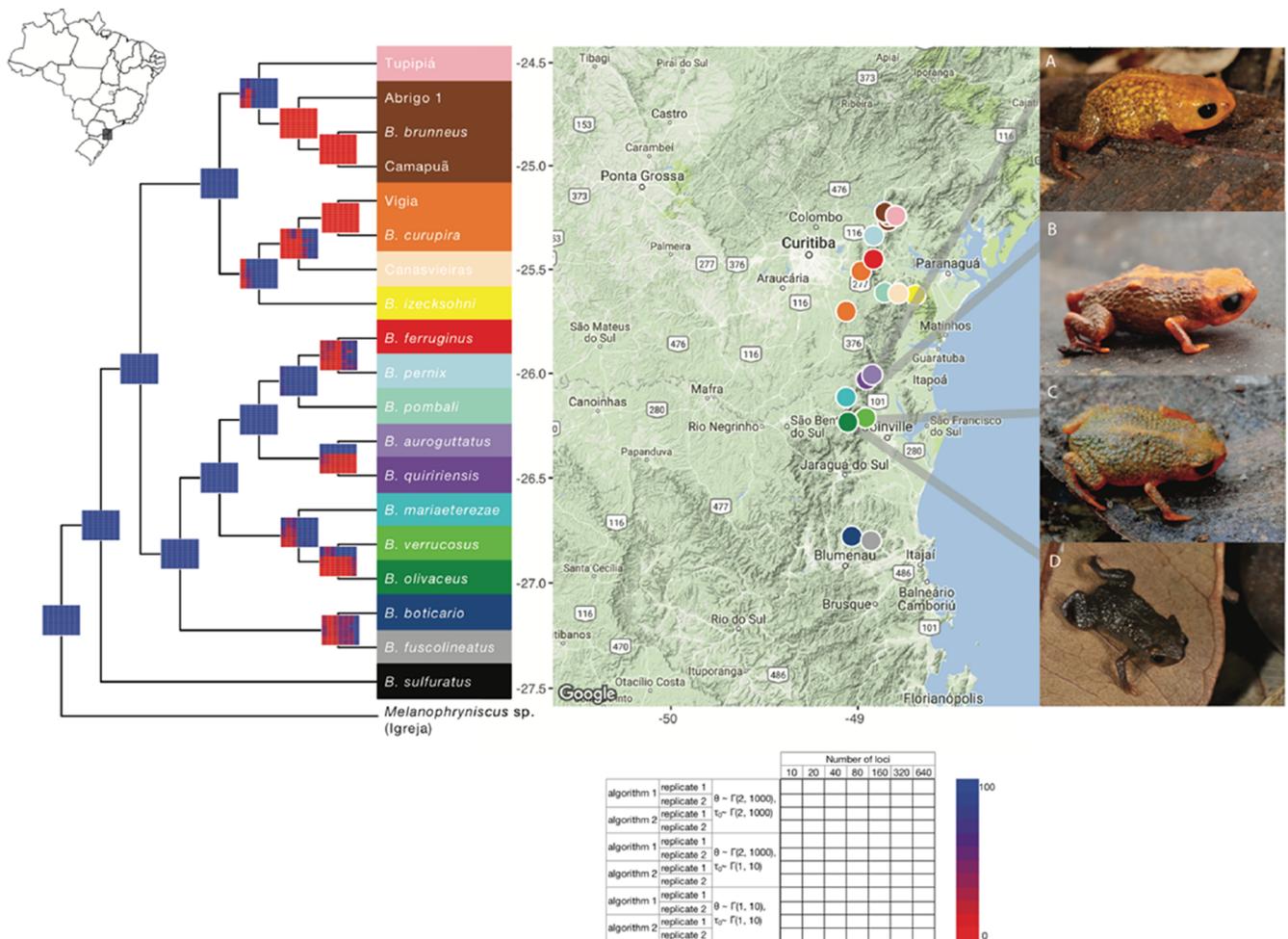


**Fig. 2.** Species delimitation analyses of *Melanophryniscus* from the southern Brazilian Atlantic Forest. Heat maps correspond to differences in posterior probability for a given node between analyses according to variation in priors, algorithms, replicates, and the number of loci (see heat map legend in figure). Colors on the tip labels correspond to the best-supported delimitation scheme. Colors that only include locality names correspond to putative undescribed species. Background image from Map data ©2017 Google. A) *M. alipioi*; B) *M. sp.* “Igreja”; C) *M. sp.* “Boa Vista”; and D) *M. milanoi*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

computational limitations led the authors to analyze fewer than 100 loci simultaneously. Our results extend prior work by demonstrating that species delimitation with large numbers of loci is possible using UCE data in Anura, with clear delimitation schemes that are robust to a variety of prior settings and levels of locus informativeness. This adds to the utility of UCEs for anuran phylogenetics (e.g. Alexander et al., 2017).

Our results also demonstrate that locus number is a more important factor affecting BPP results than locus information content. In particular, if we consider the full 640 locus dataset as a benchmark, the resolution of recalcitrant nodes was obtained asymptotically as additional loci were included, whereas prioritizing more informative loci had a relatively minor impact on the inferred posterior probabilities. Previously, Hime et al. (2016) used several analyses of two data sets to demonstrate that fine-scale delimitation improved with increasing number of loci, a result that is also supported by simulation studies (e.g. Hird et al., 2010; Camargo et al., 2012). In addition to providing guidance to researchers seeking to most effectively collect data, this result also suggests that MSC species delimitation programs like BPP may not show the same negative effects of including low information-content loci as seen in some MSC-based, species tree methods such as STAR (Liu et al., 2009), ASTRAL (Mirarab and Warnow, 2015) and MP-EST (Liu et al., 2010), for which the inclusion of those loci often leads to the recovery of inconsistent topologies (e.g. Hosner et al., 2016; Manthey et al., 2016; Meiklejohn et al., 2016).

The use of genetic data for species delimitation has been recently criticized by Sukumaran and Knowles (2017), who argued that species delimitation based on the MSC, particularly as implemented in BPP, diagnoses genetic structure, not species, and that this species delimitation procedure cannot statistically distinguish structure associated with population isolation versus species boundaries. The main concern raised by Sukumaran and Knowles (2017) is the possibility of taxonomic inflation if species are described based on genetic data alone (see also Olave et al., 2014). We believe that their concern is highly relevant, in theory, but a relatively minor point, in practice, for two main reasons. First, although many studies in recent years have used genetic species delimitation methods, very few actual species descriptions have been based on genetic data alone (cf. Leaché and Fujita, 2010). Rather, nearly all studies using molecular species delimitation that led to actual species descriptions incorporated other sources of data (morphological, behavioral, ecological) prior to making their determination (e.g. Bauer et al., 2010; Rittmeyer and Austin, 2012; Solís-Lemus et al., 2015). This is the integrative approach we have taken. Second, although conservation and management efforts ordinarily use species numbers as their main currency, such that taxonomic inflation could potentially lead to flawed conclusions, the opposite problem—failure to recognize cryptic species as distinct entities—is equally important (Bickford et al., 2007). Molecular species delimitation is often used precisely when diagnosis using phenotypic traits alone is difficult. It is therefore ironic that the recommendation is to return to the phenotype, which is tacitly



**Fig. 3.** Species delimitation analyses of *Brachycephalus* from the southern Brazilian Atlantic Forest. Heat maps correspond to the differences in posterior probabilities for the presence of a given node between analyses according to variation in priors, algorithms, replicates, and the number of loci (see heat map legend in figure). Colors on the tip labels correspond to the best-supported delimitation scheme. Colors that only include locality names correspond to putative undescribed species. Background image from Map data ©2017 Google. A) *B. auroguttatus*; B) *B. quiririensis*; C) *B. verrucosus*; and D) *B. olivaceus*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

assumed to be more reliable. One might easily forget that phenotypic traits can be affected by the same sources of uncertainty as those indicated for molecular data, for example the difficulty in finding fixed diagnostic traits among potentially distinct species (Wiens and Servedio, 2000), and the challenge of discriminating between differentiated populations and “good” species.

Some of the criticism of molecular species delimitation methods likely derives from naive optimism of early efforts that heralded the approach as an objective means of defining species (as in the early days of molecular taxonomy). However, the practice of taxonomic research has already demonstrated that species delimitation will never be an automated task, with or without phenotypic traits. In the end, it is important to keep in mind that cryptic species do exist, and that their biological reality should not be negated because other axes of divergence have not been identified.

An indication that BPP is delimiting lineages at the species level, in this case, is that our results corroborate some prior species descriptions that used phenotypic data alone (e.g. Bornschein et al., 2015, 2016b; Pie and Ribeiro, 2015; Ribeiro et al., 2015). This is significant, given that many of those species — particularly in the case of *Brachycephalus* — have been diagnosed largely based on coloration, a trait often considered unreliable for anuran taxonomy. In addition to the corroboration of prior species descriptions, our analyses found evidence for two putatively undescribed species of *Brachycephalus* and they suggest that

three currently recognized *Melanophryniscus* species (i.e. *M. alipioi*, *M. xanthostomus*, and *M. milanoi*) actually represent species complexes involving a total of seven species. Interestingly, three pairs of closely related species (e.g. *B. ferruginus* × *B. pernix*, *B. auroguttatus* × *B. quiririensis*, and *B. verrucosus* × *B. olivaceus* – see Fig. 3) did not reach 1.0 posterior probability in all analyses, despite having clear morphological diagnoses (including color variation), suggesting that BPP may be more conservative in its delimitation of species compared to phenotypic evidence. Alternatively, one could envision that this result is due to violations in the assumptions of BPP in those cases. However, it seems unlikely here, given that all of those species share similar life-histories, habitats, and environmental conditions, such that it seems unlikely that the assumptions would only be violated in those particular lineages.

In conclusion, our study provides guidance for researchers seeking to delimit species with genomic data, and our empirical results have important implications for the conservation of these Brazilian Atlantic Forest frogs. Cloud forests, which are the habitat of most of the species in the *pernix* group, are among the most threatened ecosystems globally (Doumenge et al., 1995; Aldrich et al., 1997; Toledo-Aceves et al., 2011). This is of particular concern, not only due to the key role played by these forests in hydrological cycle maintenance, but also because they are reservoirs of endemic biodiversity (Toledo-Aceves et al., 2011). Many of the species in this study are categorized as threatened or as data deficient, yet any level of formal governmental protection

necessarily involves the availability of a species name. Given their microendemic distribution and highly threatened habitats, one could argue that commission errors (considering them as different species) would be preferable in relation to omission errors (lumping them as single species). In practice, urgent management efforts should be enforced to ensure their long-term survival.

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## Declaration of Competing Interest

None.

## Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ympev.2019.106627>.

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