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In the shadows: Phylogenomics and coalescent species delimitation unveil cryptic diversity in a Cerrado endemic lizard (Squamata: *Tropidurus*)

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ABSTRACT

The recognition of cryptic diversity within geographically widespread species is gradually becoming a trend in the highly speciose Neotropical biomes. The statistical methods to recognise such cryptic lineages are rapidly advancing, but have rarely been applied to genomic-scale datasets. Herein, we used phylogenomic data to investigate phylogenetic history and cryptic diversity within *Tropidurus itambere*, a lizard endemic to the Cerrado biodiversity hotspot. We applied a series of phylogenetic methods to reconstruct evolutionary relationships and a coalescent Bayesian species delimitation approach (BPP) to clarify species limits. The BPP results suggest that the widespread nominal taxon comprises a complex of 5 highly supported and geographically structured cryptic species. We highlight and discuss the different topological patterns recovered by concatenated and coalescent species tree methods for these closely related lineages. Finally, we suggest that the existence of cryptic lineages in the Cerrado is much more common than traditionally thought, highlighting the value of using NGS data and coalescent techniques to investigate patterns of species diversity.

1. Introduction

The Linnean shortfall (Brown and Lomolino, 1998) was initially envisioned to describe the lack of taxonomic knowledge and the impediments it brings to biological studies, but this shortfall was soon developed to acknowledge the problem in terms of conservation practices (Possingham et al., 2007). Similarly, there is a lack of knowledge concerning the distribution of species and its associated issues, which has been termed the Wallacean shortfall (Whittaker et al., 2005). Both shortfalls are correlated and can strongly restrict action on conservation measures, especially in biodiversity hotspots (Bini et al., 2006). Nonetheless, even if both issues are resolved, information concerning phylogenetic relationships would still be lacking for many species in the world, and this Darwinian shortfall (Diniz-Filho et al., 2013) would further hinder conservation efforts. Not surprisingly, the three shortfalls are highly accentuated in the Neotropics, where the high biodiversity and remoteness of several areas make it difficult for researchers to overcome these knowledge gaps (Balian et al., 2007; Kier et al., 2005; Schipper et al., 2008; Silva et al., 2014).

Overcoming the above-mentioned shortfalls is not a trivial task. Recent attention has been devoted to the study, discovery, and description of cryptic species, which, given the correct technical and analytical tools, has the potential to address the three shortfalls at once (Beheregaray and Caccone, 2007; Bickford et al., 2007; Pfenninger and Schwenk, 2007). Coalescent species delimitation has emerged as a useful tool for resolving taxonomic issues and informing conservation decisions (Fujita et al., 2012). However, the use of coalescent species delimitation is still in its infancy compared to many other phylogenetic methods, with only a few examples of its application in Neotropical studies (e.g., Camargo et al., 2012; Domingos et al., 2014; Gamble et al., 2012; Gehara et al., 2014; Smith et al., 2014a). Moreover, to the best of our knowledge, there is only one study of Neotropical organisms

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(on rainforest birds; Smith et al., 2014a) that combined Next-Generation Sequencing (NGS) data with coalescent species delimitation methods. Increasing the number of loci used for species delimitation can assist in the detection of different lineages in non-model organisms (Pante et al., 2015), avoid problems associated with not sampling the possibly different demographic histories retrieved by different loci (Garrick et al., 2015), and reduce the probability of delimitation errors when using coalescent species delimitation methods (Zhang et al., 2014; Zhang et al., 2011). Thus, sampling a large number of nuclear loci using NGS technology could notably improve the power of coalescent species delimitation analyses when investigating highly diverse lineages, such as in Cerrado endemic lizards.

The Cerrado, arguably the most species rich savanna in the world (Castro et al., 1999; Furley, 1999; Oliveira and Marquis, 2002), has been the focus of an increasing number of squamate species descriptions in recent years (e.g., Colli et al., 2003a; Colli et al., 2003b; Colli et al., 2009; de Freitas et al., 2011; Rodrigues et al., 2007; Teixeira et al., 2013). Similarly, several studies have recently recognised cryptic lineages among previously described Cerrado endemics (Domingos et al., 2014; Gamble et al., 2012; Giugliano et al., 2013; Recoder et al., 2014; Werneck et al., 2012). In fact, the simple activity of visiting and collecting biological specimens in previously unexplored regions of the Cerrado has recently resulted in the discovery of new species (Diniz-Filho et al., 2006; Diniz-Filho et al., 2008), even for large-bodied lizard species (Giugliano et al., 2013; Nogueira and Rodrigues, 2006).

Many species of *Tropidurus* are well-studied in terms of ecology (see Carvalho, 2013 for a comprehensive literature review) and have also been the subject of systematics (Frost et al., 2001; Harvey and Gutberlet, 2000), biogeography, and conservation studies (Carvalho, 2013), making them a broadly studied group of organisms in South America. There are presently 29 recognised species of *Tropidurus* distributed across South America (Carvalho, 2016), 11 of which can be found in the Cerrado (Morais et al., 2014), and five are endemic to the biome (Nogueira et al., 2011). A few *Tropidurus* species have been recently described (e.g.; Carvalho, 2016; Carvalho et al., 2016; Kunz and Borges-Martins, 2013; Passos et al., 2011), but there are only two published molecular phylogenetic analyses of the genus (Carvalho et al., 2016; Frost et al., 2001). Frost and colleagues (2001) used mtDNA and morphological data to propose phylogenetic hypotheses for the genus, using the recognised *Tropidurus* species at the time of publication. Almost 15 years later, the first multilocus phylogenetic reconstructions concerning *Tropidurus* species was published (Carvalho et al., 2016). The lack of a modern taxonomic revision and the limited sampling of earlier molecular phylogenetic reconstructions preclude comprehensive evolutionary studies on this iconic Neotropical genus. Furthermore, morphological and molecular variation across and within species suggests that unrecognised species complexes exist (Matos et al., 2016; Rodrigues, 1987; Werneck et al., 2015), which currently hinders a thorough attempt of reconstructing the phylogenetic history of the genus (Carvalho et al., 2016). Thus, cryptic diversity within nominal species of *Tropidurus* might be more common than the recent rate of species description suggests (Carvalho et al., 2016).

The taxon targeted in this study, *Tropidurus itambere* Rodrigues (1987), is diagnosed by the presence of a deep mite pocket in the inguinal region, and another on the side of the neck (Rodrigues, 1987). However, there is great variation in the depth and form of mite pockets between populations, as well as in anterior and posterior limb lengths that might be associated with different degrees of specialization for the use of rock crevices. Although there is clear variation among localities, the underlying pattern of variation does not seem to be geographically clustered (Domingos and Colli, pers. obs.). Therefore, it is currently unknown if all populations that can be morphologically assigned to *T. itambere* actually belong to the same species.

Here, we applied a coalescent species delimitation method and a series of phylogenetic analyses to test whether the morphological and geographical variation observed among Cerrado *T. itambere* populations reflects genome-wide divergences and to assess if inferred evolutionary distinctiveness are consistent with the presence of species-level cryptic diversity. We clarify phylogenetic relationships among lineages and discuss the potential advantages of applying coalescent species delimitation methods to a robust dataset of ~400 loci obtained by an anchored hybrid enrichment phylogenomic approach (Lemmon et al., 2012). Our results suggest that *T. itambere* populations can be assigned to 5 well-supported cryptic species. We also found that the coalescent species tree estimated using BPP retrieve a slightly different topology compared to concatenated and other coalescent phylogenetic reconstruction methods for these closely related species. To the best of our knowledge, this is the first evolutionary study applying NGS data to investigate such questions in Cerrado vertebrates.

2. Material and methods

2.1. Sampling and genetic protocols

First, we obtained mitochondrial DNA (mtDNA) data for 103 *Tropidurus itambere* and outgroups individuals from 29 localities. Our choice of outgroup taxa was based on Frost et al. (2001) and included *T. guarani*, *T. hispidus*, *T. insulanus*, *T. oreadicus*, *T. psamonastes*, *T. torquatus*, three undescribed *Tropidurus* species (see Results) and two other tropidurid lizards, *Plica plica* and *Uranoscodon superciliosus*. We extracted genomic DNA using a modified salting-out technique (Sunnucks and Hales, 1996) and used polymerase chain reaction (PCR) to amplify fragments of the mtDNA cytochrome *b* (cytb). Primers and PCR cycle protocols are in Supplementary Table 1. We assembled and visually inspected chromatograms using SEQUENCHER v4.9 (Gene Codes Corporation, Ann Arbor, MI USA). Sequences were codon aligned using MUSCLE (Edgar, 2004) applying a gap open penalty of 3 and a gap extension penalty of 1, implemented in MEGA v5.2.2 (Tamura et al., 2011).

Secondly, we obtained nuclear sequence data from hundreds of loci for a subset of 34 individuals representing different mtDNA clades (putative cryptic species); these taxa were selected based on geographic criteria and Neighbour-Joining phylogenetic trees (Supplementary Fig. 1) in an attempt to maximise sampling of divergent clades and to cover the known range of the nominal species (Fig. 1). To generate the nuclear phylogenomic data set, we employed anchored hybrid enrichment (AHE; Lemmon et al., 2012). Data were collected at the Center for Anchored Phylogenomics at Florida State University (www.anchoredphylogeny.com). In short, each genomic sample was sonicated to ~300–800 bp fragment size on a Covaris E220 Focused-ultrasonicator using Covaris microTUBES. Libraries were prepared and indexed on a Beckman-Coulter Biomek FXp liquid-handling robot following a protocol modified from Meyer and Kircher (2010). One modification is a size-selection step using SPRIselect beads (Beckman-Coulter Inc.; 0.9x ratio of bead to sample volume) after blunt-end repair. Indexed libraries were pooled in equal quantities (~12–16 samples per pool), and enrichments were performed on each pool via an Agilent Custom SureSelect kit (Agilent Technologies, Inc.), designed for use in amniotes (Prum et al., 2015; Ruane et al., 2015; Tucker et al., 2016). After enrichment, each set of three enrichment reactions were pooled in equal quantities and sequenced on an Illumina HiSeq2000 PE150 lanes. Sequencing was performed in the Translational Science Laboratory in the College of Medicine at Florida State University.

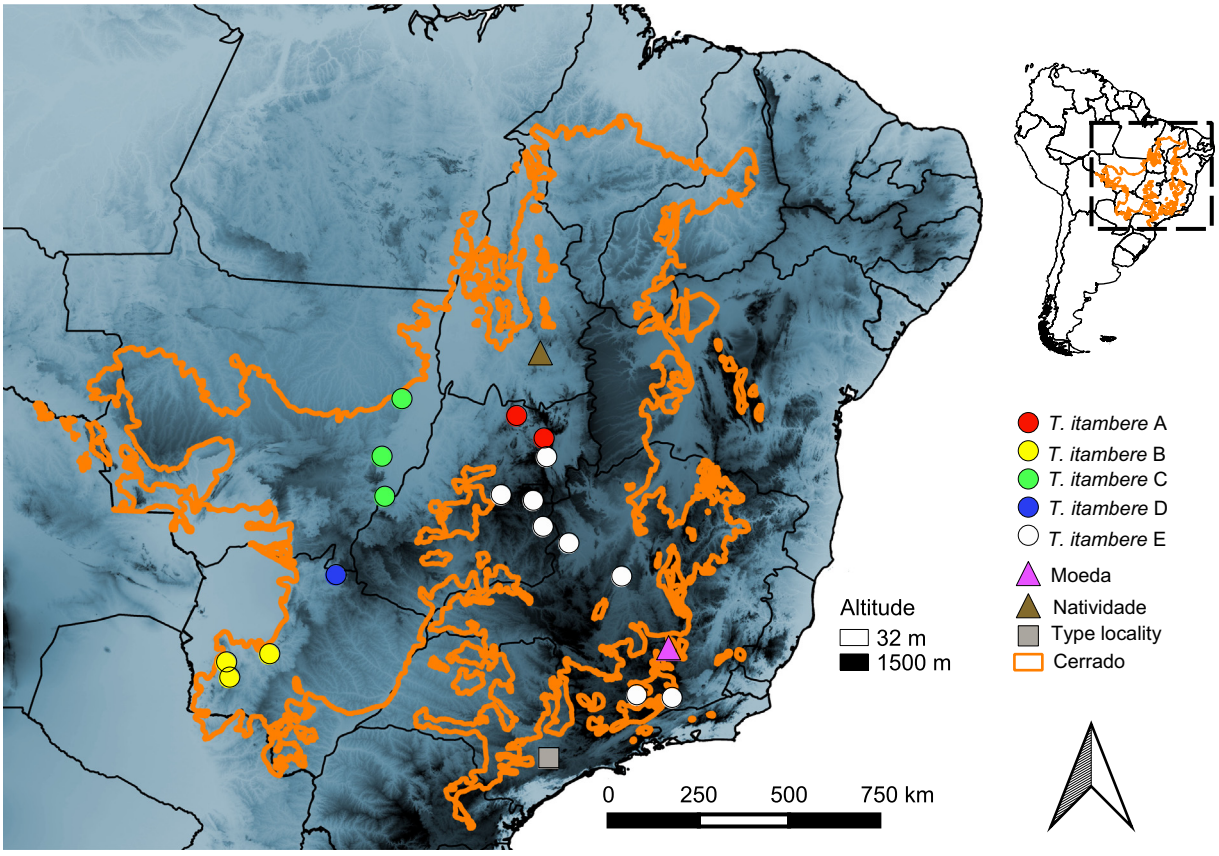

Fig. 1. Partial map of Brazil with *Tropidurus itambere* sample sites in the context of the distribution of the Cerrado. Different colours indicate clades (species hypotheses) used in BPP analyses.

Overlapping reads were merged following the protocol described in Rokyta et al. (2012), and reads were assembled using *Anolis carolinensis* and *Calamaria pavimentata* as references (Lemmon et al., 2012; Ruane et al., 2015; Tucker et al., 2016). Consensus bases were called from assembled clusters as follows. Unambiguous bases were called for assembly sites either containing only one type of base, or containing a polymorphism that could be explained as sequencing error (assuming a binomial probability model with the probability of error equal to 0.01 and alpha equal to 0.05). Note the rejection of sequencing error as a possible explanation typically requires 10× coverage under these parameters. If the polymorphism could not be explained as sequencing error, the appropriate ambiguous base corresponding to all of the observed bases at that site was called.

Finally, full coalescent calculations, like the one implemented in the software Bayesian Phylogenetics and Phylogeography (BPP; Yang, 2015), require alleles as input data and the AHE loci had to be phased prior to analyses. Hence, alleles were phased in a Bayesian statistical framework using read overlap information and alignments were trimmed to remove ambiguously aligned regions, as described in Pyron et al. (2016). To allow for direct comparisons between the phylogenetic and species tree methods, we use the phased dataset in all analyses described below. Because of computational constraints, we only used one random allele per individual in all downstream phylogenetic and species delimitation analyses. Information about individuals sampled for cytb and AHE, and their collection sites is provided in Supplementary Table 2. The cytb dataset was only used to select individuals for AHE sequencing, whereas the AHE dataset was used for all phylogenetic and species delimitation analyses.

2.2. Phylogenetic relationships

We used concatenated Maximum Likelihood and Bayesian phylogenetic analyses to infer relationships among lineages. While species trees based on the multispecies coalescent may yield better accuracy than traditional concatenated approaches (Heled and Drummond, 2010; Xi et al., 2014), they are computationally demanding and most methods cannot be applied to phylogenomic datasets (O'Neill et al., 2013). Therefore, we used two multispecies coalescent approaches that incorporate information from previously estimated gene trees in a coalescent framework (Song et al., 2012), and one full coalescent approach implemented in BPP (Yang, 2015), and compared their results to the concatenated estimations. All analyses were run using the high performance computer facilities (HPCF) *Colossus* or *Phoenix* (Molecular Ecology Lab) at Flinders University. *Colossus* has 1160 cores and 4.25 TB of RAM, whereas *Phoenix* has 40 AVX-512 cores and 512 GB of RAM.

Partition schemes are generally guided by knowledge on the genes and codon positions, however, because the AHE loci lack these obvious sequence structures, we used the automatic *k-means* selection implemented in PartitionFinder v2 (Frandsen et al., 2015; Lanfear et al., Forthcoming). Unlike previous versions of PartionFinder (Lanfear et al., 2012; Lanfear et al., 2014), this algorithm selects a partition scheme by dividing the sequence into subsets of sites that have similar evolutionary rates (Frandsen et al., 2015). All downstream concatenated phylogenetic analyses used this selected partition scheme.

We implemented Maximum Likelihood (ML) phylogenetic analyses in RAxML v8.1.1 (Stamatakis, 2014) using rapid hill-climbing

searches, and estimated bootstrap support values using 1000 replicates with the RELL bootstrap option (Minh et al., 2013). We also ran phylogenetic analyses using Bayesian inference implemented in Exabayes v1.4.1 (Aberer et al., 2014). Starting from a parsimony tree, we conducted two independent runs with four parallel Markov Chain Monte Carlo (MCMC) chains for at least 1 million generations (sampled every 500th), and set to automatically stop when the average standard deviation of split frequencies was below 0.05 (indicating good convergence). We used a minimum acceptable effective sample size (ESS) of 200 for each parameter and checked the potential scale reduction factor (PSRF, ~1.0) using the postProcParam and extractBips programs distributed with Exabayes v1.4.1. Branch lengths were linked across partitions, while substitution rates, character state frequencies, gamma shape parameters and proportion of invariable sites were all unlinked. An extended majority-rule consensus tree was obtained using the "consense" program distributed with Exabayes v1.4.1, discarding 25% of the initial samples as burn-in. The GTR model with gamma shape distribution (GTRGAMMA) was used on all partitions for the Bayesian analyses. This was carried out because over-parameterising (overfitting) the evolution model on Bayesian analyses has little influence in the resulting topology (Huelsenbeck and Rannala, 2004), especially when numerous and long loci are used (Lemmon and Moriarty, 2004), and to avoid highly intense computations. The same strategy was adopted for the ML analyses implemented in RAxML.

2.3. Species tree estimations

Species tree reconstructions based on the coalescent are very computationally intensive and most methods are apparently unable to deal with phylogenomic datasets (Leache and Rannala, 2011; O'Neill et al., 2013; Pyron et al., 2014). These species-tree approaches may fail to identify the correct topology over competing tree hypotheses (Lischer et al., 2014) and even show decreasing resolution and lineage support as more loci are included (O'Neill et al., 2013). Nonetheless, a few methods may overcome this limitation (Kubatko et al., 2009; Liu et al., 2009), mainly because they incorporate already estimated gene trees and treat them under coalescent models. Indeed, the coalescent species-tree methods MP-EST (Liu et al., 2010) and NJst (Liu and Yu, 2011) have performed well using phylogenomic datasets (Pyron et al., 2014). We used the web-server STRAW (Shaw et al., 2013) to estimate both MP-EST and NJst species-trees for the three study taxa. Individual gene trees were generated using RAxML v8.1.1 (Stamatakis, 2014) performing 100 rapid bootstrap inferences and a thorough ML search, under a GTR evolution model with gamma shape distribution.

For the sake of comparability, we used exactly the same data for all phylogenetic analyses. Accordingly, all loci for which the selected outgroup was not captured (sequenced) by AHE were excluded from analyses, so that individual gene trees used for STAR and NJst could be generated (even thought they could have been used as missing data for the ML and Bayesian concatenated analyses). Thus, the final dataset for the phylogenetic analyses differed slightly from that used for the coalescent species delimitation described below, for which all loci were used (Supplementary Table 3).

We also implemented a full coalescent species tree estimation using the software BPP (Rannala and Yang, 2015). Although BPP can handle the AHE data and achieve convergence in a reasonable amount of time, it can only implement the simplistic Jukes-Cantor substitution model (JC69). This assumption might influence BPP's ability to estimate phylogenetic relationships of closely related species (Jia et al., 2014; Wu et al., 2013), although this limitation has not been empirically addressed. Details on the BPP analyses are given in the coalescent species delimitation section below.

Finally, we chose not to estimate divergence times within *Tropidurus itambere* lineages because of the lack of tropidurid or closely related fossils from the Neogene (Albino and Brizuela, 2014), when presumably many living South American squamates originated (Domingos et al., 2014; Guarnizo et al., 2016; Werneck et al., 2012), and because we currently have no reasonable account on the evolutionary rate of the AHE data.

2.4. Coalescent species delimitation

To investigate species limits within *T. itambere* lineages we used the software BPP v3.2 (Rannala and Yang, 2015; Yang and Rannala, 2014), running analysis type 'A11' (Yang, 2015), which simultaneously estimates a species tree while running the reversible-jump MCMC species delimitation algorithm (Yang and Rannala, 2010). We separated individuals into monophyletic groups (species hypotheses) based on the previously estimated phylogenetic trees and on the geographic distribution of clades (Figs. 1, 2). Specimen affiliation to these clades was the same among all four estimated phylogenies for all species, i.e. specimen assignment to a species hypothesis agreed among all phylogenetic analyses (concatenated ML and Bayesian, MP-EST and NJst). Thus, BPP analyses contained 12 potential species – 5 *T. itambere* clades, and the 7 outgroup species. After initial trials testing different parameters, we used a gamma prior of ~G (21,000) for population size (θs) and the age of the root in the species tree (τ_0), and the Dirichlet prior (Yang and Rannala, 2010: Eq. (2)) for other divergence time parameters. Alternative prior choices had no effect on the results, and our choice seems to reflect a realistic assumption for the species (Van-Sluys, 1993). We ran analyses for 5×10^5 MCMC generations, sampled every five generations and using 1×10^4 burn-in generations. Because we had a considerable amount of gaps and ambiguous sites, and to make sure we were getting consistent results, we ran analyses multiple times with alternative settings: (1) using both available reversible-jump MCMC species delimitation algorithms (Algorithms 0 and 1, Yang and Rannala, 2010), and (2) with or without the "cleandata" option. Cleandata = 1 means the program will remove all columns in the alignment that have gaps or ambiguity characters, and cleandata = 0 means that those will be used in the likelihood calculation. For each analysis type, we ran BPP at least twice (with random starting trees) to check that our results were consistent across runs.

BPP can handle any number of missing individuals per locus; thus, we excluded all individuals with more than 30% missing data for a given locus from the alignment. The number of excluded sequences range from zero to eight with a mean of 1.9 excluded sequences per alignment. We empirically decided this threshold after visually inspecting the data and running initial BPP trials: most individuals with more than 30% missing data also had a very high number of ambiguous and undetermined sites, i.e. they were mainly low quality captures. Excluding them from the loci alignments was necessary to avoid an unwarranted influence of these individuals on the likelihood calculations.

2.5. Summary statistics

We calculated population genetics summary statistics for each AHE locus (Supplementary Table 3) using Arlequin v3.5 (Excoffier and Lischer, 2010) and, for comparative reasons, obtained the same statistics for cytb from individuals used in the AHE protocol (Table 1). Additionally, we used MEGA v5.2.2 to calculate cytb net between-group distances for BPP-delimited species (Tamura et al., 2011). Specifically, we used all individuals for which cytb was available (Supplementary Table 2) and computed both uncorrected *p*-distances and ML corrected distances with standard error estimates calculated using 1000 bootstrap replicates.

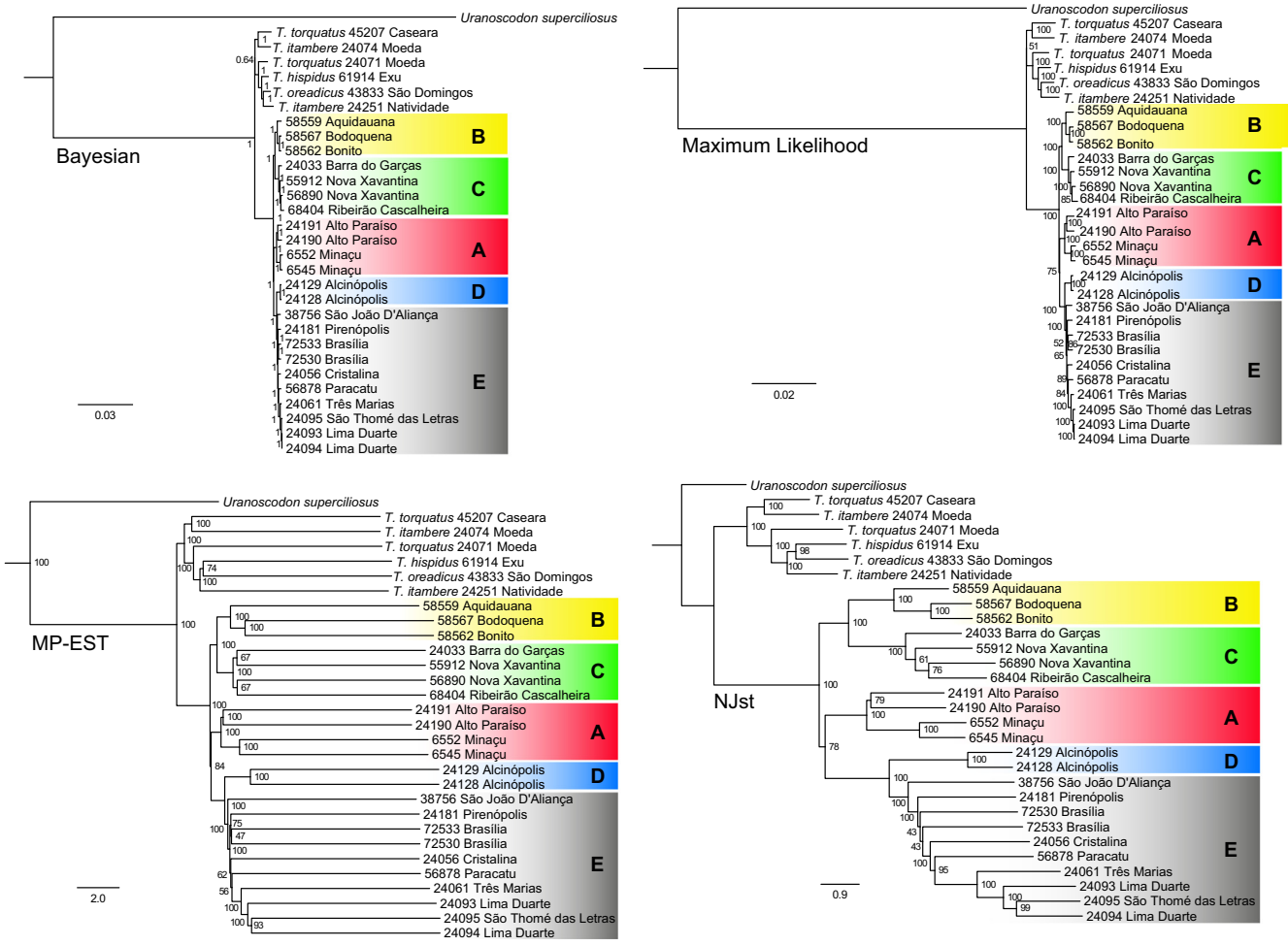

Fig. 2. Phylogenetic relationships among *Tropidurus itambere* lineages estimated by Bayesian, Maximum Likelihood, and coalescent methods MP-EST and NJst. Numbers in nodes denote posterior probabilities for the Bayesian analyses, and bootstrap scores for all others. MP-EST branch lengths are in coalescent units. NJst branch lengths are the average number of internodes. Grouping colours and letters refer to clades used on BPP coalescent species delimitation analyses, according to Fig. 1.

Table 1
Mean values (±SD) of *Tropidurus itambere* population genetics summary statistics (Watterson's θ, pairwise nucleotide diversity (θ_π), and Tajima's *D*) from anchored hybrid enrichment (AHE) and cytb alignments. Cytb statistics were calculated from the same individuals used for AHE. All statistics were calculated after excluding outgroups and using unphased data.

AHE			Cytb		
Watterson's θ	Nucleotide diversity θ_π	Tajima's *D*	Watterson's θ	Nucleotide diversity θ_π	Tajima's *D*
7.09 (3.63)	5.19 (3.03)	−1.02 (0.54)	55.14	59.12	0.29

3. Results

3.1. Sampling and population genetic summary statistics

Out of 103 *Tropidurus itambere* and outgroup specimens sequenced for cytb, we performed AHE sequencing for 34, but only 30 were actually captured (Supplementary Material 2). Cytb alignments were 801 bp long, and the final AHE alignment after cleaning and pruning contained 422 loci and was 607,948 bp long (cytb and AHE alignments are available in DRYAD repository doi: 10.5061/dryad.1hs2m). Only 4.5% of the AHE sites were gaps or undetermined (missing) sites in the final alignments.

All loci were polymorphic, although number of polymorphic sites per locus varied extensively (Supplementary Material 3). As expected, population genetics summary estimates were much lower for the AHE dataset compared to the faster-evolving mitochondrial cytb (Table 1). Tajima's D estimates were not significantly different from zero (Tajima, 1989).

3.2. Phylogenetic relationships and species trees

The major inferred clades were all strongly supported (Bayesian posterior probability = 1, Bootstrap values >85) by all approaches. Differences between concatenated versus coalescent approaches are expected, especially for short internodes (Pyron et al., 2014). Both concatenated analyses, MP-EST and NJst, recovered an identical topology for *T. itambere* lineages (Fig. 2), but the BPP species tree analyses retrieved a slightly different topology for the *T. itambere* ingroup and also for the outgroups (Fig. 3). The main topological difference was that clade A is sister to all other

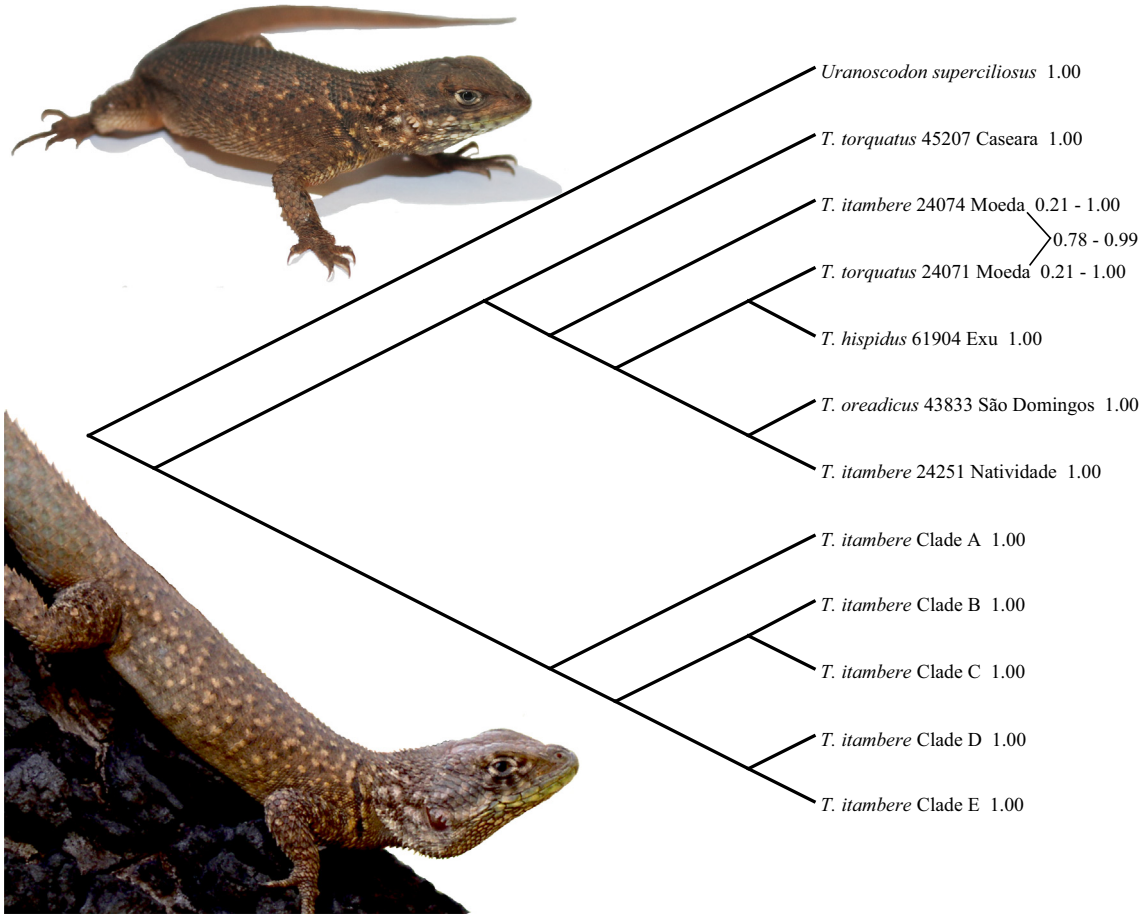

Fig. 3. Species tree, delimited species and their posterior probabilities as estimated by BPP v3.2 on different runs for *Tropidurus itambere*. Posterior probabilities shown for the Moeda specimens (24071 and 24074) indicate runs when they were recovered as the same species or as separated species (posterior probabilities ⩽0.01 not shown). Photos are both from *T. itambere* Clade A specimens (Brasília). Upper photo by Carlos J. S. Morais, and lower photo by Davi L. Pantoja.

species in the BPP species tree (Fig. 3), whereas clade B + C is sister to the A + D + E clade in the concatenated, MP-EST and NJst trees (Fig. 2).

As can be noted from Figs. 2 and 3, two *T. itambere*-like species were recovered as outgroups by the phylogenetic analyses ("Moeda" and "Natividade" populations), which makes *T. itambere* a paraphyletic species. These are yet undescribed species, within the distribution of *T. itambere*, and from which diagnosable morphological characters are, at this stage, indistinguishable from the described diagnosis of *T. itambere* (see Discussion on these individuals on Section 4.1).

3.3. Coalescent species delimitation

BPP species delimitation results indicated the existence of 5 cryptic species within *Tropidurus itambere*, using both the complete alignment (no cleandata) and the one excluding sites with missing and ambiguous characters (cleandata). Although all *T. itambere* clades (A–E) and most outgroup species were consistently recovered as distinct species with a posterior probability of 1, this was not the case for two sympatric outgroups: *Tropidurus torquatus* and a *T. itambere*-like species from Moeda which were sometimes recovered as a single species (posterior probability ranging from 0.78 to 0.99) (Fig. 3).

Cytb levels of uncorrected sequence divergence among *Tropidurus itambere* cryptic species retrieved by BPP ranged from 3.1% to 8.3% and ML corrected distances from 3.3% to 9.7% (Table 2).

4. Discussion

The Darwinian shortfall represents a problem not only for conservation biology (Diniz-Filho et al., 2013; Forest et al., 2015; Redding et al., 2014), but it also prevents adequate assessments of evolutionary and biogeographic hypotheses (Monnet et al., 2014; Rangel et al., 2015). Overcoming this shortfall is essential to provide the basis for in-depth investigations on the evolutionary history of many understudied Neotropical species. Here, we used a powerful anchored phylogenomics dataset to investigate phylogenetic relationships and cryptic diversity within Neotropical lizards endemic to the Brazilian Cerrado. Under the Generalised Lineage Concept (de Queiroz, 2007), our results indicate that *T. itambere* populations are split in 5 cryptic species and suggest that the existence of cryptic diversity in the Cerrado might be relatively common. More broadly, our study highlights the value of using NGS data and coalescent techniques to investigate patterns of diversity in understudied Neotropical regions.

4.1. Cryptic species in the Tropidurus itambere complex

The species delimitation analyses generated straightforward results. There were 5 inferred cryptic lineages, all with high support values in every BPP run. Importantly, the cryptic lineages were also clearly geographically structured (Fig. 1), which will facilitate their future taxonomic descriptions. The type locality of *T. itambere* (Sorocaba, São Paulo state; Fig. 1), although unsampled, is within the distribution of our Clade E in the southeast part of the Cerrado.

Table 2
Net among group distances between *Tropidurus itambere* cryptic species for cytb data. ML corrected distances using the Tamura-Nei model are above the diagonal, and uncorrected p-distances below. Standard error estimates, calculated using 1000 bootstrap replicates, are shown in brackets.

	Clade A	Clade B	Clade C	Clade D	Clade E
Clade A	–	0.078 [0.009]	0.077 [0.009]	0.092 [0.011]	0.085 [0.011]
Clade B	0.065 [0.007]	–	0.059 [0.008]	0.093 [0.011]	0.078 [0.010]
Clade C	0.065 [0.006]	0.050 [0.006]	–	0.097 [0.012]	0.087 [0.011]
Clade D	0.079 [0.008]	0.080 [0.009]	0.083 [0.009]	–	0.033 [0.006]
Clade E	0.073 [0.008]	0.067 [0.008]	0.074 [0.008]	0.031 [0.006]	–

However, a preliminary analysis of Carvalho et al. (2016) based on cytb sequences from Piedade (São Paulo state), the closest available sample from the type locality, showed that this sample did not cluster with either Clade D or E (Supplementary Fig. 1). Given that only one locus from a single population is available for comparison with our data, it is not possible at this stage to determine if any of our cryptic species actually corresponds to the nominal taxon.

The topological pattern recovered within clade E (our best sampled lineage) suggests the influence of isolation by distance, with populations structured in a NW-SE direction, similarly to what was observed for the Cerrado frog *Hypsiboas punctatus* (Prado et al., 2012) and the lizard *Norops meridionalis* (Guarnizo et al., 2016). Clade A is at the northernmost distribution of the species, and the remaining ones are located in the western portion of the Cerrado. In addition, Clades B and C were always retrieved as sister species, even though Clade D's range intersects their distribution (Figs. 1 and 2).

Noteworthy, individuals from two populations (Moeda and Natividade), morphologically diagnosable as *T. itambere*, appear not to belong to the *T. itambere* species group (Figs. 2 and 3). This reinforces the limitations of current morphological diagnoses for species of *Tropidurus*, often based on only a few characters (e.g., mite pockets). The published phylogeny of the genus is based only on samples from the type localities of each species and thus lacks broad geographical sampling (Carvalho et al., 2016). The modest geographic sampling of earlier *Tropidurus* phylogenies precludes a detailed investigation of its morphological characters and hinders an informed taxonomic revision of this group. A detailed multilocus phylogenetic analysis that includes described and cryptic lineages (e.g., Matos et al., 2016; Werneck et al., 2015; this study) of the genus *Tropidurus* is highly warranted, and will help clarify aspects of morphological evolution and facilitate taxonomical decisions. Furthermore, *Tropidurus torquatus* and the *T. itambere*-like species from Moeda were sometimes recovered as a single species in the BPP species delimitation analyses (Fig. 3). Interestingly, these two species were never retrieved as sister in the phylogenetic analyses (Fig. 2). Individuals from these taxa were collected in close range to each other (Supplementary Table 2) and are very distinct morphologically. This might suggest a historical introgression event between these two sympatric species, calling for fine-scale population genetic studies in this geographic region.

4.2. Cryptic speciation in the Neotropics

It would be feasible to consider, given the relatively high number of cryptic lineages revealed by our analyses, that BPP is over-splitting species. However, both simulations (Zhang et al., 2011) and empirical tests (Camargo et al., 2012; Hime et al., 2016) demonstrated that BPP can correctly recover species limits under different evolutionary scenarios. The main limitation of species delimitation analyses using BPP is the number of available loci; less than 20 loci might not have enough power to correctly delimitate species (Hime et al., 2016; Yang and Rannala, 2014). Given the large number of loci used here, none of these problems are likely to affect our species delimitation analyses. Moreover, there is also an increasing number of cases where Neotropical cryptic lineages are recognised using different approaches (and not BPP) (Beheregaray et al., 2015; Fouquet et al., 2014, 2007; Gamble et al., 2012; Gehara et al., 2014; Prado et al., 2012; Recoder et al., 2014; Werneck et al., 2012). The Brazilian lizard fauna is one of the most diverse in the world (Costa and Bérnils, 2014), but with few exceptions (Giugliano et al., 2013; Recoder et al., 2014), recent species descriptions rely exclusively on morphological information (e.g. Arias et al., 2014a,b; Teixeira et al., 2014; Teixeira et al., 2013). In hyperdiverse regions such as the Neotropics, the sole use of morphological data on the recognition of new species can be problematic, because taxonomists might be confounded by morphological stasis when trying to separate different biological entities (Bickford et al., 2007).

In other lizard study systems where detailed phylogenies are available, it is not uncommon to recognise an enormous diversity of closely related species with relatively restricted, and even overlapping distributions. Examples exist for Australian groups such as *Gehyra* (Hutchinson et al., 2014; Sistrom et al., 2013), *Heteronotia binoei* (Fujita et al., 2010), *H. spelea* (Pepper et al., 2013), and *Diplodactylus* (Pepper et al., 2006); for Melanesian lizards *Cyrtodactylus* (Oliver et al., 2012); Brazilian *Coleodactylus* (Geurgas et al., 2008); and for west African *Hemidactylus* (Leaché and Fujita, 2010), among others. Expanding on Brazilian studies, it was reported that the lizard *Gymnodactylus darwinii* from the Atlantic Forest show strong clinal morphological variation (Freire, 1998), and indeed several cryptic species were later reported to occur within that species group (Pellegrino et al., 2005). The same is true for the *Phyllopezus pollicaris* complex (Gamble et al., 2012; Werneck et al., 2012), and *Norops meridionalis* (Guarnizo et al., 2016), although no morphological studies were conducted for these species so far. In addition, cryptic lineages with strong morphological support were described for *G. amarali* (Domingos et al., 2014). The Cerrado comprises a massive area of ~2 million km^2 and much of its species diversity is still to be uncovered (Diniz-Filho et al., 2006). That taxonomists have not recognised different lineages among *T. itambere* in the past strengthens the importance of applying modern analytical tools for the recognition of cryptic biodiversity. Hence, an increasing number of cryptic species in the Neotropics should be revealed through the use of modern NGS data and coalescent species delimitation analyses. Likewise, population-level studies, and integrative analytical frameworks that assess the speciation process itself may prove useful for testing hypotheses about cryptic species diversity (Andrew et al., 2013; Arnegard et al., 2014; Faria et al., 2014; Smouse et al., 2015).

4.3. Phylogenetic and species tree reconstruction methods

Out of the five different tree reconstruction methods used, three directly estimated phylogenies based on the genetic data. These are the concatenated Bayesian and ML inferences, and the coalescent species tree method implemented in BPP. The other two coalescent species tree methods (MP-EST and NJst) estimate the species tree topology based on previously estimated gene trees. Although the MP-EST and NJst species tree analyses are based on

the coalescence, they recovered the same topology for the *T. itambere* cryptic species ingroup estimated by the concatenated methods, whereas BPP recovered a slightly different topology (Figs. 2 and 3).

The short branch length leading from *T. itambere* clades B + C to all other clades in the Bayesian and ML trees (Fig. 2) suggests this might be a hard to resolve topology, even though the support values are high. High support values may be found even in incorrect topologies due to different methodological issues, such as incorrect substitution model selection (Sullivan and Joyce, 2005; Wu et al., 2013), and poor partition schemes (Kainer and Lanfear, 2015). The MP-EST branch lengths are in coalescent units (Liu et al., 2009), and the branch lengths in the NJst are the average number of internodes (Liu and Yu, 2011). Thus, branch lengths of both analyses are not realistic representations of sequence divergence distance. Nonetheless, given the strong congruence between the four methods, this short branch length likely depicts a rapid diversification event in a short period of time. Therefore, incomplete lineage sorting (Camargo et al., 2012; Sistrom et al., 2014; Song et al., 2012), and loci with different evolutionary rates (Xi et al., 2014) probably account for the topological incongruence between these four trees (Fig. 2) and the one estimated by BPP (Fig. 3). If that is indeed the case, the multi-species coalescent model implemented in BPP appears to be the best species tree reconstruction method currently available for phylogenomic data, since it is able to detect topological incongruences between gene trees even for closely related species (Leaché et al., 2015).

Phylogenomic studies on different taxa recovered the same tree topology using concatenated and species tree methods when investigating relationships among distantly related species (Leaché et al., 2015; Ruane et al., 2015), but the same is not true for rapid radiations (Brandley et al., 2015; Pyron et al., 2014). Our results suggest that these incongruences may arise even between different coalescent species tree methods when species are closely related, i.e. when cryptic diversity is present or when dealing with phylogeographic structure. Future studies investigating phylogeographic patterns and evolutionary processes underpinning the cryptic diversification of *T. itambere* may further elucidate reasons accounting for these methodological discrepancies.

5. Conclusion

The problem of identifying how many species there are in the world is substantially augmented by the current biodiversity crisis (Costello et al., 2013), and accompanying losses of ecosystem functions (May, 2011). In this study, we applied coalescent methods of species delimitation using a powerful anchored phylogenomics dataset, compared phylogenetic reconstruction methods, and provide indications of the usefulness of such data and analytical approaches for species delimitation hypothesis testing. Whether or not the patterns of morphological variation observed within *T. itambere* corresponds to the species boundaries we propose remains to be investigated. The same is true for assessing the relative roles of geography and ecology in the generation of diversity in our system. Our lizard survey adds to a growing list of recent studies with amphibians (Fouquet et al., 2014; Funk et al., 2012) and birds (Smith et al., 2014b) that suggest that hidden vertebrate diversity is common in Neotropical biomes. Identification of cryptic lineages will allow for eventual species descriptions and is important for formulating conservation strategies in the Cerrado (Silva et al., 2014). At this stage, even though the *Tropidurus* cryptic species are yet to be described, information about the geographically-delimited evolutionary lineages reported here is already available for management purposes and can be promptly used for direct conservation planning (Niemiller et al., 2013; Silva et al., 2014).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2016.12.009.

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