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Out of the deep: Cryptic speciation in a Neotropical gecko (Squamata, Phyllodactylidae) revealed by species delimitation methods

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ABSTRACT

Levels of biodiversity in the Neotropics are largely underestimated despite centuries of research interest in this region. This is particularly true for the Cerrado, the largest Neotropical savanna and a formally recognized biodiversity hotspot. Molecular species delimitation methods have become essential tools to uncover cryptic species and can be notably robust when coupled with morphological information. We present the first evaluation of the monophyly and cryptic speciation of a widespread Cerrado endemic lizard, *Gymnodactylus amarali*, using phylogenetic and species-trees methods, as well as a coalescent-based Bayesian species delimitation method. We tested whether lineages resulting from the analyses of molecular data are morphologically diagnosed by traditional meristic scale characters. We recovered eight deeply divergent molecular clades within *G. amarali*, and two additional ones from seasonally dry tropical forest enclaves between the Cerrado and the Caatinga biomes. Analysis of morphological data statistically corroborated the molecular delimitation for all groups, in a pioneering example of the use of support vector machines to investigate morphological differences in animals. The eight *G. amarali* clades appear monophyletic and endemic to the Cerrado. They display several different properties used by biologists to delineate species and are therefore considered here as candidates for formal taxonomic description. We also present a preliminary account of the biogeographic history of these lineages in the Cerrado, evidence for speciation of sister lineages in the Cerrado–Caatinga contact, and highlight the need for further morphological and genetic studies to assess cryptic diversity in this biodiversity hotspot.

1. Introduction

Biodiversity in the Neotropical region has been a matter of great interest of biologists for centuries (Humboldt, 1849; Rull, 2011; Spix and Martius, 1824). The levels of biodiversity in this region remain relatively unknown (Fouquet et al., 2007, 2014; Scheffers et al., 2012) and a large amount of species is still waiting to be discovered (Costello et al., 2013; Mora et al., 2011; Wheeler et al., 2012). The Brazilian Cerrado is the largest Neotropical savanna (Eiten, 1972; Oliveira and Marquis, 2002) and one of the world's formally recognized biodiversity hotspots (Myers et al., 2000). However, most of its area lacks adequate sampling efforts (Costa et al., 2007, 2010), which makes the discovery of new taxa not uncommon. Considering that only 2.2% of the Cerrado is under legal protection (Klink and Machado, 2005), one of the first steps towards the conservation of this biome is to investigate the taxonomic diversity and phylogenetic relationships of its endemic biota.

In early studies, the Cerrado herpetofauna was considered impoverished compared to surrounding biomes, such as the Caatinga (seasonally dry tropical forests – SDTF) and the Amazon (Vanzolini, 1948, 1976; Vitt, 1991). This paradigm has changed substantially with improved sampling efforts (Colli et al., 2002), and currently 267 squamate species (39% endemics) are recognized to occur in the Cerrado (Nogueira et al., 2011). Moreover, the number of species descriptions is still increasing (e.g. Colli et al., 2009; Giugliano et al., 2013; Nogueira and Rodrigues, 2006; Rodrigues et al., 2007, 2008; Teixeira et al., 2013), as well as the recognition of previously unknown cryptic lineages (Gamble et al., 2012; Giugliano et al., 2013; Prado et al., 2012), mostly in the light of new data from populations previously assigned to the same species.

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Cryptic lineage recognition can be severely impacted by morphological stasis (Pfenninger and Schwenk, 2007) and as such it is not surprising that the majority of recent cryptic species studies rely largely on genetic data (Beheregaray and Caccone, 2007; Bickford et al., 2007). Coalescent-based methods have recently become popular to assist in species delimitation (Carstens et al., 2013; Fujita et al., 2012; Knowles and Carstens, 2007; Yang and Rannala, 2010), especially regarding cryptic speciation in biodiversity hotspots (Nair et al., 2012). Despite the unquestionable value of those methods in assessing cryptic diversity (Leaché and Fujita, 2010), it is advisable to use independent morphological or ecological data to corroborate molecular-based hypotheses of cryptic diversification (Bauer et al., 2011; Burbrink et al., 2011; Sistrom et al., 2012). In this context, morphological data can be used to test the placement of individuals within the reconstructed molecular clades and evaluate the validity of such lineages (Hebert et al., 2004; Sistrom et al., 2013; Tan et al., 2010). This integrative approach can provide valuable support when delimiting 'candidate species' for conservation management strategies (Morando et al., 2003; Padial et al., 2010).

The gecko *Gymnodactylus amarali* Barbour, 1925 is a Cerrado endemic with a wide distribution in the central and northern portions of the biome (Vanzolini, 2005). It was synonymized with *Gymnodactylus geckoides* Spix, 1825 by Vanzolini (1953), but later resurrected and restricted to the 'Alto Parnaíba' region (close to the type locality of Barbour, Vanzolini, 2005). A new species, *Gymnodactylus carvalhoi* Vanzolini, 2005, was described as the widespread form in the Cerrado (Vanzolini, 2005), but soon synonymized with *G. amarali* after Cassimiro and Rodrigues (2009) rechecked the type specimen and found that the diagnostic characters were highly variable within all *Gymnodactylus* sampled in the Cerrado. Thus, five species of *Gymnodactylus* are currently recognized, all within the Brazilian territory: *G. amarali,* endemic to the Cerrado; *Gymnodactylus darwinii* (Gray, 1845), endemic to the Atlantic Rainforest; *G. geckoides*, endemic to the Caatinga; and two other species restricted to the Espinhaço mountain range, known only from the surroundings of their type localities: *Gymnodactylus guttulatus* Vanzolini, 1982, in the southernmost segment of the Espinhaço, and *Gymnodactylus vanzolinii* Cassimiro and Rodrigues, 2009, in the northern portion. Nevertheless, only one study evaluated phylogenetic relationships within *Gymnodactylus*, addressing the phylogeography and cryptic speciation of *G. darwinii* (Pellegrino et al., 2005). The *G. darwinii* species group is monophyletic in relation to at least *G. geckoides* from Caatinga (Pellegrino et al., 2005), and *G. vanzolinii* appears to be more closely related to *G. guttulatus* (Cassimiro and Rodrigues, 2009). Apart from these two assertions, no other evolutionary relationships among *Gymnodactylus* species are known.

The evolution of groups sharing a Caatinga–Cerrado distribution remains a poorly understood subject in South American biogeography (Werneck, 2011) and few studies have implemented molecular techniques to investigate the relationship between those biomes (Almeida et al., 2007; Faria et al., 2013; Moraes et al., 2009; Recoder et al., 2014; Werneck et al., 2012). Dissimilarities noted between *G. amarali* and *G. geckoides* include ecological differences such as clutch and egg sizes (Colli et al., 2003), karyological differences in chromosome number and type (Pellegrino et al., 2009), and morphological differences in pholidosis and coloration patterns (Cassimiro and Rodrigues, 2009; Vanzolini, 1953, 2005). Nonetheless, it remains unclear whether the widespread Cerrado populations of *G. amarali* form a monophyletic group in relation to its Caatinga counterpart. In fact, it was proposed that relict populations of *G. geckoides* might be present in the core of the Cerrado region (Pellegrino et al., 2009), because the karyotype of one specimen was identical to the karyotype observed in *G. geckoides* populations. In addition, extensive chromosomal polymorphism has been observed within and between *G. amarali* populations (Pellegrino et al., 2009), as well as great morphological variation among populations (Cassimiro and Rodrigues, 2009; Vanzolini, 1953, 2005). As such, it is possible that populations treated under the name *G. amarali* are paraphyletic in relation to *G. geckoides* and, moreover, that differences among *G. amarali* populations reflect the existence of cryptic species. Thus, in order to investigate the evolution of *G. amarali* in the Cerrado it is also important to assess its contact zone with *G. geckoides*, accounting for the shared evolutionary history of the two biomes.

Here we investigate the potential cryptic diversity within *Gymnodactylus amarali* by implementing a framework that includes molecular and morphological data, phylogenetic and 'species tree' methods, and coalescent-based Bayesian species delimitation approaches. Our hypotheses are that (1) *G. amarali* in the Cerrado is a monophyletic group; (2) there are relict *Gymnodactylus geckoides* populations inside the Cerrado biome, and (3) several cryptic species exist within *G. amarali*. Evolutionary relationships are reconstructed using samples spanning the entire distribution of *G. amarali* in the Cerrado, *Gymnodactylus* populations from SDTF enclaves in the contact zone between Cerrado and Caatinga, and populations of *G. geckoides* as outgroups. This framework enabled testing for an important contact zone between two understudied biomes and allowed us to conduct the first assessment of how molecular diagnosis predicts morphology-based cryptic divergence in a vertebrate endemic to the Cerrado.

2. Material and methods

2.1. Taxon sampling

We obtained samples of *Gymnodactylus amarali* from 24 sites in the Cerrado, as well as two populations inhabiting SDTF enclaves (Fig. 1). Three New World gecko species (Phyllodactylidae) were used as outgroups: *Gymnodactylus darwinii*, *Gymnodactylus geckoides* and *Phyllopezus pollicaris* (Spix, 1825). Specimens were curated at the 'Coleção Herpetológica da Universidade de Brasília' (CHUNB) and 'Museu de Zoologia da Universidade de São Paulo' (MZUSP). Our final dataset consisted of 83 *G. amarali*, 4 *G. geckoides*, 4 *G. darwinii* and 3 *P. pollicaris*. Voucher numbers, localities, geographical information and GenBank accession numbers are in Supplementary Table 1.

2.2. Molecular methods and analyses

We extracted genomic DNA using a modified salting-out technique (Sunnucks and Hales, 1996) and used PCR to amplify fragments of the mitochondrial DNA (mtDNA) cytochrome b (cytb) and the exonic locus Kinesin Family Member 24 (KIF24). Primers and PCR cycle protocols are in Supplementary Table 2. The PCR products were visualized on a 1.5% agarose gel and sequenced using Big Dye v3.1 on an ABI 3130xl at the Flinders Sequencing Facility, SA Pathology. We assembled and visually inspected chromatograms using SEQUENCHER 4.9 (Gene Codes Corporation, Ann Arbor, MI USA). Sequences were codon aligned using MUSCLE (Edgar, 2004) as implemented in MEGA 5.2.2 (Tamura et al., 2011) applying a gap open penalty of 3 and a gap extension penalty of 1. Prior to analyses, we tested for third codon saturation using Xia et al. (2003) index of substitution saturation as implemented in DAMBE5 (Xia, 2013). The index suggested that saturation was negligible (cytb: Iss 0.165 < Issc 0.810, $p < 0.001$; KIF24: Iss 0.36 < Issc 0.792, $p < 0.001$) and we proceeded with analyses using the complete alignments.

Our molecular hypothesis-testing framework aimed to concomitantly test the monophyly of *G. amarali* and identify possible

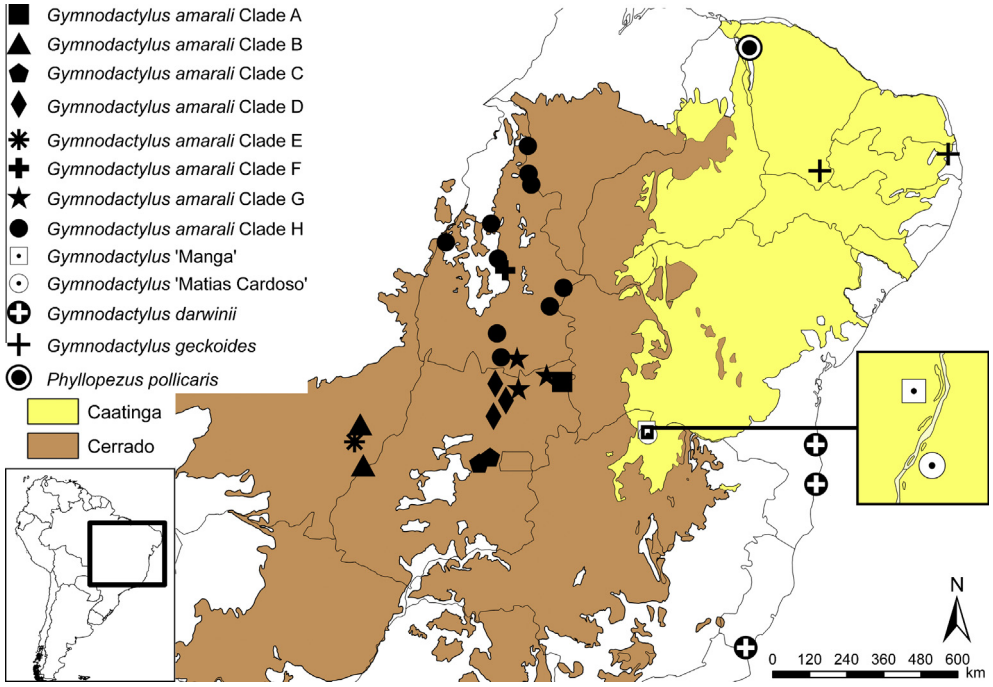

Fig. 1. Partial map of Brazil with *Gymnodactylus* and outgroup samples, in the context of the distribution of the Cerrado and Caatinga biomes. Symbols indicate clades selected by the GMYC analysis. Inset map detail show the populations of 'Manga' and 'Matias Cardoso' separated by the São Francisco River.

cryptic lineages within the species, based on the following approach: (1) reconstructing phylogenetic trees with the concatenated dataset using two methods; (2) building a 'species tree' that incorporates individual gene genealogies using a coalescent method; and (3) test the fit of the data from both genes to different evolutionary hypotheses generated by the previous analyses via a Bayesian coalescent species delimitation method.

2.2.1. Phylogenetic reconstructions

All downstream phylogenetic analyses used the partition strategies and models of sequence evolution selected based on the Bayesian Information Criterion (BIC) in PartitionFinder (Lanfear et al., 2012, 2014). Partition strategies and evolution models were separately estimated for the concatenated and individual locus alignments, and for each phylogenetic software used (MrBayes, RAxML or Beast). Selected evolution models and partitions are in Supplementary Table 3.

We used Bayesian inference implemented in MrBayes v3.2.2 (Ronquist et al., 2012), to investigate phylogenetic relationships with the concatenated dataset and separately for each gene fragment. We conducted two independent runs using four parallel Markov Chain Monte Carlo (MCMC) chains for 5 million generations, sampling every 500th generation. Substitution rates, character state frequencies, gamma shape parameters and proportion of invariable sites were all unlinked. We used a minimum acceptable effective sample size (ESS) of 200 for each parameter, and assessed stationarity and convergence of Bayesian analysis respectively by plotting MCMC generations versus the log-likelihood values of the data and checking the potential scale reduction factor in MrBayes. Stationarity and convergence were also visually inspected by plotting likelihood values in Tracer v1.5 (Rambaut and Drummond, 2009). *Phyllopezus pollicaris* was used as outgroup.

We also implemented a maximum likelihood (ML) phylogenetic analysis on the concatenated dataset using RAxML (Stamatakis, 2014), with unlinked partitions as selected by PartitionFinder. We used 1000 bootstrap replicates in a rapid bootstrap analysis, and a thorough search for the best-scoring ML tree.

2.2.2. Species discovery methods and species tree

We explored the performance of two coalescent species discovery methods (sensu Carstens et al., 2013). First, we used spedeSTEM discovery (Satler et al., 2013), a method that uses information theory to compare models of lineage composition through Akaike Information Criterion (AIC) and returns the ranked 'species tree' models. The spedeSTEM software takes as input gene trees that we separately estimated for both genes using RAxML (as above), and converted to ultrametric trees using package *APE* (Paradis et al., 2004) in R v3.0.1 (R Core Team, 2013). It also requires a $\theta = 4N_e\mu$ value that we estimated with Migrate-n v3.6.1 (Beerli and Felsenstein, 2001). We ran Migrate-n using a random starting tree and four multiple Markov chains for 1×10^7 generations sampled every 20th generation, discarding 10% as burn-in. Second, we used the Generalized Mixed Yule Coalescent (GMYC), a method especially developed for only one mitochondrial locus (Pons et al., 2006). Using unique haplotypes of cytb (Supplementary Table 1) we built an ultrametric phylogenetic tree in BEAST v1.7.5 (Drummond et al., 2012) required to run the GMYC algorithm. This algorithm estimates the number of "species" by classifying the branching rates of a phylogram as being the result of interspecific or intraspecific lineage branching patterns (Pons et al., 2006).

We implemented two versions of the GMYC algorithm: the originally proposed ML-based calculation in package *splits* (Fujisawa and Barraclough, 2013; Pons et al., 2006), and a Bayesian implementation that accounts for uncertainty in phylogenetic estimation in package *bGMYC* (Reid and Carstens, 2012), using R v3.0.1 (R Core Team, 2013). In BEAST, we ran phylogenetic analysis under a strict molecular clock set to an evolutionary rate of 1.0 (i.e., no attempt to estimate divergence time) considering a coalescent tree with constant population size, using an UPGMA starting tree, and with 1×10^7 Markov Chain Monte Carlo (MCMC) generations sampled every 1,000th generation. We implemented three independent runs and combined results using LogCombiner v1.7.5 (Drummond et al., 2012), burning the first 10% of the samples and subsequently used Tracer v1.5 (Rambaut and Drummond,

2009) to check for minimum adequate ESS (200) and visually inspect stationarity and convergence by plotting likelihood values. We summarized the resulting trees into a target maximum clade credibility tree to use in the ML implementation, and alternatively kept 100 random trees for the Bayesian implementation, using TreeAnnotator v1.7.5 (Drummond et al., 2012). For the ML-GMYC we also performed a log-likelihood ratio test of the fitted model against a null model of no distinct species clusters, and calculated AIC-based support values for the species clusters with a $p < 0.05$ (Fujisawa and Barraclough, 2013).

To investigate the phylogenetic relationship between the species retrieved by the GMYC analyses in a multilocus perspective, and also estimate divergence times between the putative species, we ran a *Beast analysis (Heled and Drummond, 2010) as implemented in BEAST v.1.7.5. The lack of *Gymnodactylus* fossils prevented a more robust calibration, and we estimated divergence times based in the putative substitution rate of 2% changes million/year (Johns and Avise, 1998). We used the evolutionary models selected for each locus under a relaxed lognormal molecular clock set for cytb, and the KIF24 evolution rate dependent on cytb. We selected a Yule process prior for the tree using an UPGMA starting tree and performed the analysis with 5×10^7 MCMC generations sampled every 1,000th generation. We implemented three independent runs and combined results using LogCombiner v1.7.5 (Drummond et al., 2012), burning the first 10% of the samples. We summarized resulting trees into a target tree using TreeAnnotator v1.7.5 (Drummond et al., 2012), and used Tracer v1.5 (Rambaut and Drummond, 2009) to check for minimum adequate ESS (200) and visually inspect stationarity and convergence.

We also calculated cytb and KIF24 net between-group distances using lineages selected by the GMYC analysis with MEGA 5.2.2 (Tamura et al., 2011). We computed both uncorrected *p*-distances and ML corrected distances with standard error estimates calculated using 1,000 bootstrap replicates.

2.2.3. Bayesian coalescent species delimitation

We used a coalescent approach implemented in the software Bayesian Phylogenetics and Phylogeography (BPP) (Yang and Rannala, 2010) to test the performance of different 'species trees' by assessing their posterior probabilities considering both loci. This method accommodates the species phylogeny, as well as lineage sorting due to ancestral polymorphism, by comparing the posterior probability of an *a priori* user-specified phylogenetic ('species') tree with the posterior probability of all possible variations of the same tree when branches of a particular node are collapsed (Yang and Rannala, 2010). After initial trials testing different parameters (Supplementary Material), we used a gamma prior of G(2,1000) for population size (θs) and the age of the root in the species tree (τ_0), and the Dirichlet prior (Yang and Rannala, 2010: Equation 2) for other divergence time parameters. We ran analyses for 5×10^5 MCMC generations, taking samples every five and using 1×10^4 burn-in generations. To check for consistency of results, we conducted three independent runs, starting at two random tree models, and the fully resolved tree model, using both available reversible-jump MCMC species delimitation algorithms (Yang and Rannala, 2010). We repeated this process for three different 'species trees': (1) the one generated by spedeSTEM, and two considering the GMYC groups – (2) with the *Beast topology and, (3) the tree topology generated by the concatenated dataset (ML and Bayesian analysis).

2.3. Morphological analyses

We performed analyses to evaluate whether divergence patterns based on morphology were concordant with the retrieved molecular lineages. From the 94 samples used in the molecular analyses we had access to 81 preserved museum specimens. Because we did not have access to the same *Gymnodactylus darwinii* specimens, we generated data from other three available specimens (Supplementary Table 1). Thus, our total morphological dataset comprised 84 specimens from all the cryptic and described species. With the aid of a stereomicroscope, a single person (FMCBD) processed all specimens and generated the data to avoid bias. Morphological characters were selected in order to maximize variation among *G. amarali* morphotypes. The data consisted of 21 pholidosis variables and 8 categorical variables (see Appendix A for a detailed description of characters). From a total of 2,436 observations (29 characters of 84 specimens), 255 (10.5%) were missing values because of damaged specimens. In multivariate approaches, a missing value usually means that the whole case should be omitted, resulting in loss of information (Rubin, 2003) and biased evolutionary estimations (Nakagawa and Freckleton, 2008). To avoid such problems, we imputed missing values through chained equations using a predictive mean matching algorithm implemented in R package *mice* (Buuren and Groothuis-Oudshoorn, 2011). All morphological analyses were carried out in R v3.0.1 (R Core Team, 2013).

Because specimen-lineage affiliation retrieved by the GMYC and all phylogenetic analyses were exactly the same (see Section 3.2), we could assign each individual to a unique 'species' in the following analyses. In a multivariate space, to statistically classify and predict cases belonging to different groups, one would generally employ a Discriminant Function Analysis (DFA) (Quinn and Keough, 2002). However, the DFA linear analytical process assumes normality, no collinearity, and homoscedasticity; in addition, it cannot be applied when the number of cases is smaller than the number of variables (Quinn and Keough, 2002). Meristic characters are known not to be normally distributed (Houle, 1992), and for some clades we had a maximum number of two individuals. To overcome those limitations, we employed a Support Vector Machine (SVM), which is a sophisticated model-training approach for classifying and predicting sample-affiliation based on learning theory (Schölkopf et al., 2000). The SVM builds a kernel function that maps cases into a high-dimensional space, subsequently finding a "margin" in the hyperspace that maximizes the separation between the groups (Cortes and Vapnik, 1995). Although successfully used in computational biology (Ben-Hur et al., 2008), some areas of molecular biology (Park and Kanehisa, 2003; Xue et al., 2005), and ecological distribution modeling (Giovanelli et al., 2010; Kelly et al., 2007), to the best of our knowledge, this is the first time that SVM is used to investigate morphological segregation in animals.

We performed the SVM analysis using R package *e1071* (Meyer et al., 2014). Initially, we implemented a manual search for the best fine tune parameters for the model, i.e. the ones that minimized the error-rate estimated via cross-validation (Chang and Lin, 2011). We then trained the model using the fine tuned *C*-classification SVM algorithm on the whole morphological dataset, setting 'species' to be explained by the 29 morphological characters. Lastly, we tested the predictive power of the generated model using the default *predict.svm* function of the package, which predicts case affiliation to groups (individual to 'species') based in the model trained by the SVM. More details about the SVM analysis and implementation are in the Supplementary Material.

3. Results

3.1. Taxon sampling and molecular data

We sequenced both fragments for all *Gymnodactylus amarali* ($n = 83$) and *G. geckoides* (4) specimens, and downloaded GenBank

sequences for *Phyllopezus pollicaris* (3). We did not have access to *G. darwinii* tissue samples and available cytb sequences were obtained from GenBank ($n = 4$). The aligned cytb fragment was 749 bp long from which 369 were variable sites, and KIF24 was 486 bp with 123 variable sites (i.e. 1,235 aligned base pairs in the concatenated dataset). A few contiguous deletions comprising different numbers of codons were found in KIF24: *P. pollicaris* presented two gaps, one with two codons and another with three codons; the two *Gymnodactylus* populations from SDTF enclaves ('Manga' and 'Matias Cardoso') presented different non-shared patterns of deletions, where 'Matias Cardoso' had two gaps of two codons each, and 'Manga' had only one gap of four codons in another position, the latter shared by *G. geckoides*. All specimens of *G. amarali* presented no deletions.

3.2. Monophyly of Gymnodactylus amarali and cryptic species recognition in the G. amarali species group.

All phylogenetic analyses (using both the concatenated dataset and the two genes separately) supported the monophyly of *Gymnodactylus amarali* from the Cerrado region, excluding the two populations from SDTF enclaves ('Manga' and 'Matias Cardoso') (Fig. 2, Supplementary Fig. 1). Bayesian and ML phylogenetic analyses of the concatenated dataset returned the same topology (Fig. 2).

The ML-GMYC analysis returned 14 ML entities ('species'), including outgroups, with a confidence interval from three to 36. The log-likelihood ratio test was significant ($p = 0.037$), i.e. the null hypothesis of a single species was rejected. Most 'species' nodes had $p < 0.05$ in the AIC based support value of the ML-GMYC analysis, and high posterior probability in the bGMYC (Fig. 3). The 14 entities were: *Phyllopezus pollicaris*, two *Gymnodactylus darwinii* clusters, 'Matias Cardoso', *G. geckoides*, 'Manga', and eight clusters for *G. amarali*, which were named A to H (Fig. 3).

'Matias Cardoso' and 'Manga' were very divergent from the *Gymnodactylus amarali* clusters: cytb uncorrected distances ranged from 19.1% to 21.4% and ML corrected distances from 27.4% to 32.1% for the former and 18.3% to 21.5% and from 25.7% to 32.8%, respectively, for the latter. Cytb levels of uncorrected sequence divergence were lower among *G. amarali* clusters, ranging from 1.8% to 17.5% and ML corrected distances from 2.1% to 23.7% (Table 1). Levels of divergence were lower for KIF24: uncorrected and ML corrected distances respectively ranged from 5.8% to 8.0% and from 6.7% to 9.1% for 'Matias Cardoso', from 5.9% to 8.0% and from 6.7% to 9.1% for 'Manga', and from 0.2% to 4.2% and from 0.2% to 4.7% among *G. amarali* clusters. Interestingly, 'Manga' is more related to *G. geckoides* of the Caatinga, and 'Matias Cardoso' to *G. darwinii* of the Atlantic Rainforest (Figs. 2–4 and Supplementary Material).

The *Beast 'species tree' also supports the monophyly of *Gymnodactylus amarali* with 'Matias Cardoso' and 'Manga' nested outside the *G. amarali* clade (Fig. 4). The position of some *G. amarali* clades differed between the *Beast and the concatenated dataset phylogenetic tree (Table 2) because clades that shared specimens from geographically close localities were recovered as sister species (B and E, F and H; Fig. 1). Divergence time between the most basal clade of *G. amarali* and remaining clades was ~5 million years (MY) ago, while most other clades diverged within the last 2 MY (Fig. 4). Support was high (>0.95) for most nodes in the *Beast consensus tree. Considering that our main interest was in the relationships among *Gymnodactylus amarali* clades, although we could have used a *G. darwinii* KIF 24 alignment with nothing but missing data, because we only had two loci and the position of *G. darwinii* as the sister group of '*Gymnodactylus* Matias Cardoso' was unlikely to change, we adopted a conservative approach and omitted *G. darwini* from this analysis.

The spedeSTEM analysis returned 14 groups as the most likely model, but lineage composition was different from previous

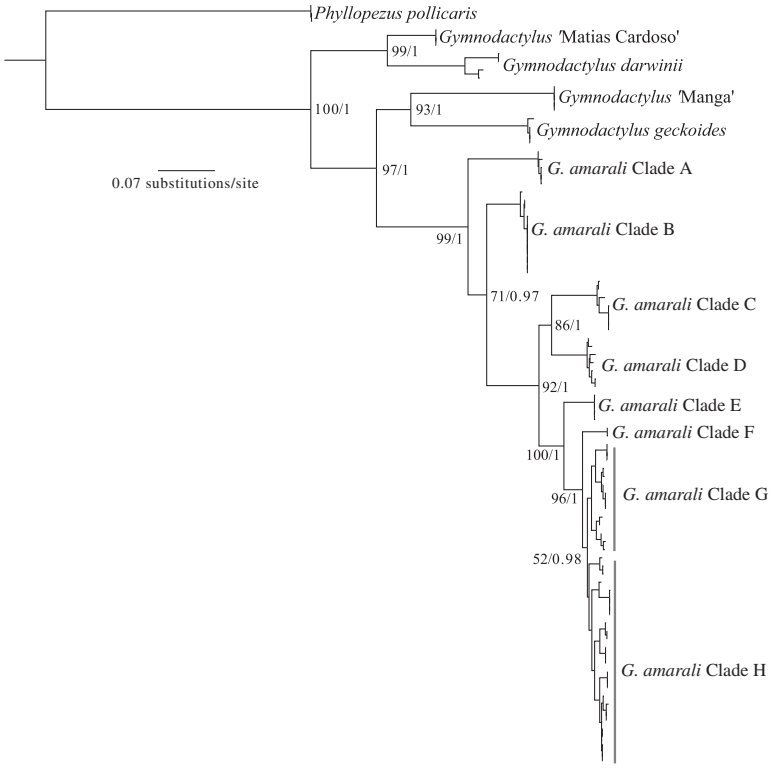

Fig. 2. Maximum likelihood tree of the concatenated dataset for all samples. Bayesian analysis returned the same topology. Numbers in nodes are ML bootstrapping scores/Bayesian posterior probabilities. Clades A to H refer to *Gymnodactylus amarali* clades identified by GMYC analysis.

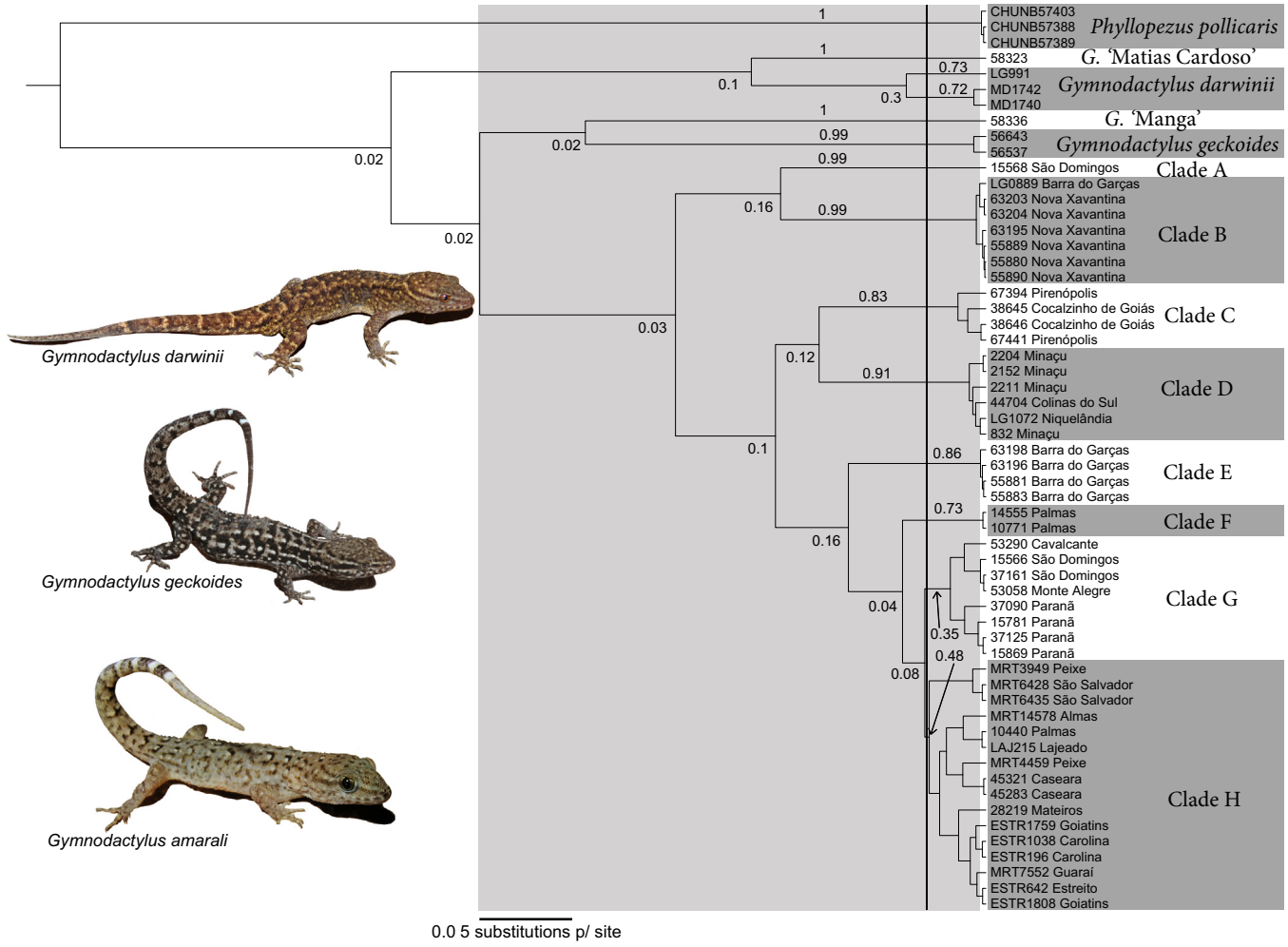

Fig. 3. Ultrametric tree of unique cytb haplotypes. Vertical line represents the limit value for ML species clusters identified by ML-GMYC analysis, and the large gray box represents the confidence interval of species level clusters (3–16). Numbers below nodes are *p* values of the AIC based support of the ML-GMYC analysis. Posterior probability for each species from bGMYC analysis is shown above branches, or indicated by arrows. Species-level clusters are enclosed by boxes, and *Gymnodactylus amarali* 'candidate species' are named clusters A to H. Photos of *G. darwinii* and *G. geckoides* by MTR, and *G. amarali* by Daniel Velho.

analyses. Only three of the eight *G. amarali* groups identified by the GMYC were also retrieved (Supplementary Material). The posterior probability of the full model calculated by BPP was very low, and no other model showed higher probability (Supplementary Table 4). As such, we considered the GMYC results as the best lineage diversification hypothesis (Fig. 3) and proceeded with morphological analyses and discussion without considering the spedeSTEM results.

The other two lineage relationship hypotheses tested using BPP returned slightly different results considering the posterior probabilities of the fully resolved tree model (Table 2). These results support the placement of individuals within the defined GMYC lineages, considering that models with collapsed versions of the tree had very small posterior probabilities. Thus, combining those lineages in the same species would not reflect the best evolutionary hypothesis from a coalescent perspective.

In summary, the results support the monophyly *of Gymnodactylus amarali* distributed within the Cerrado, and point to the existence of eight well-defined clades that could represent different cryptic species. Furthermore, the two populations in the contact zone between Cerrado and Caatinga (SDTF enclaves), 'Matias Cardoso' and 'Manga', belong respectively to *G. darwinii* and *G. geckoides* species groups, and likely represent cryptic lineages of those two groups.

3.3. Morphological support of lineages

The analysis of morphological data corroborated the retrieved evolutionary lineages, with a very low rate of specimens misidentification (3%) returned by the model prediction. Assignment errors were observed only between *G. amarali* clades B, D and H, where one specimen was incorrectly assigned in each group. All other *G. amarali* clades (A, C, E, F and G), 'Matias Cardoso', 'Manga', and the outgroup species were correctly assigned. From all *G. amarali* specimens sequenced for cytb and KIF24, only nine (9) were not available for pholidosis and could not be included in the morphological analysis (Supplementary Table 1). The morphological characters can therefore reliably discriminate the seven *G. amarali* lineages, as well as 'Manga' and 'Matias Cardoso', and can be used to diagnose those clades (Supplementary Table 5).

4. Discussion

Biologists have used morphological data for centuries to describe and infer relationships among species. The advent of molecular tools has drastically changed this activity (Wiens, 2007) and molecular data overcame the use of traditional characters to reconstruct lineage relationships. Not surprisingly,

Table 1
Net among-group distances between GMYC clades 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 parentheses.

Taxa	*G. amarali* Clade A	*G. amarali* Clade B	*G. amarali* Clade C	*G. amarali* Clade D	*G. amarali* Clade E	*G. amarali* Clade F	*G. amarali* Clade G
G. amarali Clade A	–	0.158 (0.019)	0.237 (0.027)	0.230 (0.026)	0.255 (0.028)	0.233 (0.026)	0.211 (0.024)
G. amarali Clade B	0.122 (0.011)	–	0.220 (0.026)	0.174 (0.021)	0.203 (0.023)	0.192 (0.022)	0.178 (0.022)
G. amarali Clade C	0.163 (0.012)	0.153 (0.013)	–	0.131 (0.017)	0.162 (0.020)	0.157 (0.019)	0.141 (0.018)
G. amarali Clade D	0.161 (0.012)	0.131 (0.012)	0.102 (0.010)	–	0.154 (0.018)	0.150 (0.019)	0.127 (0.016)
G. amarali Clade E	0.175 (0.013)	0.149 (0.013)	0.122 (0.011)	0.119 (0.011)	–	0.122 (0.015)	0.092 (0.012)
G. amarali Clade F	0.166 (0.013)	0.142 (0.012)	0.119 (0.011)	0.115 (0.011)	0.097 (0.010)	–	0.054 (0.010)
G. amarali Clade G	0.150 (0.012)	0.131 (0.011)	0.106 (0.010)	0.099 (0.010)	0.075 (0.009)	0.047 (0.007)	–
G. amarali Clade H	0.144 (0.012)	0.133 (0.011)	0.103 (0.010)	0.100 (0.010)	0.079 (0.009)	0.044 (0.006)	0.018 (0.003)
Manga	0.211 (0.014)	0.188 (0.013)	0.215 (0.014)	0.183 (0.014)	0.215 (0.013)	0.211 (0.014)	0.189 (0.013)
Matias Cardoso	0.194 (0.014)	0.204 (0.014)	0.212 (0.014)	0.202 (0.015)	0.212 (0.015)	0.214 (0.015)	0.191 (0.014)
G. geckoides	0.195 (0.012)	0.183 (0.013)	0.187 (0.013)	0.173 (0.012)	0.199 (0.013)	0.189 (0.013)	0.182 (0.013)
G. darwinii A	0.212 (0.015)	0.208 (0.015)	0.222 (0.015)	0.200 (0.014)	0.223 (0.014)	0.214 (0.015)	0.183 (0.014)
G. darwinii B	0.211 (0.014)	0.204 (0.014)	0.226 (0.014)	0.197 (0.014)	0.222 (0.014)	0.203 (0.014)	0.185 (0.014)
Phyllopezus pollicaris	0.261 (0.015)	0.253 (0.015)	0.239 (0.014)	0.243 (0.015)	0.265 (0.015)	0.256 (0.015)	0.246 (0.015)

Taxa	*G. amarali* Clade H	Manga	Matias Cardoso	*G. geckoides*	*G. darwinii* A	*G. darwinii* B	*Phyllopezus pollicaris*
G. amarali Clade A	0.203 (0.023)	0.310 (0.030)	0.274 (0.029)	0.280 (0.028)	0.315 (0.032)	0.312 (0.033)	0.417 (0.041)
G. amarali Clade B	0.187 (0.022)	0.263 (0.026)	0.299 (0.030)	0.257 (0.026)	0.308 (0.030)	0.294 (0.029)	0.397 (0.040)
G. amarali Clade C	0.139 (0.018)	0.328 (0.032)	0.321 (0.033)	0.270 (0.027)	0.351 (0.037)	0.358 (0.038)	0.369 (0.036)
G. amarali Clade D	0.132 (0.016)	0.257 (0.027)	0.307 (0.034)	0.246 (0.026)	0.300 (0.032)	0.291 (0.031)	0.381 (0.040)
G. amarali Clade E	0.099 (0.013)	0.319 (0.029)	0.312 (0.031)	0.290 (0.029)	0.344 (0.033)	0.335 (0.032)	0.429 (0.041)
G. amarali Clade F	0.050 (0.009)	0.310 (0.030)	0.319 (0.032)	0.272 (0.028)	0.323 (0.033)	0.293 (0.030)	0.403 (0.040)
G. amarali Clade G	0.021 (0.004)	0.269 (0.026)	0.274 (0.028)	0.264 (0.027)	0.265 (0.027)	0.265 (0.027)	0.389 (0.039)
G. amarali Clade H	–	0.281 (0.027)	0.286 (0.029)	0.266 (0.027)	0.285 (0.030)	0.282 (0.029)	0.417 (0.042)
Manga	0.192 (0.013)	–	0.275 (0.028)	0.248 (0.026)	0.278 (0.028)	0.284 (0.029)	0.430 (0.043)
Matias Cardoso	0.192 (0.014)	0.194 (0.014)	–	0.262 (0.026)	0.163 (0.018)	0.172 (0.020)	0.368 (0.035)
G. geckoides	0.180 (0.012)	0.178 (0.013)	0.186 (0.013)	–	0.273 (0.027)	0.270 (0.027)	0.408 (0.041)
G. darwinii A	0.189 (0.014)	0.195 (0.014)	0.126 (0.012)	0.190 (0.014)	–	0.061 (0.009)	0.393 (0.038)
G. darwinii B	0.190 (0.013)	0.199 (0.014)	0.132 (0.012)	0.190 (0.013)	0.055 (0.008)	–	0.403 (0.038)
Phyllopezus pollicaris	0.252 (0.015)	0.266 (0.016)	0.241 (0.016)	0.254 (0.015)	0.248 (0.015)	0.255 (0.015)	–

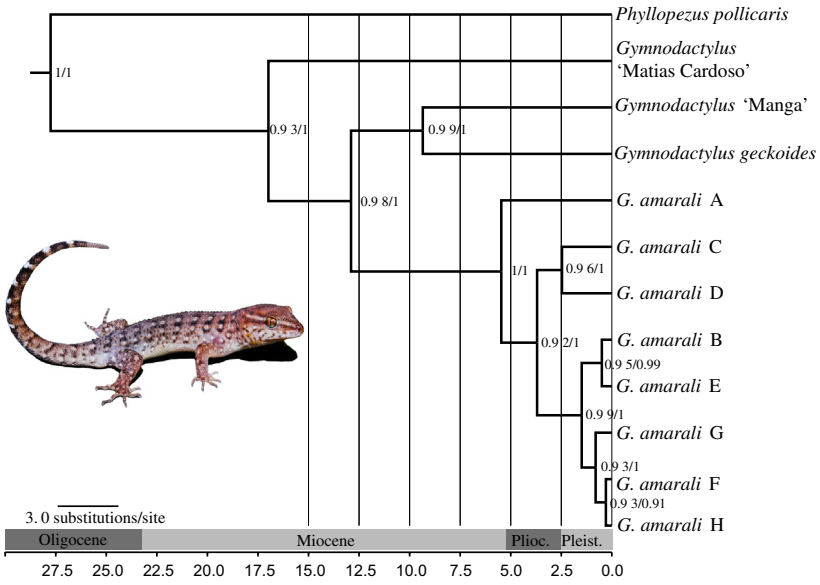

Fig. 4. Phylogenetic relationships and divergence times between *Gymnodactylus* clades estimated using a Bayesian 'species tree' coalescent analysis with *Beast. Numbers inside nodes indicates Bayesian posterior probabilities/posterior probabilities of the species splits estimated by BPP. Clades A to H refer to *Gymnodactylus amarali* clades identified by GMYC analysis. Photo of *G. amarali* by Daniel Velho.

the use of molecular tools also became the major approach to recognize cryptic species (Bickford et al., 2007). On the other hand, morphological data are still essential for species description and play an important role to make uncovered cryptic lineages identifiable and available for scientific and conservation purposes (Beheregaray and Caccone, 2007).

Under the Generalized Lineage Concept (De Queiroz, 2007) we presented species hypotheses using two lines of evidence, genetic and morphological, and provided the first example of cryptic species identified by coalescent lineage delimitation analyses in a Cerrado endemic vertebrate. Moreover, no 'species' are shared between the Cerrado and the Caatinga, reinforcing a still

Table 2
Different 'species trees' used in the Bayesian species delimitation analysis (BPP), based on the groups defined by the GMYC analysis. The lowest and highest posterior probability of the model for different BPP runs is shown. Phy = *Phyllopezus pollicaris*, Dar = *Gymnodactylus darwinii*, MaC = 'Matias Cardoso', Gec = *Gymnodactylus geckoides*, Ma = 'Manga', A to H = *G. amarali* clades A to H.

Analysis with concatenated dataset	Resulting species tree	Posterior probabilities of full model
Bayesian and ML	(Phy,((MaC,(DarA,DarB)),((Ma,Gec),(A,(B,((C,D),(E,(F,(G,H))))))))))	0.963–0.982
*Beast	(Phy,(MaC,((Ma,Gec),(A,((C,D),((E,B),(G,(F,H))))))))	0.891–0.914

understudied evolutionary pattern between these two biomes (Werneck, 2011; Werneck and Colli, 2006).

4.1. Monophyly of *Gymnodactylus amarali* in the Cerrado

Molecular and morphological results corroborate the monophyly of *Gymnodactylus amarali* within the Cerrado region and the existence of multiple cryptic lineages within this taxon. The two populations sampled in the contact zone between Cerrado and Caatinga, SDTF enclaves, do not belong to the *G. amarali* species group but are recovered as sister groups to *G. geckoides* ('Manga') and *G. darwinii* ('Matias Cardoso').

Our samples cover the whole distribution of the species, which suggests that the herein recognized *G. amarali* species complex is the only *Gymnodactylus* lineage to inhabit the Cerrado biome. We found no evidence for the existence of *G. geckoides* populations in the Cerrado as previously suggested by Pellegrino et al. (2009). These authors found one specimen in 'Barra do Garças' (one of our sampled locations in central Cerrado) to have an identical karyotype to *G. geckoides*, and suggested it was a potential relict population of *G. geckoides*. Considering the heterogeneous landscape that characterizes the region of 'Barra do Garças' and that two different clades of *G. amarali* (B and E) inhabit the area, the presence of an additional species remains to be tested. Additional sampling and chromosome data from 'Barra do Garças' as well as a complete phylogeny of the genus are critical to understand geographical patterns of karyotypic evolution in *Gymnodactylus*. Testing phylogenetic hypotheses for the genus would require a multilocus dataset for all five currently described taxa, as well as for different cryptic species recognized for *G. darwinii* (Pellegrino et al., 2005) and *G. amarali* (this study). Nonetheless, our results suggest a (*G. darwinii*, (*G. geckoides*, *G. amarali*)) topology.

4.2. Cryptic species in the *Gymnodactylus amarali* species complex

We uncovered eight cryptic lineages within *Gymnodactylus amarali*. Levels of mtDNA divergence between recovered clades (2.1–23.7%, Table 1) were higher than usually observed between species of lizards or other vertebrate groups (Avise et al., 1998; Fouquet et al., 2007; Oliver et al., 2009) and consistent with those recently reported for cryptic lineages of New World geckos (Gamble et al., 2012; Werneck et al., 2012).

Regarding lineage relationships, the phylogenetic and 'species tree' methods resulted in different placements of some clades (Table 2). This is a common issue comparing 'gene trees' and 'species trees' (Degnan and Rosenberg, 2009; Pamilo and Nei, 1988) and can probably be suppressed by the use of more loci (but see Degnan and Rosenberg, 2006).

Despite this topological disagreement between the results of the two methods, the Bayesian species delimitation analysis performed by BPP resulted in similar posterior probabilities for both hypotheses (Table 2). From a coalescent perspective, these results imply that every option where different clades are collapsed into one would be a worse diversification scenario. A similar result was found for geckos of the *Hemidactylus fasciatus* complex, where BPP also returned very limited differences between different phylogenetic hypotheses (Leaché and Fujita, 2010). Based on a series of simulations and different *a priori* phylogenetic trees, the authors concluded that when divergent populations are placed as sister taxa, large divergences among the species are artificially created, and the algorithm interprets those divergences as speciation events. This suggestion likely reflects the trend of our results, and reinforces the placement of the eight different clades as 'candidate species' in the *G. amarali* complex (Fig. 4, Supplementary Fig. 2). Another simulation study showed that even when only one individual is sampled, the accuracy of BPP using two loci is almost as good as using 10 loci (Camargo et al., 2012). Divergence times and migration rates also did not substantially influence the performance of the algorithm (Camargo et al., 2012), and we believe our results depict a real trend in the evolution of *G. amarali*, in spite of our limited number of loci and the fact that we had as few as 2 individuals for at least one 'species'.

Incomplete lineage sorting (Degnan and Rosenberg, 2009) and gene flow among lineages (Leaché et al., 2014) are also known to affect 'species tree' reconstruction. These two processes would have an effect on the input phylogenetic tree to be used in BPP, interfering with the species delimitation algorithm (Leaché and Fujita, 2010; Yang and Rannala, 2010). On the other hand, concordant reciprocal monophyly between lineages in different gene trees is not essential when delimiting species (Knowles and Carstens, 2007) and the morphological analyses supported the placement of individuals in the clades using a different dataset. This suggests that our hypotheses testing framework was strong enough to support the recovered clades as distinct evolutionary lineages. Finally, given that the multi-species coalescent is more likely to recover a pattern of diversification than gene genealogies (McVay and Carstens, 2013), we suggest that the *Beast topology is a better provisionary arrangement for the *G. amarali* 'candidate species', and discuss the evolution of the group considering this phylogenetic hypothesis below.

4.3. Evolution of *Gymnodactylus amarali* in the Cerrado

This study was not aimed at reconstructing the biogeographic history of *Gymnodactylus amarali* but it has enabled a number of inferences about the evolution of the species in the Cerrado. The fact that *G. amarali* inhabiting the Cerrado form a monophyletic group suggests that they diversified within this biome, likely influenced by landscape evolution of the Cerrado (Prado et al., 2012; Werneck et al., 2012). Moreover, the two populations from SDTF enclaves in the border of Cerrado are clearly distinct lineages, supporting the view that *G. amarali* does not occur in SDTF physiognomies. The transition between the Caatinga and Cerrado is not marked by topographical barriers (Ab'Sáber, 1974, 1998), indicating that environmental filters are probably responsible for the absence of *G. geckoides* from the Cerrado and the absence of *G. amarali* from the Caatinga (Colli et al., 2003).

Traditional hypotheses for the origins of the high Neotropical biodiversity include vegetation refugia created by Pleistocene climatic fluctuations (Vanzolini, 1968; Williams and Vanzolini, 1966), a scenario suggested to account for the diversification of forest animals (Fouquet et al., 2012; Haffer, 1969; Moraes-Barros

et al., 2006) and SDTF endemic *Drosophila* species (Franco and Manfrin, 2013; Moraes et al., 2009). However, recent studies point towards ancient events of lineage diversification for Cerrado vertebrates, dating back to the Neogene (Giugliano et al., 2013; Prado et al., 2012; Werneck et al., 2012). Three main Neogene vicariant events were proposed to influence the diversification of endemic herpetofauna in the Cerrado: the formation of a latitudinal temperature gradient in the early Paleogene, the Miocene marine transgression, and the final uplift of the Central Brazilian Plateau in the Miocene-Pliocene transition (Colli, 2005). The latter event is responsible for the major compartmentalization currently observed in the Cerrado landscape: a mosaic of plateaus separated by valleys excavated by river drainages (Ab'Sáber, 1998; King, 1956). It is possible that an ancestral *G. amarali* lineage was distributed over the landscape before the compartmentalization, which is consistent with our estimated divergence times starting at approximately five MY ago (Fig. 4). This assumption is also corroborated by estimated divergence times for other Cerrado vertebrates (Giugliano et al., 2013; Prado et al., 2012) and Neotropical geckos (Werneck et al., 2012).

Clades A, D and C are distributed in different plateaus and show deep divergences (Figs. 1 and 4). Clade E lizards were collected in a plateau ~630 m above sea level that is only ~50 km apart from the ~300 m valley inhabited by lizards from a sister clade (clade B). These two groups showed a cytb genetic distance of 20% and only 0.02% for the nuclear gene KIF24. Similarly, sister clades F and H, (5% divergent at cytb and 0.08% at KIF24), were also distributed across different elevations (650 m and mostly 150–350 m, respectively; Supplementary Table 1). The above results might reflect ancient events of gene flow during early stages of landscape compartmentalization, a pattern still apparent in the slower evolving nuclear gene (Supplementary Fig. 1). Gene flow estimation using statistical phylogeography are beyond the aims of this study and would be an ideal tool to evaluate such a pattern (Knowles and Maddison, 2002).

4.4. Status of *Gymnodactylus amarali* species group and conservation in the Brazilian Cerrado

Using 'species tree' reconstructions based on molecular data and a Bayesian species delimitation method we identified ten novel clades in the genus *Gymnodactylus* in a pattern concordant with morphology. In addition, the low assignment error (3%) of the SVM analysis shows that these lineages are morphologically distinguishable. We acknowledge that prompt descriptions of identified cryptic species are needed to avoid delays of taxonomic availability (Schlick-Steiner et al., 2007), but assessing morphological diagnostic characters is essential when proposing taxonomic revisions (Bauer et al., 2011). Because species' descriptions can be time consuming and laborious, we argue that the uncovered clades should be referred to as 'candidate species' for conservation delineation and management purposes (Bickford et al., 2007; Whittaker et al., 2005). Moreover, knowledge on the evolutionary relationship between newly discovered lineages can efficiently improve potential conservation initiatives (Diniz-Filho et al., 2013). To our knowledge, only two other studies (Giugliano et al., 2013; Recoder et al., 2014) focused on squamate cryptic species recognition in the Cerrado using both molecular and morphological datasets. We suggest that using both types of data should be a priority in studies on squamate diversity in the Cerrado.

The rate of species description in the Brazilian Cerrado is biased by unequal distribution of sampling efforts across the biome (Diniz-Filho et al., 2005, 2008). Even large-bodied cryptic squamate species were recently described following expeditions to previously unsampled regions (Giugliano et al., 2013; Nogueira and Rodrigues, 2006). Sampling in remote areas is an expensive activity (Costa et al., 2010) and we suggest that funding should be directed towards research projects that combine faunal inventories with collection of data useful for assessing putative cryptic diversification. This is especially important if we seek to understand the evolution of the endemic biota and to inform conservation management strategies.

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Appendix A. Morphological characters of *Gymnodactylus*

For every specimen used in the morphological analyses the following meristic (1–21) and qualitative (22–29) variables were recorded:

1. Number of scales in canthus rostralis, counted from post nasal to the eye
2. Number of scales above and in contact with the supralabials, counted from frontonasal to last supralabial
3. Number of scales below and in contact with the infralabials, counted from mental to last infralabial
4. Number of supralabials (sum of both sides)
5. Number of infralabials (sum of both sides)
6. Number of enlarged supraciliary scales
7. Number of dorsal scales, counted from rostral scale to posterior margin of thigh (before tail)
8. Number of keeled scale rows in tail
9. Number of keeled scales in one row in tail, counted in the third keeled scales row
10. Number of paramedian tubercles, counted from tympanum to posterior margin of thigh (before tail)
11. Number of paramedian ocelli, counted in one row from rostral to posterior margin of thigh (before tail)
12. Number of longitudinal ocelli at midbody
13. Number of longitudinal tubercles rows at midbody
14. Number of longitudinal rows of ventral scales at midbody
15. Number of scales between enlarged post mentals, in contact with mental
16. Number of subdigital lamellae on fourth finger
17. Number of transverse rows of ventral scales, counted from mental to cloaca
18. Number of femoral and tibial ventral scale rows, counted from cloaca (start of thigh) to foot at mid part of the limb
19. Number of subdigital lamellae on fourth toe

(*continued on next page*)

20. Number of granule like scales from cloaca to first enlarged subcaudal
21. Number of white bands in tail
22. Relative size of post nasals in relation to supranasal – (0): both post nasals smaller than supranasal; (1): second post nasal as large as supranasal
23. Contact between supranasals – (0): in full contact; (1): in partial contact, with distal indentation; (2): no contact, with scales in the space between them
24. Alignment between frontonasals division and the incomplete suture of rostral – (0): aligned; (1) not aligned
25. Ear opening shape – (0) circular; (1) sagitally elliptic; (2) dorsally elliptic
26. Ear opening position– (0) aligned with supralabials; (1) aligned with eye
27. Dorsal ocelli – (0): present; (1) absent
28. Ocelli in limbs – (0): present; (1) band pattern (non-round ocelli); (2) absent
29. Bands in tail – (0): present; (1) absent

Appendix B. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2014.07.022.

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