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Diversity of teiid lizards from Gran Chaco and Western Cerrado (Squamata: Teiidae)

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The Gran Chaco dry forest ecoregion corresponds to the southern portion of the South America diagonal belt of open formations, being one of the most threatened subtropical woodland savannas in the world. The area is still poorly known biologically and has been suffering with impressively high forest cover loss in the last 10 years. Integrating morphological and molecular data, we detected and describe a cryptic new species of lizard genus *Ameivula* endemic from the eastern part of this ecoregion, the called Humid Chaco. *Ameivula apipensis* sp nov. is characterised by a whitish brown vertebral stripe in adults and juveniles, a lateral field without ocelli and with overlapping spot, presence of an interfrontoparietal scale in 46.2% of the specimens, 12–17 femoral pores, an hemipenis without lateral sac, five xiphisternal ribs, and by a combination of meristic features as confirmed by discriminant analysis. The new species was recovered sister to a clade from Western Cerrado in our analysis, the first phylogenetic hypothesis for the *Ameivula* and *Glaucomastix* genera based on 1977 base pairs of three mitochondrial (12S, 16S and cyt-*b*) and one nuclear (c-mos) genes, including all the recognised species at the moment. Maximum parsimony and Bayesian inference recovered the monophyly of *Ameivula* and *Glaucomastix* with strong support. Reinforcing previous studies, our results suggest the presence of additional cryptic species in *Ameivula* from the Western Cerrado.

1 | INTRODUCTION

The Gran Chaco is the largest dry forest in South America and the southern component of the continent′s Cis-Andean diagonal biomes of open formations (Caatinga, Cerrado and Chaco) separating the Amazonia and Atlantic Forests. It occupies territories in the North of Argentina, West of Paraguay and South-east of Bolivia and enters Brazil as a narrow strip parallel to the Paraguay river in Mato Grosso do Sul (TNC et al. 2005). Its geographic area covers between 800,000 and 1,000,000 m^2 (Prado, 1993), extending from tropical latitudes (18°S) to subtropical regions (31°S). Climatic and edaphic gradients determine two main assemblages with stable plant compositions: the dry Chaco (D-Ch) and the humid Chaco (H-Ch), separated by the Paraguay-Paraná river in the centre (Spichiger, Bise, Calenge, & Chatelain, 2006; Werneck, 2011). These divisions were corroborated by zoogeographic studies of mammalian (López-González, 2004; Myers, 1982; Willig, Presley, Owen, & López-González, 2000) and avian groups (Short, 1975), where the west (mesic) and eastern (xeric) Chaco have distinct faunal compositions, geology and soil patterns delimited by the river.

One of the most characteristic lizards of this huge area is the genus *Ameivula*, previously known as the *Cnemidophorus*

ocellifer complex, but proposed by Harvey, Ugueto, and Gutberlet (2012) to solve the poliphyletism of *Cnemidophorus*. These species were previously assigned to two morphological groups (Arias, de Carvalho, Rodrigues, & Zaher, 2011a): (i) *Ameivula littoralis* group formed by *A. abaetensis*, *A. littoralis*, *A. venetacauda* and *A. cyanura*, and (ii) the *Ameivula ocellifera* group assembling *A. confusioniba*, *A. cipoensis*, *A. jalapensis*, *A. mumbuca*, *A. xacriaba*, *A. nigrigula*, *A. pyrrhogularis*, *A. abalosi* and *A. ocellifera*. More recently, in a molecular study of Teioidea (Goicoechea et al., 2016), the new genus *Glaucomastix* comprising all species of the former *A. littoralis* group was proposed to eliminate the paraphyletism they recovered for *Ameivula*. Controversially, Tucker et al. (2016), using 316 loci, found that *A. littoralis* and *A. abaetensis* are sister to a clade formed by different populations of the *A. ocellifera* group, with high support (bootstrap = 100). Currently, *Ameivula* has fourteen recognised species (Arias, Teixeira, et al., 2014), distributed throughout the Cis-Andean open areas south of the Amazon River (Arias, de Carvalho, Rodrigues, & Zaher, 2011b; Harvey et al., 2012; Silva & Ávila-Pires, 2013). Like other teiid lizards, they generally occur in open habitats with sandy soils and are active at high temperatures, in very distinct landscape physiognomies (for more detail see table 1 in Arias, Teixeira, et al., 2014), such as Caatinga, Cerrado, Pantanal, Chaco and "restingas."

Throughout the Gran Chaco region, the widespread species is *A. abalosi* Cabrera (2012), distributed from central Argentina to Paraguay, inhabiting both the dry Chaco and Humid Chaco. Along with its distribution, especially from west to east, this species shows variation in the lepidosis and colour pattern (Williams & Tedesco, 1985). The populations from the humid Chaco lack the diagnostic pholidotic characters of *A. abalosi* proposed by Cabrera (2012), neither the colour pattern described for the species. This wide variation leads us to look carefully at the specimens distributed through the humid Chaco to determine its taxonomic status.

Extensive fieldwork in Argentina, Bolivia, Paraguay and Brazil provide us with samples from different localities of dry and humid Chaco, Pantanal, Cerrado and Caatinga. By integrating a large set of external (lepidosis and colour pattern) and internal (osteology and hemipenis), morphological characters with molecular data (mDNA and nDNA sequences), we revealed and confirm cryptic diversity in *Ameivula* lizards from Gran Chaco region and Western Cerrado. Based on our results, we describe a new species of *Ameivula* for the humid Chaco region, characterised by the morphology of hemipenis, colour pattern, osteology, lepidosis and divergence in mtDNA.

Moreover, by combining the molecular data obtained in this study (both mitochondrial DNA and nuclear DNA), with sequences from previous studies on teiids (Giugliano, Nogueira, Valdujo, & Collevatti, 2013; McCranie & Hedges, 2013; Pellegrino, Rodrigues, Yonenaga-Yassuda, & Sites, 2001; Reeder, Cole, & Dessauer, 2002), we present the first hypothesis of relationships that includes all species of *Ameivula* and *Glaucomastix* know to date, to assess the monophyly of these genera and the relationship among their species.

2 | MATERIALS AND METHODS

2.1 | Taxon sampling

Our analyses include morphological and molecular data of all current species of *Ameivula* and *Glaucomastix* from different localities from Argentina, Bolivia, Paraguay and Brazil (for more detailed see Figure S5).

Most specimens were captured with the hand and killed by intraperitoneal injection of a 2% xylocaine solution. Individuals were fixed in 10% formalin and transferred to 70% ethanol for permanent storage. Before fixation, a piece of liver or tail was taken from specimens and preserved in 95% ethanol for posterior molecular analyses. The specimens collected were deposited in the Herpetological Collections of the Universidad Nacional del Nordeste, Corrientes, Argentina (UNNEC), Colección Herpetologica del Museo de Ciencias Naturales-Salta, Argentina (MCN) and Museu de Zoologia da Universidade de São Paulo (MZUSP).

2.2 | Morphological analyses

We analysed 76 specimens of the new species of 15 localities from humid Chaco (H-Ch), both Argentina and Paraguay; 75 specimens of *A. abalosi* of 20 localities from dry Chaco (D-Ch), of Argentina, Bolivia and Paraguay; and 39 specimens of *Ameivula* aff. *ocellifera* of six localities of the West Cerrado, from Mato Grosso do Sul and Mato Grosso. For comparisons, we used morphological data from 10 currently recognised species of *Ameivula* (Appendix S1) taken during this and previous studies (Arias, de Carvalho, Zaher, & Rodrigues, 2014; Arias, Teixeira, et al., 2014). Specimens examined are deposited in the following institutions: Colección Herpetológica de la Fundación Miguel Lillo, Tucumán, Argentina (FML); Colección Herpetológica de la Universidad Nacional del Nordeste, Corrientes, Argentina (UNNEC); Colección Herpetológica Museo de Ciencias Naturales, Salta, Argentina (MCN); Museo Argentino de Ciencias Naturales Bernardino Rivadavia, Buenos Aires, Argentina (MACN); Museu de Zoologia da Universidade de São Paulo, Brazil (MZUSP); Museu Nacional do Rio de Janeiro, Brazil (MNRJ); Coleção Herpetologica Universidade Federal de Mato Grosso (UFMG); Museu Paraense Emilio Goeldi (MPEG); American Museum of Natural History (AMNH); Field Museum of Natural History (FMNH); California State University (CSUN); Carnegie Museum of Natural History (CM).

Scale nomenclature, scales count, measurements and colour patterns used in morphological analyses follow Harvey et al. (2012), Arias, de Carvalho, et al. (2014) and Arias, Teixeira, et al. (2014).

We recorded the following continuous characters: number of supraocular and supraciliary scales on right side (SUP); number of granules of the circumorbitals on right side (SUB); number of supralabials (SL) and infralabials (IL); number of gular scales (GUL), counted along the midline, from postsymphysal scale to mesoptychial scales; number of femoral pores (POR) on both sides; longitudinal rows of ventral scales (LVT); transverse rows of ventral scales, from gular fold to anterior margin of hindlimbs (TVT); scales around midbody (AMB), counted midway between fore- and hindlimbs excluding ventrals; scales around tail (AT), counted on the fourth transverse row; dorsal scales (D), counted along the midline, from occiput to first transverse row of scales around tail; number of dorsal scales between paravertebral stripes (DPV); and subdigital lamellae under fourth finger (FFL) and fourth toe (FTL). Moreover, we recorded the contact between the first supraocular and canthal; first supraocular and second supraocular; first subocular and first supraciliary; postnasal and prefrontal; presence or absence of keel in subdigital lamellae both in the hand or foot; and presence or absence of patch of tibiotarsal spurs in the males. In addition, we took the following morphometric characters using a digital caliper to the nearest 0.02 mm: snout–vent length (SVL); trunk length (TRL) and head length (HL); head width (HW) and head height (HH); humeral length (HUL), forearm length (FAL), tibia length (TL), femur length (FL), foot length (FTL), total hindlimbs length (HLL) and tail length (TAL). Scale observation and measurements were made under a stereomicroscope (10–40×).

Colour pattern included the followings features: presence of vertebral strip, stripe in the temporal region (continuous or discontinuous), ocelli in the lateral flank, spots in the forelimbs and colour in life in males and females.

For the hemipenial morphology comparisons, we prepared the hemipenis of the new species (UNNEC 09753, UNNEC 09766 and UNNEC 11099) and that of *A. abalosi* (MCN 1145, MCN 1144, MCN 1108, MCN 1113 and MCN 1143), following the procedures described by Manzani and Abe (1988), subsequently modified by Pesantes (1994) and Zaher (1999; see also Zaher & Prudente, 2003). Moreover, we registered the hemipenial morphology of specimens deposited in collections with the organs already everted from Western Cerrado and Pantanal (UFMT 1356; UFMT 1352; UFMT 1358). In addition, comparisons were also carried out with the hemipenial morphology of *A. confusioniba*, *A. pyrrhogularis*, *A. venetacauda*, *A. cipoensis* and *A. xacriaba* (Arias, de Carvalho, et al., 2014; Arias, Teixeira, et al., 2014; Silva & Ávila-Pires, 2013).

For osteological comparisons, we cleared and stained skeletons from adult specimens of the new species (UNNEC 9766, FJA 25) and *A. abalosi* (MCN 1107, MCN 1111, MCN 1112, MCN 1116, MCN 1118), following protocols by Wassersug (1976). We extend the comparisons to the skeleton of other species of *Ameivula* prepared by Arias (2012): *A. mumbuca* (MZUSP 94107), *A. xacriaba* (MZUSP 99642), *A. cipoensis* (MRT 19603), *A. nigrigula* (MZUSP 92138) and *A. ocellifera* (MZUSP 26834, MZUSP 6149).

2.3 | Molecular data and laboratory protocols

We gathered tissue samples for 74 specimens of all the nominal species of the *Ameivula* and *Glaucomastix* genera. The specimens studied were sampled from 33 different localities from Argentina, Paraguay and Brazil, including the Caatinga, Atlantic Forest, Cerrado, Pantanal and dry and humid Chaco (Table S1).

Genomic DNA was extracted using Promega DNA extraction kit. Three fragments of the mitochondrial genes 12S, 16S (rRNA) and cytochrome *b* (cyt-*b*) and one fragment of the nuclear gene oocyte maturation factor gen (c-mos) were amplified by standard PCR techniques. These loci have already been sequenced in most teiids, are substantially informative at the species- and genus-taxonomic level and easily amplified with universal primers. Amplifications were conducted with 12Sa, 12Sb, 16SaR and 16Sd primers and the same PCR condition of Reeder (1995). The nuclear gene c-mos was amplified with G73 and G74 primers as described by Saint, Austin, Donnellan, and Hutchinson (1998). Sequencing was performed using ABI Big Dye V3.1 (ABI, Foster City, USA) and resolved on an automated sequencer at IQUSP (São Paulo, Brazil). Sequences were edited and aligned with CodonCode Aligner v.3.5.2. Novel sequences were deposited in GenBank.

We performed a multiple alignment with ClustalX (Thompson, Higgins, & Gibson, 1994), under default settings. This led to a ~410 bp alignment for the 12S gene, ~570 bp for the 16S gene, ~620 bp for cyt-*b* and ~400 bp of the c-mos gene. The final alignment comprised ~1,977 bp.

For all these genes, we generated 1,977 novel DNA sequences from 74 specimens of *Ameivula* and *Glaucomastix*. Furthermore, terminals of other six genera of Teiidae were used as outgroups (*Ameiva*, *Kentropyx*, *Contomastix*, *Cnemidophorus*, *Teius* and *Dicrodon*), being *Tupinambis teguixin*, used to root the analyses.

2.4 | Species concept and species delimitation

The unified species concept proposed by de Queiroz (2007), which synthesises the common conceptual definition present

in all traditional species concepts excluding operational criteria (i.e., species are lineages united through gene flow), is herein followed; the term exclusive lineage is used instead of monophyletic, as the term monophyly is not applicable below the species level (e.g., de Queiroz & Donoghue, 1990). Species delimitation followed the tree-based approach (sensu Baum & Donoghue, 1995; Sites & Marshall, 2004) proposed by Wiens and Penkrot (2002), in which species hypotheses are formulated on the basis of well-supported, geographically consistent exclusive lineages obtained from DNA phylogenies containing at least two individuals of each focal species.

2.5 | Phylogenetic analyses

Based on molecular information (combined 16S+ 12S+ cyt-*b*+ c-mos data), we performed phylogenetic analyses under two different criterions, maximum parsimony (MP) and Bayesian inference (BI). The analyses were also performed for mitochondrial (mtDNA) and nuclear (nDNA) genes separately.

For the phylogenetic analyses with MP, we employed TNT (Tree Analysis using New Technology, Goloboff, Farris, & Nixon, 2003) with search level 50 and requesting 100 independent hits of the best length, submitting the resulting tree to a run of TBR swapping, considering gaps as a fifth state. Support information was performed by generating 50 RAS + TBR per replicate, keeping only one tree, for a total of 1,000 replicates of parsimony jackknife (JN), with 36% of removal probability. Trees were edited with WinClada (Nixon, 2002).

For Bayesian analyses, the substitution model for the mtDNA locus and c-mos codon position was selected with jModeltest v2.1.6 (Posada, 2008) under Akaike's information criterion (AIC), and the best fit models were as follows: GTR+ I+ G (12S, 16S and cyt-*b*) and GTR+ G (c-mos). The analysis performed using the MrBayes v3.2.0 (Ronquist & Huelsenbeck, 2003), consisted of two independent runs, each consisting of 10 million generations, starting with random trees and 10 Markov chains (one cold), sampled every 1,000 generations. The stabilisation of posterior probabilities was checked using Tracer v. 1.5 (Rambaut & Drummond, 2007), with the average standard of split frequencies and ESS (effective sample sizes >550) values being monitored; trees prior to stationary were discarded as burn-in (10%), with a 50% majority rule consensus tree obtained from the remaining data points. Phylogenetic trees were visualised and edited using FigTree v.1.4.2 (Rambaut, 2014).

2.6 | Genetic distance among lineages of *Ameivula*

The genetic distances among *A. abalosi*, *A.* sp nov. (described below) and the lineages of *Ameivula* from West Cerrado (recovered in the phylogenetic analyses) were estimated by pairwise comparisons of the 16S, 12S and cyt-*b* mitochondrial gene using the software Mega v.6.0 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). Additionally, pairwise comparisons were provided among the other species of the *Ameivula*.

2.7 | Statistical analyses

As our main goal was to disclose the diversity of *Ameivula* in the Gran Chaco region; in quantitative analyses, we compared the new species from humid Chaco proposed here (*Ameivula* sp nov. H-Ch), with *A. abalosi* from the dry Chaco and *Ameivula* sp. 1 from Pantanal and Western Cerrado (*Ameivula* sp. 1 W-Ce), which was recovered as sister to the new species in our molecular phylogeny (see Results).

Morphometric measurements were $\log_{10}$-transformed prior to analyses, and normality was tested with Lilliefors tests (Lilliefors, 1967). Data distribution was inspected with box plots and outliers were excluded. Differences in size (SVL) among groups and sexes were tested using two-way ANOVA, and differences in shape were tested with MANOVA and in relative tail length (TAL) using ANCOVA with SVL as covariate. Because of missing data, TAL was compared only between *A. abalosi* and *A.* sp nov. H-Ch. Differences in meristic data were tested with Kruskal–Wallis non-parametric test. Overall variation among individuals in morphology was explored using principal component analysis (PCA) for morphometric and scale counts separately. A discriminant function analysis (DFA) using morphometric and meristic data pooled was used to identify the measurements that best discriminate groups and to assess percentage of correct classification of individuals, for each sex separately. The number of subocular granules (SUBOC) was not normally distributed thus was not used in multivariate analysis. Statistical analyses were performed in R environment (R Development Core Team, 2017) and SPSS 20.0 (IBM Corp. 2011).

3 | RESULTS

3.1 | Molecular data

The concatenated molecular dataset consisted of 1,977 aligned base pairs from three mitochondrial (12S, 16S and cyt-*b*) and one nuclear locus (c-mos) for 122 terminal taxa (Table S1). The aligned 16S sequences consisted of 488 base pairs, with 155 variable sites, the aligned 12S sequences consisted of 461 base pairs, with 242 variable sites, whereas the aligned cyt-*b* sequences consisted of 623 base pairs, with 267 variable sites. The aligned c-mos nuclear sequences consisted of 406 base pairs, with 42 variable sites.

3.2 | Phylogenetic analyses

Both MP and BI analyses of the concatenated dataset recovered similar topologies, which conform with the inferred individual trees for each locus. The tree based on concatenated mitochondrial data (16S, 12S and cyt-*b*) shows high support for all clades, while the nuclear one (c-mos) is unresolved for many nodes. The MP analysis resulted in 20 optimal trees of 2,344 steps (consistency index = 0.425; retention index = 0.75).

In all trees obtained the subfamily Teiinae resulted monophyletic, within this *Glaucomastix* resulted as sister group to *Ameivula* in a strong supported clade (JN = 53%, PP = 93%; Figure 1), which is congruent with previous studies (Giugliano, 2009; Arias 2012; Tucker et al., 2016).

Our data (Figure 1) strongly support the monophyly of *Ameivula* and *Glaucomastix* (JN = 94%, PP = 99%; JN = 76%, PP = 100%, respectively); and in all cases, these genera resulted as sister taxa. *Glaucomastix* consists of two clades, one formed by *G. abaetensis* and the other node including the rest of the species, with *G. cyanura* sister to the clade (*G. littoralis* + *G. venetacauda*). The position of *G. cyanura* is controversial between BI and MP, as in MP analyses, this species was recovered sister to *G. abaetensis*, but weakly supported.

Moreover, all the nominal species of *Ameivula* were recovered with high support, excepting *A. mumbuca* and *A. confusioniba*, which were not monophyletic. *Ameivula cipoensis* was recovered sister to all other *Ameivula* in all analyses, while *A. nigrigula* is sister to all other species of *Ameivula*, but the position of these taxa is not clearly resolved (without JN support; PP = 54%).

The remaining species of *Ameivula* are grouped in two clades: the clade A (JN = 2%, PP = 81%) is split into two nodes, one of them containing specimens from the West Cerrado (Chapada do Guimarães and Barra de Garças, called here as *Ameivula* sp. 1 W-Ce) in a well-supported clade (JN = 85; PP = 100%), the other clade cluster in two groups, the first includes specimens of *A. apipensis* sp nov. (describe here) from the humid Chaco of Argentina and Paraguay with strong support (JN = 99; PP = 100%), whereas the other group is formed by exemplars from the Pantanal + southernmost Mato Grosso (called here as *Ameivula* sp. 2 W-Ce, for more details see Table S1).

The clade B (Figure 1) recovered (JN = 2; PP = 95%) specimens of *A. ocellifera* from Bahia, Sergipe and Pernambuco states sister to all other *Ameivula* which clustered in two clades highly supported. The internal one (node B1) comprises specimens of *A. abalosi* from dry Chaco (D-Ch, from Argentina and Paraguay; JN = 87; PP = 100%), whereas the other (node B2) is formed by species from Eastern Cerrado ([*A. mumbuca*, *A. confusioniba*] [*A. jalapensis*, *A. xacriaba*] [*A. pyrrhogularis*]) and Caatinga specimens from the left bank of São Francisco river. The subclade formed by *A. mumbuca* and *A. confusioniba* is strongly supported (JN = 96; PP = 100%), but the monophyly of these species is not recovered. Moreover, *A. jalapensis* nested with *A. xacriaba* in a well-supported clade (JN = 69%; PP = 100%) sister to *A. pyrrhogularis*.

3.3 | Sequence divergence

Interspecific sequence divergences for cyt-*b* between *Glaucomastix* and *Ameivula* ranged from 13% to 12%. Moreover, divergence varied between 12% and 3% among species of *Ameivula*, 9% and 7% between *A. abalosi* and *Ameivula* sp nov. H-Ch, and 7% and 4% between *Ameivula* sp nov. H-Ch and *Ameivula* sp. 2 W-Ce. For 16S, the distance between *Glaucomastix* and *Ameivula* ranged from 10% to 8%, 6% to 2% among the species of *Ameivula*, and 3% to 2% between *A. abalosi* and *Ameivula* sp nov. H-Ch, and 2% to 1% between *Ameivula* sp nov. H-Ch and *Ameivula* sp. 2 W-Ce. For 12S, the distance between *Glaucomastix* and *Ameivula* ranged from 7% to 5%, 3% to 1% among the species of *Ameivula*, and 1% between *A. abalosi* and *Ameivula* sp nov. H-Ch and 2% to 1% between *Ameivula* sp nov. H-Ch and *Ameivula* sp. 2 W-Ce. Finally, for c-mos, the distance between *Glaucomastix* and *Ameivula* is 2%, while among species of *Ameivula* varied from 3% to 0% and between *A. abalosi*, *Ameivula* sp nov. H-Ch and *Ameivula* sp. 2 W-Ce, there is no genetic distance.

3.4 | Morphological variation

Ameivula abalosi, *Ameivula* sp nov. H-Ch and *Ameivula* sp. 2 W-Ce showed significant differences in size (ANOVA, $F_{2,102} = 11.49$, $p < .01$), but sexes did not differ ($F_{1,102} = 0.64$, $p = .427$) and did not interact with group differences ($F_{2,100} = 0.28$, $p = .759$). In post hoc comparisons, *A. abalosi* is significantly larger than *Ameivula* sp nov. H-Ch and *Ameivula* sp. 2 W-Ce (Tukey's HSD Test, $p < .01$), but *Ameivula* sp nov. H-Ch did not differ significantly from *Ameivula* sp. 2 W-Ce in body size. *Ameivula* sp nov. H-Ch differed in shape (MANOVA, Wilk's Lambda = 0.377, $F_{16,190} = 7.46$, $p < .01$), and sexual dimorphism in shape was significant (Wilk's Lambda = 0.406, $F_{8,95} = 17.38$, $p < .01$). Relative tail length did not differ significantly between *A. abalosi* and *Ameivula* sp nov. H-Ch for males (ANCOVA, $F_{1,16} = 2.82$, $p = .11$) and females (ANCOVA, $F_{1,10} = 0.24$, $p = .88$).

All scale counts differed significantly among *A. abalosi*, *Ameivula* sp nov. H-Ch and *Ameivula* sp. 2 W-Ce (Kruskal–Wallis, $p < .01$).

Quantitative meristic characters show an evident separation among the three groups (*A. abalosi*, *Ameivula* sp nov. H-Ch and *Ameivula* sp. 2 W-Ce), where the number of supraocular

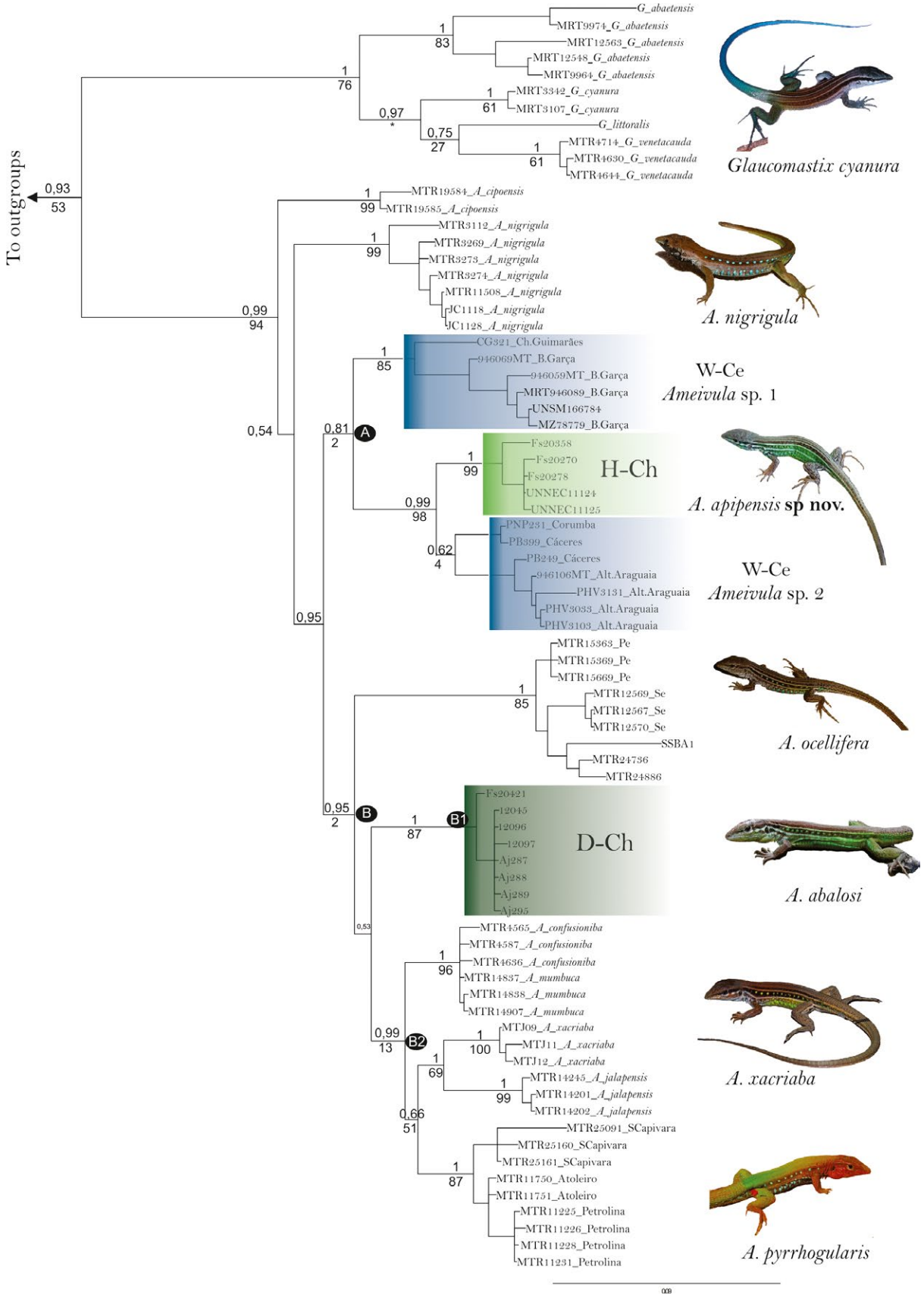

FIGURE 1 Phylogenetic relationships of *Ameivula* and *Glaucomastix* genus, recovered through a Bayesian analysis (BI) on the concatenated dataset on mitochondrial genes. Numbers on branches represent the posterior probability (>0.95), and values below branch are jackknife percentages. * indicate node without support

granules, the scales around midbody and the femoral pores are the variables that present more difference among *A. abalosi*, *Ameivula* sp nov. H-Ch and *Ameivula* sp. 2 W-Ce. Moreover, the four toe and finger lamellae, and gular granules separate *Ameivula* sp nov. H-Ch from *Ameivula* sp. 2 W-Ce.

The two-first principal components of a PCA based on the morphometric data explained 65.9% of variation among individuals. The first principal component (PC1), which accounted by 45.3% of variation, had positive correlation with all characters but highest with SVL, HL and HW, thus, representing an axis of allometric body size (Table 1). The second principal component (PC2), which accounted by 20.6% of variation, had high negative correlation with TRL and high positive correlation with TIB and FAL, thus representing an axis of contrast between body elongation and limb length (Table 1). In the morphological space delimited by the two-first principal components based on morphometric data, it is possible to detect a subtle differentiation among candidate species, especially among *A. abalosi* and the remaining groups, and also within-group variation represented by sexual dimorphism (Figure 2a).

The two-first principal components of a PCA based on scale counts explained 45.9% of variation among individuals. The first principal component (PC1), which accounted by 26.4% of variation, had high negative correlation with SUP and high positive correlation with POR (Table 2). The second principal component (PC2), which accounted by 19.5% of variation, had high positive correlation with GUL, FFL and AMB, and high negative correlation with AT (Table 1). In the morphological space delimited by the two-first principal components based on scale counts, it is possible to observe differentiation among candidate species, especially between *A. abalosi* and *Ameivula* sp nov. on PC1, and a low within-group variation (Figure 2b).

TABLE 1 Results of principal component analyses (PCA) based on morphometric and scale counts data for *Ameivula abalosi*, *Ameivula* sp nov. H-Ch and *Ameivula* sp. 2 W-Ce

	Morphometry			Scale counts	
Variables	**PC1**	**PC2**	**Variables**	**PC1**	**PC2**
SVL	**0.826**	−0.451	SUP	**−0.800**	−0.020
TRL	0.571	**−0.746**	GUL	−0.001	**0.673**
HL	**0.808**	0.142	VT	0.043	−0.326
HW	**0.784**	0.246	POR	**0.750**	−0.273
FAL	0.575	0.320	AMB	−0.587	0.494
FEM	0.536	−0.177	AT	−0.224	**−0.449**
TIB	0.681	**0.561**	D	−0.327	0.475
FOL	0.685	0.232	FFL	0.541	**0.496**
			FTL	0.613	0.455
Eigenvalues	3.825	1.342	Eigenvalues	2.373	1.759
% of variance	45.25	20.58	% of variance	26.37	19.54
Cumulative %	45.25	65.84	Cumulative %	26.37	45.91

The correlation of each variable with the two principal components, eigenvalues and percentage of variance explained for both analyses is presented. Scores with highest correlation with each principal component are highlighted in bold.

The two-first discriminant functions of DFAs performed for candidate species based on pooled morphometric and scale count data explained 100% of group variation for both males and females. For males, the first discriminant function, which accounted by 54.2%, had high positive correlation with POR and high negative correlation with SUP. The second discriminant function, which accounted by 45.8%, had high positive correlation with AT, SUP and FAL, and high negative correlation with GUL (Table 2). The first discriminant function distinguishes *A. abalosi* from *A.* sp. 2 W-Ce, and the second discriminant function distinguishes *Ameivula* sp nov. H-Ch from the remaining groups (Figure 3a). For females, the first discriminant function, which accounted by 83.4%, had high positive correlation with SUP and TIB and high negative correlation with TRL and POR. The second discriminant function, which accounted by 16.6%, had high positive correlation with POR, and high negative correlation with GUL and AMB (Table 2). The first discriminant function (Figure 3a) distinguishes *Ameivula* sp nov. H-Ch from the remaining groups and the second discriminant function distinguishes *A. abalosi* from *A.* sp. 2 W-Ce (Figure 3b). Based on the original scores derived from the discriminant functions, 100% of the individuals were correctly classified in each group for both sexes. After cross-validation using Jackknife resampling, 100% of the individuals were correctly classified in each group, except females of *A.* sp. 2 W-Ce, in which three individuals (23%) were classified as *A.* sp nov. H-Ch.

With respect to the cephalic scalation, 46.2% of the specimens analysed of *Ameivula* sp nov. H-Ch presents a scale located between the frontoparietals (Figure 4b, character 5), called here interfrontoparietal, which is not present in any other species of *Ameivula*. Also, the anterior edge of the first supraocular does not exceed the anterior edge of the frontal, while *A. abalosi* and *A.* sp. 2 W-Ce present the opposite condition (Figure 4b,d, character 4). Finally, in *A. abalosi,* the postnasal and frontoparietal are not in contact, and the lacrimal always contact the first supraciliary, characteristics that allow to easily distinguish it from the other two species, which present opposite conditions (Figure 4a,c, character 3 and 2, respectively). The specimens analysed of *Ameivula* sp. 1 and 2 W-Ce have a large frontoparietal, almost the same size of the frontal scale.

Ameivula sp nov. H-Ch present a colour pattern and coloration that allows to distinguish it from the other species, as it has a whitish vertebral line both in juveniles and adults, and lateral spots (not ocelli) that usually overlapped at the border (more detailed see below, Figure S1 and S4). Furthermore, it has a green coloration in the lateral flank and yellow in the

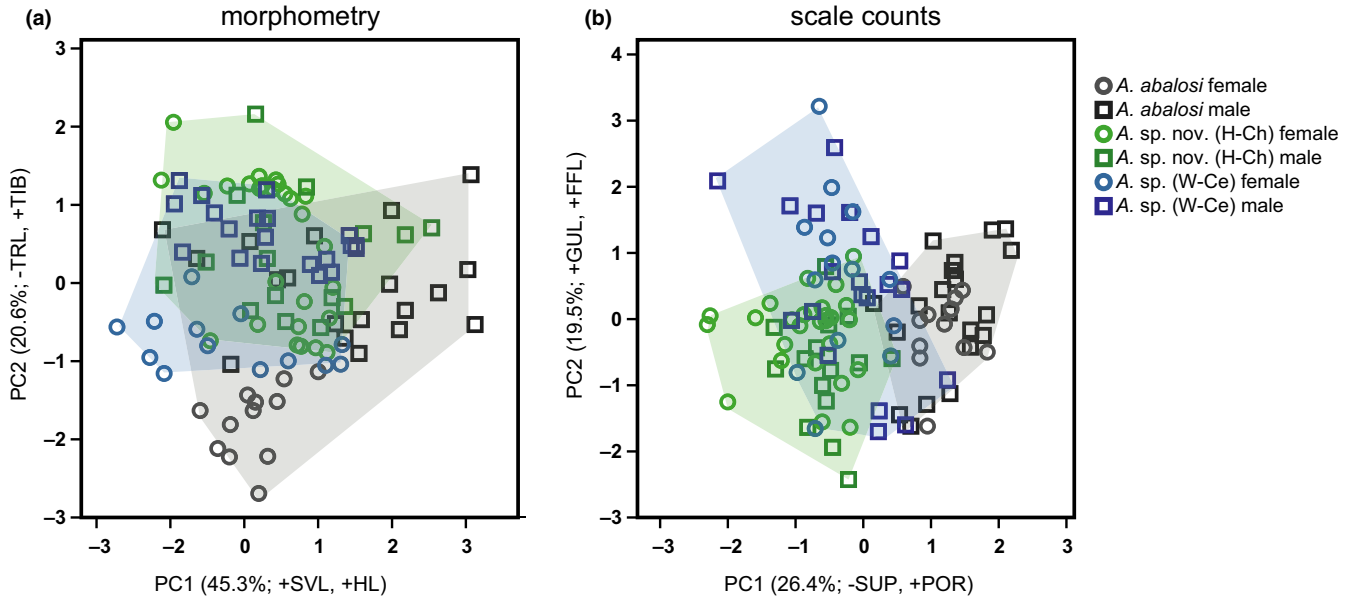

FIGURE 2 Results of a principal component analysis (PCA) showing variation among individuals of *Ameivula abalosi* (grey colour), *A.* sp nov. H-Ch (green colour) and *A.* sp. 2 W-Ce (blue colour) in the space delimited by the two principal components based on (a) morphometric data and (b) scale counts. The percentage of variation is explained, and the two characters with highest correlation are presented for each component. Males are represented by squares, and females by circles

ventral region, whereas in *A. abalosi* and *A.* sp. 1 and 2 W-Ce, the dorsal lateral colour is brown dark and the lateral flank present bright ocelli (Figure S1b).

Thus, the morphological analyses show that there are differences in morphometry, lepidosis (both in continuous and discrete characters) and coloration in specimens of *Ameivula* sp nov. from humid Chaco, which allow to diagnose it from its congeners.

Based on our molecular and morphological results, showing a deep divergence between *A. abalosi* from dry Chaco and *Ameivula* sp nov. from humid Chaco, we describe below a new species of *Ameivula* that inhabits in the humid Chaco (Taxonomic account is in Appendix S2, Figures S2 and S6).

4 | DISCUSSION

4.1 | Phylogenetic relationships

The present study is the first that present an explicit phylogenetic hypothesis including all recognised species of *Ameivula* and *Glaucomastix*. Currently, *Glaucomastix* is composed by four species, *G. abaetensis*, *G. littoralis*, *G. cyanura* and *G. venetacauda*. Previously, based on morphological features (lepidosis, hemipenial morphology and colour pattern), and without a phylogenetic analysis, these species were allocated in the *A. littoralis* subgroup, while the rest of the *Ameivula* species were placed in *A. ocellifera* subgroup (Arias et al., 2011a,b). Our phylogenetic hypotheses (Figure 1) reinforce the morphological subgroup, as in all the analyses, *Glaucomastix* resulted as sister group to *Ameivula*. These resulted are congruent with previous investigations, Giugliano (2009) shows that the clade *G. littoralis* + *G. abaetensis* is sister of the *A. ocellifera* complex. More recently (Tucker et al., 2016), using 316 loci find that the pair *Glaucomastix* + *Ameivula* complex is well supported (bootstrap = 100). Conversely, Goicoechea et al. (2016) show that *Ameivula* is paraphyletic, as *G. abaetensis* resulted sister to *Cnemidophorus* + *Kentropyx*, but this result was only recovered in one of the four analyses and with low support (JN = 37).

While those studies showed the phylogenetic position of *Glaucomastix* within Teiinae, they included so few members of this genus that no hypothesis of relationships within it was provided by Giugliano (2009); Tucker et al. (2016) included *G. littoralis* and *G. abaetensis*, whereas Goicoechea et al. (2016) included only *G. abaetensis* in their analysis. Our phylogenetic hypotheses show a deep split in *Glaucomastix* recovering *G. abaetensis* as sister to a clade (*G. cyanura* [*G. littoralis*, *G. venetacauda*]). For this last node, both the JN support as PP are low (JN = 27; PP = 75%), also for these analyses, we could only obtain sequences for a single individual of *G. littoralis*, and therefore, a more complete study is necessary to obtain a more robust hypothesis of relationships into this group.

4.2 | Phylogenetic relationships within *Ameivula*

While many species of *Ameivula* have been described in the last years (Arias et al., 2011a,b; Arias, de Carvalho, et al., 2014; Arias, Teixeira, et al., 2014; Rocha, Araujo, Vrcibradic,

TABLE 2 Results of a discriminant function analyses (DFAs) based on morphometric data and scale counts pooled for males and females of *Ameivula abalosi*, *Ameivula* sp nov. H-Ch and *Ameivula* sp. 2 W-Ce

	Males		Females	
Variable	**DF1**	**DF2**	**DF1**	**DF2**
SVL	0.178	0.011	−0.119	0.216
TRL	0.199	0.032	**−0.237**	0.156
HL	0.046	0.022	0.093	0.198
HW	0.131	−0.086	0.178	0.184
FAL	0.049	**0.306**	0.204	0.097
FEM	0.011	−0.132	−0.024	−0.082
TIB	0.002	−0.012	**0.233**	0.173
FOL	0.196	0.048	0.109	−0.059
SUP	**−0.309**	**0.352**	**0.354**	−0.259
GUL	−0.177	**−0.315**	−0.079	**−0.490**
POR	**0.419**	−0.103	**−0.248**	**0.493**
VT	0.084	0.116	0.043	0.148
D	−0.032	0.092	0.077	0.055
AMB	−0.186	−0.043	0.063	**−0.367**
AT	−0.070	**0.399**	0.041	0.158
FFL	0.146	−0.074	−0.153	−0.029
FTL	0.112	−0.163	−0.123	0.070
Eigenvalue	6.367	5.383	11.75	2.338
% of variance	54.18	45.82	83.40	16.60
Cumulative %	54.18	100.00	83.40	100.00

The correlation of each measurement with the two principal discriminant functions, eigenvalues and percentage of variance explained is presented. Scores with highest correlation with each discriminant function are highlighted in bold.

& Da Costa, 2000; Rocha, Bergallo, & Peccininiseale, 1997; Silva & Ávila-Pires, 2013), few studies have examined the phylogenetic relationship within the genus due to a restricted taxonomic sampling (Giugliano, 2009; Goicoechea et al., 2016; Harvey et al., 2012; Oliveira et al., 2015; Pyron, Burbrink, & Wiens, 2013). Our phylogenetic hypothesis of *Ameivula* includes all currently recognised species (*A. abalosi*, *A. cipoensis*, *A. confusioniba*, *A. jalapensis*, *A. nigrigula*, *A. mumbuca*, *A. ocellifera*, *A. pyrrhogularis* and *A. xacriaba*). Congruent with previous work (Giugliano, 2009; Harvey et al., 2012; Tucker et al., 2016), our result shows that *Ameivula* is monophyletic with strong support (Figure 1).

Results presented here show that all species of *Ameivula* are monophyletic, with the exception of *A. confusioniba* that it is nested in the same clade of *A. mumbuca* (Figure 1). Incomplete lineage sorting and gene flow among populations or species are evolutionary processes notably known to cause gene trees discordance, especially among populations or closely related species with low levels of divergence (Maddison & Knowles, 2006). Despite the not monophyly of *A. confusioniba*, there are conspicuous morphological characters (scalation, coloration and morphometrics) that allow distinguish it from *A. mumbuca* (Arias et al., 2011a,b). Therefore, we kept the species as valid waiting the results of future studies, including more samples and markers across its distribution, to solve this matter.

Surprisingly, the Cerrado species included in our analyses were not genetically clustered, the terminal from Eastern Cerrado (*A. mumbuca, A. jalapensis* and *A. xacriaba*) resulted closely related with West Caatinga terminal (*A. pyrrhogularis*, from left bank of São Francisco River), whereas the Western Cerrado terminals (*A.* sp. 1 and *A.* sp. 2 W-Ce) were clustered with *A. apipensis* sp nov. H-Ch. This result is congruent with previous studies for other lizard groups, such as *Phyllopezus pollicaris* species complex (Werneck, Gamble, Colli, Rodrigues, & Sites, 2012) and *Vanzosaura* (Recoder et al., 2014), which are co-distributed with *Ameivula* across the diagonal of dry Biomes of South American (Rodrigues, 1996; Vanzolini & De Carvalho, 1991).

Also interesting is the monophyletic clade that grouped *A. jalapensis* and *A. xacriaba*. A previous study (Werneck, 2011) has indicated that there may be a reciprocal monophyly between species associated with plateaus and depressions within the Cerrado. The result of the mitochondrial phylogenetic analysis of this study reinforces this proposal as *A. jalapensis* inhabit in a depression zone of the Cerrado, in Jalapão region, whereas *A. xacriaba* is apparent endemic of the Planalto dos Gerais, a plateau with an elevation of ~ 900 m (Arias, Teixeira, et al., 2014). This is also the case of *Ameiva jacuba* which inhabits the plateau and its co-generic *Ameiva ameiva* occur in depression zone (Giugliano et al., 2013). Similarly, Fabricius et al. (2014) show that populations of the *Gymnodactylus amarali* complex that inhabit a Cerrado plateau (630 m above sea level) are distinct from those inhabiting adjacent valleys (300 m) although separated themselves by only 50 km.

On the other hand, the phylogeographic analyses made by Oliveira et al. (2015) indicated that the genetic diversity of *Ameivula* in the Caatinga (their North-west lineage) is low and represented by a single widespread species. Based on their results, they concluded that *A. pyrrhogularis* is a junior synonym of *A. ocellifera*. They also indicated that, other Caatinga species, as *A. nigrigula*, were not genetically distinguishable from the North-west lineage, so the taxonomic status of these species was considered problematic. Nevertheless, in the trees obtained under Bayesian inference of these authors, the populations that corresponded to *A. pyrrhogularis* and *A. nigrigula* were recovered as monophyletic. Based on our results, morphological and molecular, we disagree with Oliveira et al. (2015), as that

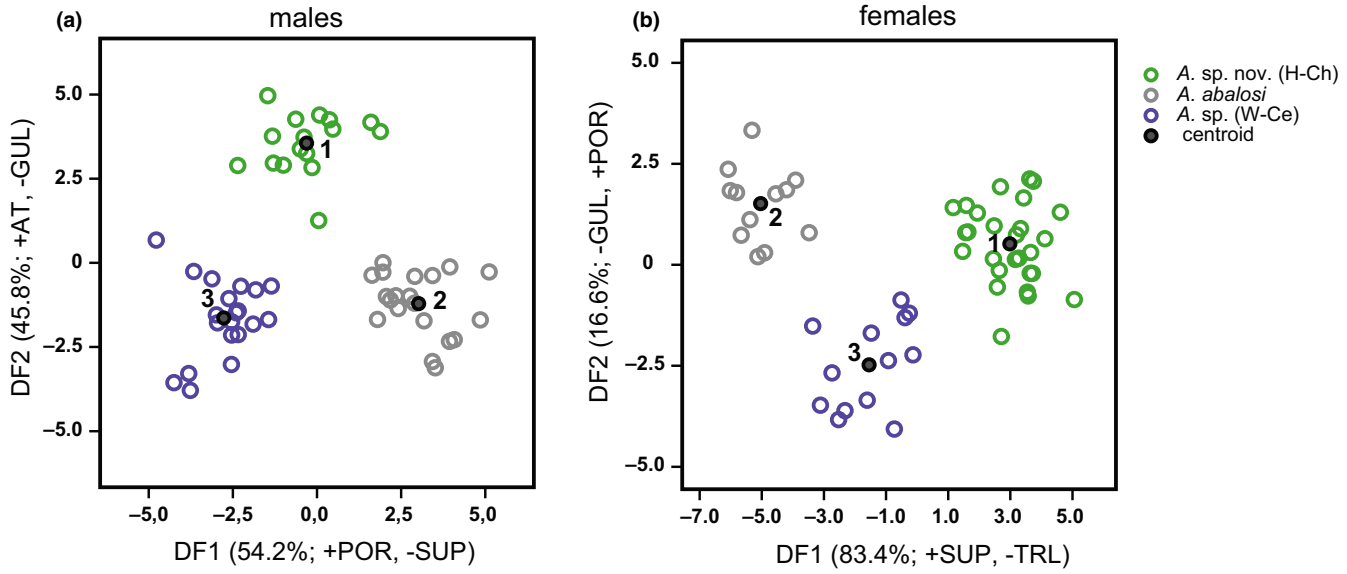

FIGURE 3 Results of a discriminant analysis (DFA) based on pooled morphometric data and scale counts, showing variation among *A.* sp nov. H-Ch (1, green colour), *Ameivula abalosi* (2, grey colour) and *A.* sp. 2 W-Ce (3, blue colour) in the space delimited by the two principal discriminant functions for (a) males and (b) females. The percentage of variation is explained, and the two characters with highest correlation are presented for each discriminant function

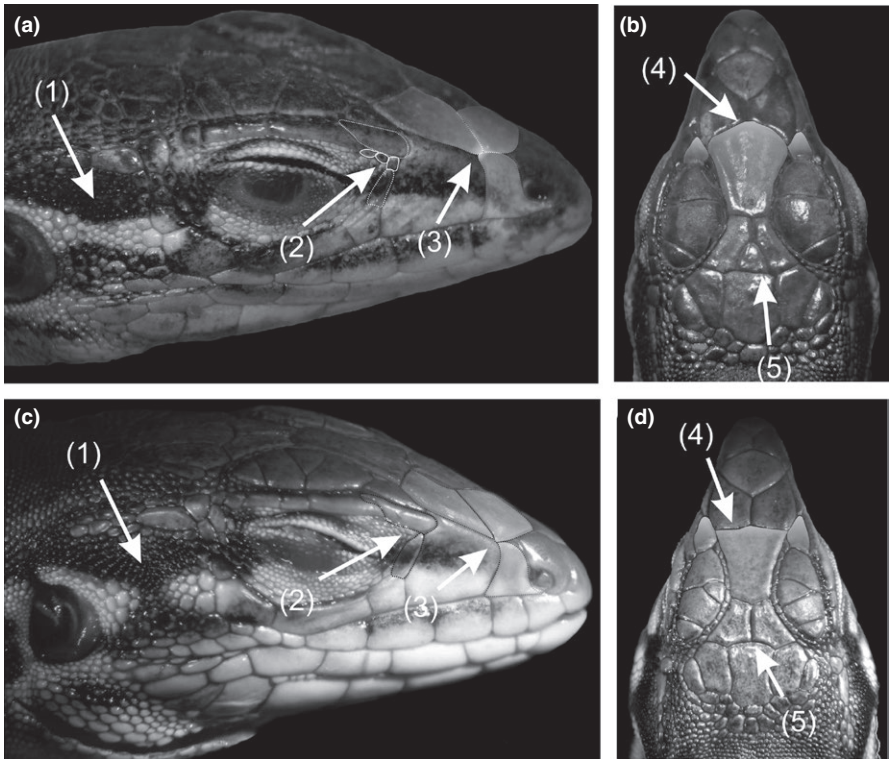

FIGURE 4 Details of the head lepidosis characters of *Ameivula apipensis* sp nov. (a,b) and *Ameivula abalosi* (c,d). Character (1) shows the continuous/discontinuous lateral line; character (2) shows the contact/no contact between the supraciliary and lacrimal scales; character (3) shows the contact/no contact between the prefrontal and postnasal; character (4) shows the anterior level of the frontal, exceed/no exceed the first supraocular scale; character (5) interfrontoparietal, present/absent

the evidence indicates that there are at least three species of *Ameivula* occurring in the Caatinga, therefore *A. pyrrhogularis* and *A. nigrigula* should be treated as valid species.

The topology of our molecular tree shows that the *A. ocellifera* clade is formed by two nodes, one nested terminals of Sergipe (MTR 12569_Se, MTR 12567_Se and 12570_Se) and Bahia (SSBA1, MTR 24736 and MTR 24886), the other one formed by terminals from Pernambuco (MTR 15363_Pe, MTR 15369_Pe and MTR 15669_Pe) (Figure 1). Moreover, the Pernambuco node shows variations in the color pattern and lepidosis, therefore we consider it as "*species candidate*" (Arias & Rodrigues, in prep.).

Although in all our phylogenetic analyses, *A. cipoensis* and *A. nigrigula* resulted basal to the rest of the species, and the position of these taxa is poorly supported (Figure 1). We think that it is necessary to include more samples covering its distribution and add more molecular markers to test the position of these species.

While we advance the most complete phylogenetic hypothesis of interspecific relationships within *Ameivula* to date, increasing the number of individuals sampled per lineage across its range in combination with additional informative markers is necessary to obtain a more robust phylogenetic hypothesis and combine this with other kind of analyses, such as estimating a molecular clock and analysing its biogeography, to understand the evolutionary history of this group.

4.3 | Cryptic *Ameivula* species diversity in the Gran Chaco and Western Cerrado

Ameivula abalosi was described as widely distributed through of the Gran Chaco (Cabrera, 2012) that encompasses strongly contrasting environmental conditions and the large gap in the range. As expected, significant variation in morphology and phylogenetic structure was observed between *Ameivula* populations from dry and humid Chaco. It became clear that a taxonomic re-evaluation was necessary for this species, a task that could only be accomplished under a quantitative framework to address the increased availability of study material. Here, we analysed several populations from the Gran Chaco, the results show that there exist strong differences both in morphology and genetics level between *A. abalosi* (dry Chaco) and *A. apipensis* sp nov. (humid Chaco). The type series and nearby populations from Argentina and Paraguay of *A. apipensis* sp nov. are readily diagnosed from *A. abalosi* by cephalic lepidosis (postnasal-prefrontal in contact, lacrimal-first supraciliar not contacting), continuous characters (gular scales, supraocular granules and femoral pores), coloration (vertebral stripe, absence of ocelli, dorsolateral stripe continuous, and between the eye and ear), hemipenial morphology (absence of lateral sac, Figure S3) and osteology (five postxiphisternal ribs, presence of palatine teeth and no commissure in the articular). Furthermore, the molecular data show that between these two species, there is a mDNA distance of 9%, while the topology of our trees show that the Chacoan *Ameivula* is paraphyletic, as *A. apipensis* sp nov. is closely related with terminals from Pantanal (PNP 231_Corumbá) + W-Ce. Similar results were obtained for *Vanzosaura* (Recoder et al., 2014) where the topology of the molecular tree shows that the humid Chaco + Pantanal populations formed a monophyletic clade. Unfortunately, specimens of *Vanzosaura* from dry Chaco were not included in their molecular phylogeny, but the morphological analyses showed that there are morphometric differences between populations of *Vanzosaura* from dry and humid Chaco, separated by the Paraná-Paraguay river, a similar results to those obtained here for *Ameivula*. Other teiid lizards that were considered as widely distributed in the Gran Chaco is *Kentropyx lagartija*, formerly composed by two subspecies *K. largartija lagartija* and *K. lagartija viridistriga*; that were elevated to species level (Tedesco & Cei, 1997) due to morphological differences found between populations from dry (*K. lagartija*) and humid Chaco (*K. viridistriga*).

In the topology of our trees (Figure 1), *A. apipensis* sp nov. resulted sister to a clade formed by specimens from Corumbá, Cáceres and Alto de Araguaia (*A.* sp. 2W-Ce) and both recovered sister to a clade assembling specimens from Chapada dos Guimarães and Barra do Garças, also from W-Ce (*A.* sp. 1 W-Ce). Between the species described here and Pantanal + W-Ce populations (called *Ameivula* sp. 2), there is a mtDNA distance of 7%. Furthermore, we found differences in lepidosis (fewer gular scales, frontoparietal smaller that the frontal), coloration pattern (lateral flank green, without ocelli) and hemipenial morphology (absence of lateral sacs). Therefore, we hypothesise that the clades that include the populations from Corumbá, Cáceres and Alto de Araguaia (*Ameivula* sp. 2), and the one formed by specimens from Chapada dos Guimarães and Barra do Garças (*Ameivula* sp. 1), should be considered as "candidate species," according to Padial, Miralles, De la Riva, and Vences (2010). We do not suggest formal names for any of these lineages because a careful morphological revision, a molecular analysis including additional markers and a better taxon sampling across the range of these candidate species is needed before considered them full species. At present, studies tackling most of these challenges are in progress in our laboratory.

It is interesting to notice that adjacent populations of *Ameivula* from West Cerrado and Pantanal have a molecular difference, as there is no apparent geographic barrier. Niche conservatism may lead populations to diverge when isolated by intervening unsuitable environmental conditions, as in the case of populations occurring in high elevations separated by lowlands (Wiens & Graham, 2005). This pattern of speciation in the West Cerrado also was observed in other lizard groups; Teixeira et al. (2015) showed that populations of *Stenocercus* from lowlands and high elevations show molecular divergences that are consistent with the timing of reported environmental shifts in this region as a result of Andean orogeny. Lowlands between the Andean foothills and the Brazilian shield underwent large-scale landscape transformations between 11.8 and 8 myr, with shifts in the course and contacts of major rivers, marine incursions coming from both north and south, and the establishment and vanishing of large lacustrine or marine systems (Cook, Chao, & Beheregaray, 2012; Hernández et al., 2005; Hoorn, 2006; Lovejoy, Albert, & Crampton, 2006; Lundberg et al.,

1998; Räsänen, Linna, Santos, & Negri, 1995). As well as the formation of large water bodies seems to have isolated the ancestor of *Stenocercus* and other lizard groups from West Cerrado (Teixeira et al., 2015; Werneck et al., 2012), the same phenomenon could have provoked the diversification in *Ameivula*.

4.4 | The Gran Chaco conservation

The Gran Chaco is strongly affected by extensive livestock raising, extractive forestry and poorly planned agricultural expansion (The Nature Conservancy et al. 2005).

Initial estimates revealed roughly 45% of selective or intensive degradation for the Paraguayan Chaco, with protected areas covering only about 1% of the total area (Redford, Taber, & Simonetti, 1990). A massive contraction of forest occurred at the southern edge of the Chaco forest, where 85% of the original subtropical dry forests (ca. 1.2 million ha) were converted to agriculture, pasture or secondary succession lands in only 30 years (Steininger et al., 2001; Zak, Cabido, & Hodgson, 2004). Only 9% of the Gran Chaco is located in protected areas and that, in Argentina, where the major section of the Gran Chaco is located, the conservation units represent 3.25% of the Argentinean Chaco (The Nature Conservancy (TNC) et al., 2005).

Recently, it has been shown that the loss of the landscape vegetation composition of this region affects the reproduction of *Boa constrictor occidentalis* (Cardozo & Chiaraviglio, 2008), an endemic snake of the dry forest of the Gran Chaco region. Besides, Nori et al. (2016) show that endemic vertebrates, such as amphibians, birds and mammals, may be key point for conservation planning.

Here, we show that *A. apipensis* sp nov. is apparently a species endemic of the "humedales" of the fluvial system of Paraná-Paraguay river and that *A. abalosi* is an endemic species of dry Chaco, distributed from Central Argentina, crossing Paraguay and probably in Santa Cruz, Bolivia. Our results may be important for formulating conservation strategies in the Gran Chaco ecoregion, where is necessary an efficient control of deforestation, protection of forest remnants and establishment of corridors.

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