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*Front cover: Male green lizard (*Lacerta bilineata*) photographed near the village of Saint-Denis-du-Payré in western France, part of the study population described in the article on page 20.*

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## Herpetological Journal 2020 and beyond – Issue 30(1)

During the past decade we have seen significant advancements in our understanding of herpetological diversity, ecology, behaviour and conservation. Depressingly, many amphibian and reptile species, however, have become extinct or undergone large declines over this period. Of all vertebrates, amphibians are one of—if not the—organismal group of most conservation concern, with 41 % of species threatened with extinction (IUCN, 2019) and some estimates suggest that an additional ca. 1,000 of the IUCN's Data Deficient species are threatened with extinction (González-del-Puerto et al., 2019).

Unfortunately, it is likely that these declines will increase over the next decade. *The Herpetological Journal* has played a pivotal role in building our understanding of all elements of herpetological biology, consequently informing appropriate conservation actions to be taken.

The current issue of *The Herpetological Journal* epitomises the range of studies in reptile and amphibian biology and spans the many topics that the journal covers. This issue has articles discussing taxonomic and phylogenetic relationships of snakes (Eskandarzadeh et al., 2020), how captivity affects skin colour in frogs (Passos et al., 2020), population changes in long term datasets of lizards (Meek, 2020), amphibian distribution and endemism in South America (Cabral et al., 2020; Ribeiro Morais et al., 2020), and a global review of mono- and bispecific genera of amphibians (Amori et al., 2020).

*The Herpetological Journal* welcomes manuscripts about any aspect of herpetology, that are of high scientific standard and of general interest, including taxonomic papers. Previously we have been accepting the following manuscript types: full length papers, short notes, reviews, mini-reviews and opinion/perspective pieces. Moving forward we will be maintaining these formats but will be adding policy-based manuscripts, given the importance of setting correct policy-based procedures for conservation.

As well as scientific advancements, the past decade has increasingly seen authors choosing to publish using Open Access. At the British Herpetological Society, we strongly encourage Open Access to allow scientific knowledge to be freely available to the public and as such, to our knowledge, we are the cheapest Open Access journal for herpetology, globally. Open Access is available free to members (membership rates from as little as £18 pa — can join during submission process) or £97 for non-members. We strongly encourage authors to use the Open Access option as a way of attracting attention and therefore impact (citations) for your articles.

Here is to another 10 years of herpetological advancements!

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Simon Maddock  
Faculty of Science and Engineering, University of Wolverhampton, WV1 1LY, UK  
[s.maddock@wlv.ac.uk](mailto:s.maddock@wlv.ac.uk)  
Scientific Editor



## Revised classification of the genus *Eryx* Daudin, 1803 (Serpentes: Erycidae) in Iran and neighbouring areas, based on mtDNA sequences and morphological data

Naeimeh Eskandarzadeh<sup>1</sup>, Nasrullah Rastegar-Pouyani<sup>1</sup>, Eskandar Rastegar-Pouyani<sup>2</sup>, Fatemeh Todehdehghan<sup>3</sup>, Mahdi Rajabizadeh<sup>4,5</sup>, Mohammad Zarrintab<sup>6</sup>, Fadhil Abbas Rhadi<sup>7</sup> & Haji Gholi Kami<sup>5</sup>

<sup>1</sup>Department of Biology, Faculty of Science, Razi University, Kermanshah, Iran

<sup>2</sup>Department of Biology, Faculty of Science, Hakim Sabzevari University, Sabzevar, Iran

<sup>3</sup>Department of Venomous Animals and Antivenin Production, Razi Vaccine & Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran

<sup>4</sup>Department of Biodiversity, Institute of Science and High Technology and Environmental Sciences, Graduate University of Advanced Technology, Kerman, Iran

<sup>5</sup>Department of Biology, Faculty of Science, Golestan University, Gorgan, Iran

<sup>6</sup>Department of Environmental Sciences, Faculty of Natural Resources, University of Tehran, Karaj, Iran

<sup>7</sup>Al-Qasim Green University, Babylon, Iraq

Several attempts have recently been made to elucidate taxonomic status and phylogenetic relationships among the species and subspecies of sand boas of the genus *Eryx* throughout their distribution range, with no stable consensus about their taxonomy. Here the phylogenetic relationships among the populations of *Eryx* in Iran and adjacent areas are studied based on two mitochondrial markers (*cytb* and 16S). Sixteen morphological characters were examined for evaluation of morphological differences among major populations. Ecological niche modeling was applied to demonstrate the potential distribution of the populations in Iran. ENMtools was also used to measure the degree of niche overlap among the major populations in Iran. Based on phylogenetic reconstruction and considering the genetic distances with specimens from type localities, *E. tataricus* is a junior synonym of *E. miliaris* and the subspecies rank for *E. m. nogaiaorum* seems to be invalid. Considering the genetic distance of populations in western Iran and Iraq, and the habitat and morphological differences among the populations of *Eryx* in western Iran, Iraq and Egypt, the population of *Eryx* in western Iran is suggested as a different species from *E. jaculus*, named here as *Eryx* sp. and the ones from Iraq as *Eryx* cf. *jaculus*. Here, the evaluation and revision of taxonomic status, distribution ranges and descriptions of morphological characters of the studied species have been done.

**Keywords:** *Eryx*, taxonomy, phylogeny, mitochondrial markers, morphology, niche modeling, niche overlap

### INTRODUCTION

Based on the most recent study, the family Erycidae comprises 13 named species: *Eryx borrii* Lanza & Nistri, 2005, *Eryx colubrinus* (Linnaeus, 1758), *Eryx conicus* (Schneider, 1801), *Eryx elegans* (Gray, 1849), *Eryx jaculus* (Linnaeus, 1758), *Eryx jayakari* Boulenger, 1888; *Eryx johnii* (Russell, 1801), *Eryx miliaris* (Pallas, 1773), *Eryx muelleri* (Boulenger, 1892), *Eryx somalicus* Scortecchi, 1939, *Eryx tataricus* (Lichtenstein, 1823), *Eryx vittatus* Chernov, 1959 and *Eryx whittakeri* Das, 1991. These are distributed in north and east Africa, Europe, the Middle East, and south and central Asia (Pyron et al., 2014). During recent decades, several attempts have been made for the taxonomic modification among the members within this family, based on morphological traits and molecular data (Tokar, 1990; 1991; Kluge, 1993; Campbell, 1997; Eskandarzadeh et al., 2013; Pyron et al., 2013; 2014; Rastegar-Pouyani et al., 2014; Reynolds et

al., 2014; Zarrintab et al., 2017). However, no consensus has been made about their taxonomic status.

It has been difficult to distinguish *E. miliaris* from *E. tataricus* either by morphological (Sorensen, 1988; Tokar, 1990; Eskandarzadeh et al., 2013; Zarrintab et al., 2017) or molecular evidence (Pyron et al., 2013; Rastegar-Pouyani et al., 2014; Reynolds et al., 2014). *Eryx miliaris* (type locality: north shore of the Caspian Sea between the Volga and Ural Rivers) is distributed across south-western Russia, east to China and Mongolia, south to Iran and Afghanistan (Wallach et al., 2014; Reynolds & Henderson, 2018). *Eryx tataricus* (type locality: Aral Sea region) is distributed from the eastern shore of the Caspian Sea south to Pakistan and Iran, and east to China and Mongolia (Wallach et al., 2014). The lack of a comprehensive study, especially on the type specimens of these two species and their subspecies, causes chaos in their taxonomic status. *Eryx miliaris* consists of two subspecies, the nominate form and *E. m. nogaiaorum*

(Nikolsky, 1910) (distribution range: western part of the distribution range of the species) (Ananjeva et al., 2006). Ross & Marzec (1990) elevated the taxonomic rank of the black sand boa, *E. m. nogaorum*, to a distinct species, but without any references to document the change (Kluge, 1993). According to Harrison (2003), because of the trouble in defining *E. tataricus*, determining the distribution range of *E. tataricus* is confusing. He also announced that some authors have had problems with distinguishing *E. tataricus* from both *E. miliaris* and *E. jaculus*. According to Tokar (1990), *E. miliaris* and *E. tataricus* form a complex.

Differentiation of *E. elegans* from *E. jaculus* is also problematic in north-east Iran (Eskandarzadeh et al., 2013; Rastegar-Pouyani et al., 2014). *Eryx elegans* (type locality: Afghanistan) is distributed in the north of Iran and Afghanistan, and southern parts of Turkmenistan (Wallach et al., 2014; Reynolds & Henderson, 2018). *E. jaculus* (type locality: Egypt) distributed in south-eastern Europe, south-western Asia and north of Africa (Wallach et al., 2014). Boulenger (1893) described the holotype of *E. elegans* as follows: "Agrees in every respect with *E. jaculus*, except that the scales are larger, in 36 rows in the middle of the body". The number of mid-body scales in the type specimen of *E. jaculus* from Egypt is 48 and in other studied specimens from Egypt varied from 43 to 51 (Anderson, 1898).

The number of dorsal scales at the mid-body is the most prominent and sometimes the only distinguishing character between *E. elegans* and *E. jaculus*. However, this number is uncertain in different works of literature (Table S1) (Boulenger, 1892; 1893; Tzarewsky, 1916; Terentyev & Chernov, 1965; Leviton & Anderson, 1970; Bannikov et al., 1977; Latifi, 1991; Zarrintab et al., 2017). In studied specimens of *E. jaculus* and *E. elegans* from north-eastern Iran, the minimum and maximum number of dorsal scales at mid-body were 36 and 46, respectively (36-41 for *E. elegans* and 42-46 for *E. jaculus*) (Eskandarzadeh et al., 2013).

Three subspecies have been described for *E. jaculus*: *E. j. jaculus* with three post-internasal scales, and *E. j. familiaris* (Eichwald, 1831) and *E. j. turcicus* (Oliver, 1801) each with two post-internasal scales. The subocular and supralabial scales are in contact in *E. j. familiaris* while these are separated by a row of scale in *E. j. turcicus* (Tzarewsky, 1916; Tokar, 1991; Kluge, 1993; Bannikov et al., 1977). Tokar (1991) studied 327 specimens of *E. jaculus* throughout its wide distribution range (Africa, Europe and Asia) and concluded that: "*Eryx jaculus* is a monotypic species, and the names *turcicus* and *familiaris* should be considered as its junior synonyms". According to Tokar (1991), the eastern boundary of the distribution range of *E. jaculus* in central Iran is not known, yet he named the area between Shiraz and Kerman as the extreme south-eastern extent of the range.

Ecological niche modeling (ENM) makes it possible to reveal the potential distribution of a species based on environmental (physical) conditions even with limited locality data. Because of the presence of biogeographic component within the disciplines of systematics, the role of ENM in phylogenetic research is considerable (Phillips

et al., 2006; Raxworthy et al., 2007). Some examples of such systematic applications include recognition of erroneous localities (Graham et al., 2004), discovery of new areas of endemism and new species (Raxworthy et al., 2003) and species delimitation (Wiens & Graham, 2005). One of the systematic utilities of ENM is in species delimitation and especially in recognition of cryptic species. One such example is the study of Raxworthy et al. (2007) about the genus *Phelsuma*, showing the utility of using ENM in species delimitation. Some studies have also been done using ENM for boid snakes (Di Cola et al., 2008; Eskandarzadeh et al., 2018a).

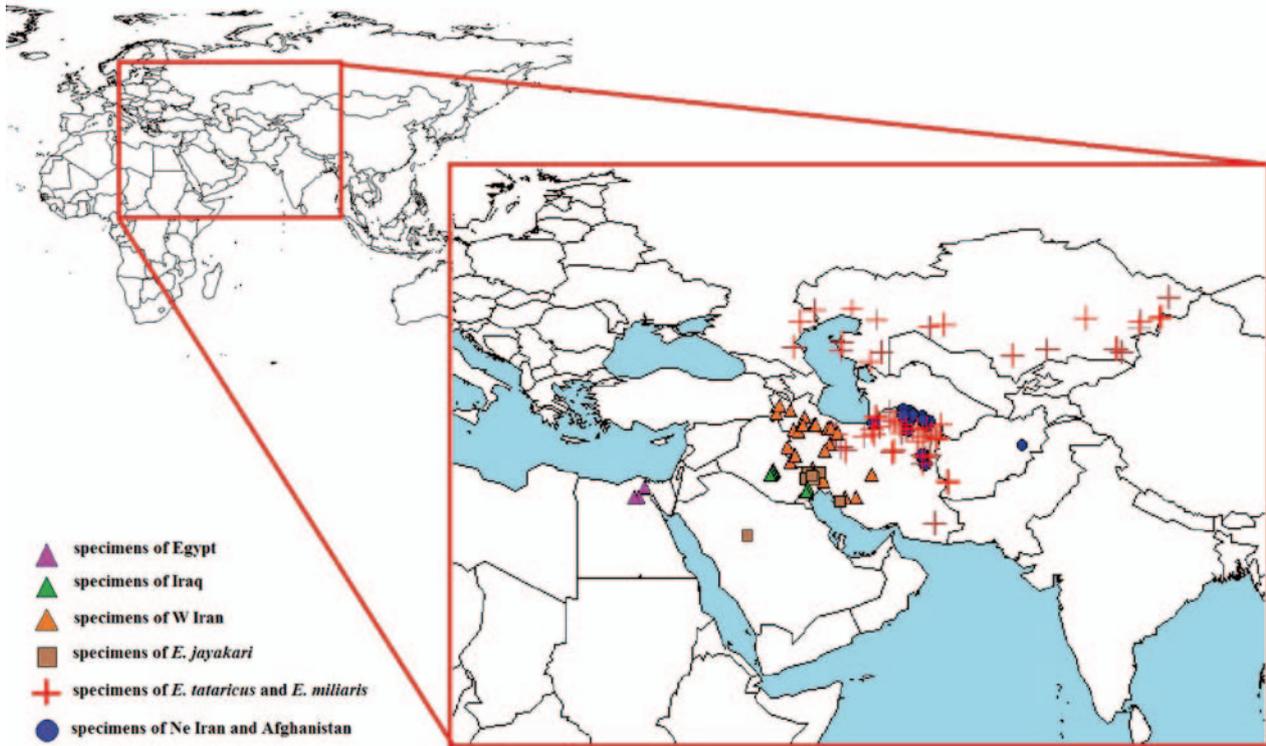
Here, we combine mitochondrial DNA and morphological data to evaluate the phylogenetic relationship and taxonomic position of populations within the genus *Eryx* throughout a wide range from Iran and adjacent areas, and apply the data of *E. tataricus* and *E. miliaris* from the type localities for the first time. We use univariate and multivariate analyses to explicate the main discriminator morphological characters among the main populations and investigate the separation of *Eryx* populations morphologically. We also apply ENM to reveal the potential distribution range of the populations in Iran and their habitat preferences. ENMtools is also used to calculate the degree of niche overlap between major populations in Iran.

## MATERIAL AND METHODS

### Phylogenetic analyses

A total of 182 sequences from two mitochondrial markers (cytochrome *b* and 16S rRNA) in 104 specimens were analysed, including 145 novel sequences produced in this study and 37 sequences retrieved from NCBI (details are presented in Fig. 1 and Table S2). Based on the reconstructed tree in the phylogenetic study of Pyron et al. (2013), *Calabaria reinhardtii* was chosen as outgroup (accession numbers are presented in Table S2). DNA extraction was done using proteinase K digestion followed by ammonium acetate extraction (Kapli et al., 2013). Amplification of the marker 16S rRNA (16S) was done using the primers 16SL (5'-CGCCTGTTTATCAAAAACAT-3') and 16SH (5'-CCGGTCTGAACTCAGATCAG-') (Palumbi et al. 1991), and cytochrome *b* (*cytb*) with the primers L14724 (5'-TGACTTGAAGAACCACCGTTG-3') and H16064 (5'-CTTTGGTTTACAAGAACAATGCTTTA-3') (Palumbi et al., 1991; Burbrink et al., 2000); and L14919 (5'-AACCACCGTTGTTATTCAACT-3') and Ei700r (5'-GGGGTGAAGGGGATTTTRTC-3') (Burbrink et al., 2000; Rastegar-Pouyani et al., 2010). The amplified fragments were sequenced on an automated sequencer ABI 3730XL (Macrogen, Seoul, South Korea).

ClustalW multiple alignment implemented in the software Bioedit sequence alignment editor 7.0.9.0 (Hall, 1999) was used to align the sequences in addition to manual checking. Mega 7 (Kumar et al., 2016) was used for checking the stop codons and align reading frames in the protein coding *cytb* gene as well as calculating the genetic distances (*p*-distance). To reconstruct the phylogenetic relationships, two phylogenetic approaches (Bayesian Inference and Maximum Likelihood) were used.



**Figure 1.** Localities of *Eryx* specimens applied in this study. Purple triangle: the specimens of Egypt (*E. jaculus*); green triangle: the specimens of Iraq (previously identified as *E. jaculus* and *E. cf. miliaris* (Rhadi et al., 2015)); orange triangle: the specimens of western Iran (morphologically identified as *E. jaculus*); brown square: the specimens of *E. jayakari* from Iran and Saudi Arabia; red cross: the specimens of *E. tataricus* and *E. miliaris*; blue circle: the specimens of north-eastern Iran and Afghanistan (morphologically identified as *E. elegans* and *E. jaculus*).

The software jModelTest 2.1.7 was used to determine appropriate models under the Akaike Information Criterion (Posada, 2008) which yielded in the model TIM2+G for 16S and TrN+G for *cytb* as the best models, respectively. Bayesian inference (BI) was performed by Mr. BAYES 3.1.2 (Huelsenbeck & Ronquist, 2001) with two runs for 10 million generations, sampling every 1000 generations and discarding 25 % of the initial samples as burn-in. Maximum likelihood (ML) analysis was done with raxmlGUI v. 1.5. with 2000 replicates under the GTRGAMMA model (Silvestro & Michalak, 2012).

### Morphological analyses

A total of 171 specimens were examined to determine the morphological divergence between major populations. Morphological data available from original descriptions and specimens from type localities were also included (Table S2). Seven meristic, two metric, and seven multistate characters were examined in the specimens (Table 1), considering the allometric variation the ratio of inter-ocular space width to distance between the posterior edge of the eye and the corner of the mouth was examined. For bilateral characters, only the right side was considered. Multistate characters were weighted numerically from 0 to 2 representing different status of these characters in the studied species. Nearest neighbor method using Minkowski implemented in SPSS 16 was used to perform cluster analysis. Based on previous studies performed on the *Eryx* spp., the most usual sexually dimorphic characters (number of

subcaudal scales, length of tail, length of body and snout-vent length) (Shine, 1978; Tokar, 1991; Eskandarzadeh et al., 2018b) were omitted and both sexes were analysed together.

Homogeneity of variance was tested using Levene's test, and normality tests were used to evaluate the normal distribution of data. ANOVA with posthoc test, and Kruskal–Wallis test, were applied to parametric and non-parametric data, respectively, implemented in the statistical package SPSS 16, to reveal significantly different characters among groups. Two multivariate analyses; principal component analysis (PCA) and discriminative cluster analysis (DCA), were used to determine the form and pattern of variation among populations. The WI/DEM value was not available for all the Egyptian and some specimens from other clades, so this character was omitted from multivariate analyses.

### Ecological niche modeling and ecological niche overlap

To reveal ecological requirements and the potential distribution for each major population in Iran, the altitude and 19 bioclimatic variables (in 30 arc-seconds resolution) were downloaded from DIVA-GIS (<http://www.diva-gis.org>) and the WorldClim database (<http://www.worldclim.org/version1>) (Hijmans et al., 2005), respectively. ENM was conducted only for the Iranian populations as data from other territories were sparse and were geographically limited (Fig.1). For each population the presence points (Table S2) and environmental layers were employed in Openmodeller v. 1.0.7. A total of 91

**Table 1.** Description of studied (A) meristic, morphometric and (B) multistate characters in populations of the genus *Eryx*.

(A)	
Abbreviation	Character
RE	Number of scales around eye
LAB	Number of supralabial scales
BE	Number of scales between the eyes
PIN	Number of scales posterior to internasal
BEN	Number of scales between the eye and nasal
DS	Number of dorsal scales (in mid-body)
VS	Number of ventral scales
WI	Width of interocular space
DEM	Distance between posterior edge of the eye and the corner of the mouth

(B)				
Character	No of states	Ch. State 0	Ch. State 1	Ch. State 2
Height of 2nd and 3rd upperlabial scales	3	2nd is higher	3rd is higher	2nd and 3rd are the same in height
The size of circum-orbital scales	2	Similar in size	One scales is bigger than the others	
Oblique line from eye to the corner of mouth	2	Present	Absent	
Connectional status of PIN with rostral scale	2	Connected	Not connected	
The status of eye position	3	Lateral	Somewhat upwarded	Upwarded
Keelness of scales	2	Scales not keeled	Scales somewhat keeled	
The shape of tail end	2	Pointed	Not pointed	

**Table 2.** Pairwise uncorrected genetic divergence ( $p$ -distance) among major clades of the genus *Eryx* from the mitochondrial markers for *cytb* (upper-right in bold-italic) and 16S (lower-left in regular).

	Clade A	Clade B	Clade C	Clade D	<i>E. jayakari</i>
Clade A		<b>0.122</b>	<b>0.116</b>	<b>0.106</b>	<b>0.162</b>
Clade B	0.044		<b>0.059</b>	<b>0.111</b>	<b>0.154</b>
Clade C	0.034	0.023		<b>0.101</b>	<b>0.156</b>
Clade D	0.051	0.037	0.029		<b>0.155</b>
<i>E. jayakari</i>	0.062	0.064	0.058	0.063	

points were used. The Pearson correlation coefficient was calculated with SPSS 16 to determine the final layers for modeling each population and layers; correlations > 0.75 were omitted.

The selected layers and presence points were applied by MaxEnt 3.4.1 to produce the final model with maximum entropy method (Phillips et al., 2017). Ten percent of the data were used as test and 90 % as training. The convergence threshold and maximum number of iterations were 0.00001 and 500, respectively. The model was run with 10 replicates with cross validated replicate types. The area under the receiver operating characteristic (ROC) curve (AUC) was used to evaluate the model performance. The niche overlap of concerned species was computed using Schoener's D metric implemented in ENMTools (Warren et al., 2010), ranging from 0 to 1, where the value 0 means that ecological niches do not overlap at all, and 1 means that the ecological niches are identical.

## RESULTS

### Phylogenetic analyses

Five major clades were revealed in both BI (Fig. 2) and ML (Fig. S1) phylogenetic trees. In addition to *E. jayakari*, four other major clades were revealed in the phylogenetic analyses. Clade A comprised the morphologically identified specimens of *E. elegans* and *E. jaculus* from north-east Iran (Eskandarzadeh et al., 2013; Rastegar-Pouyani et al., 2014). Clade B comprised the specimens from Iraq, previously identified as *E. jaculus* and *E. cf. miliaris* (Rhadi et al., 2015). Clade C comprised the specimens from western Iran morphologically identified as *E. jaculus*, and clade D comprised the specimens of *E. miliaris* and *E. tataricus* (from Chechnya, Kalmykia, Russia, Kazakhstan, Iran, and type specimens of *E. m. miliaris*, *E. t. tataricus*, and *E. m. nogaiaorum*).

In both trees, the specimens from Iraq (clade B) and those from western Iran (clade C) were sister clades (with posterior probability 1 and bootstrap value 78). In the BI tree, clades B and C were clustered with clade D as their

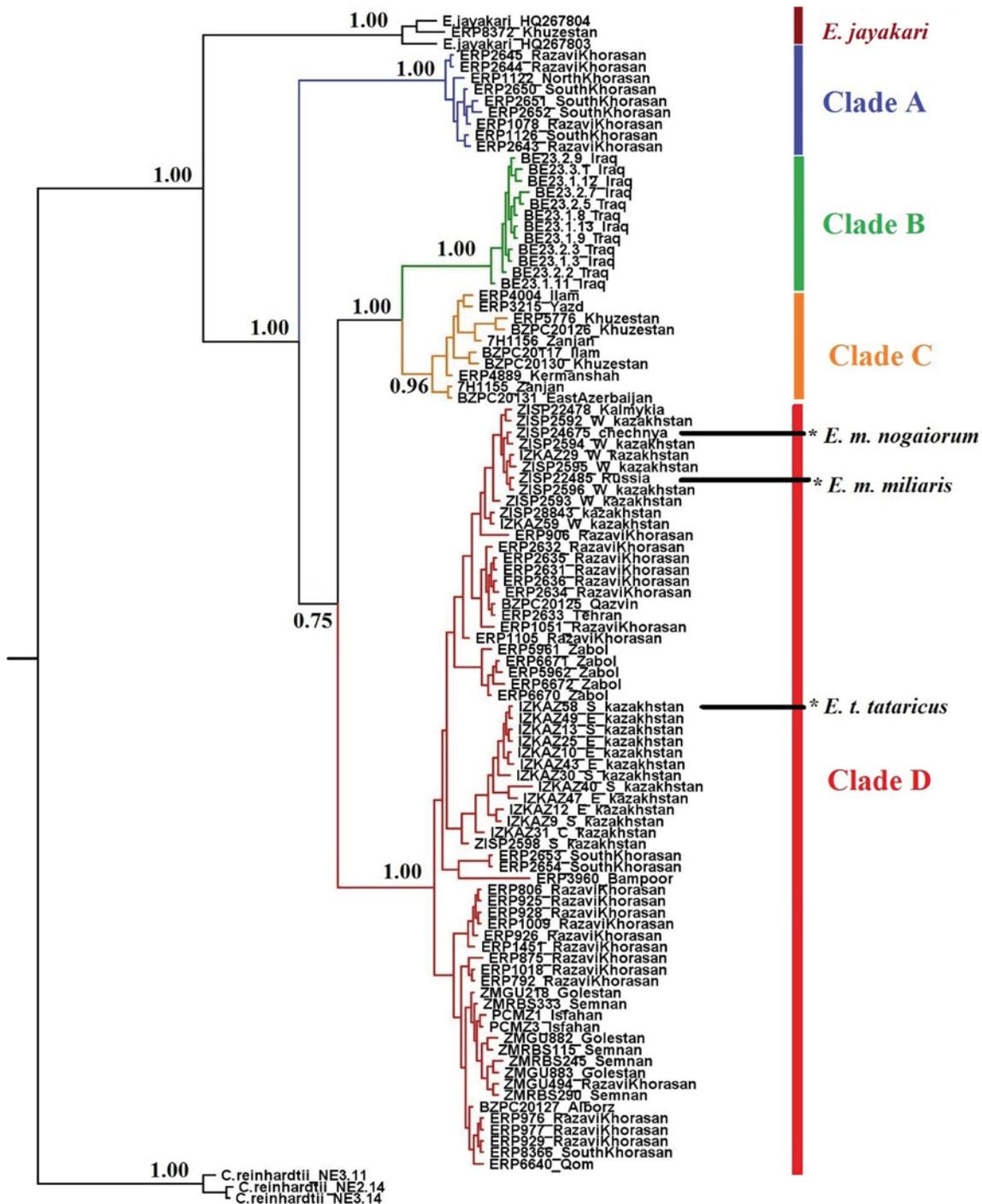
sister clade (with posterior probability 0.75), while in ML phylogram, clade D is clustered with clade A, but with a poor support value (with bootstrap value 36) (Fig. 2 and Fig. S1).

The calculated genetic divergence ( $p$ -distance) varied from 0 to 16.6 % in *cytb* and from 0 to 8.4 % in 16S sequences. The minimum and maximum genetic distances of the two mitochondrial markers between clades were 5.9 % and 16.2 % for *cytb* and 2.3 % and 6.4 % for 16S (Table 2). The genetic divergence ( $p$ -distance) within each clade is presented in Table 3.

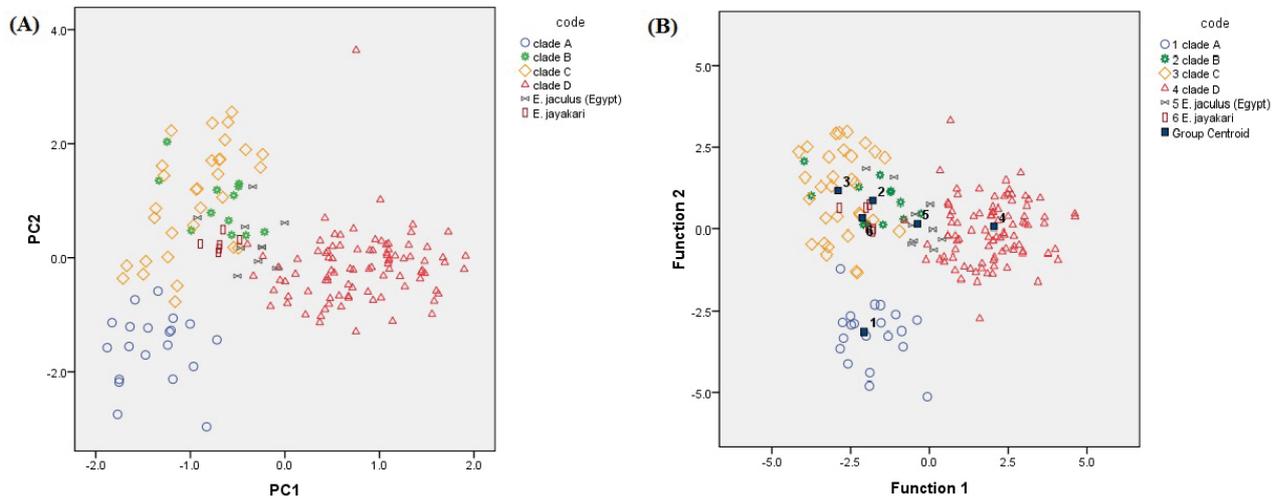
The genetic distance between *E. m. miliaris* and *E. t. tataricus* specimen from type localities was 2.2 % for *cytb* and 0.6 % for 16S and that between *E. m. miliaris* and *E. m. nogaorum* from the type locality was zero for both *cytb* and 16S.

**Morphological analyses**

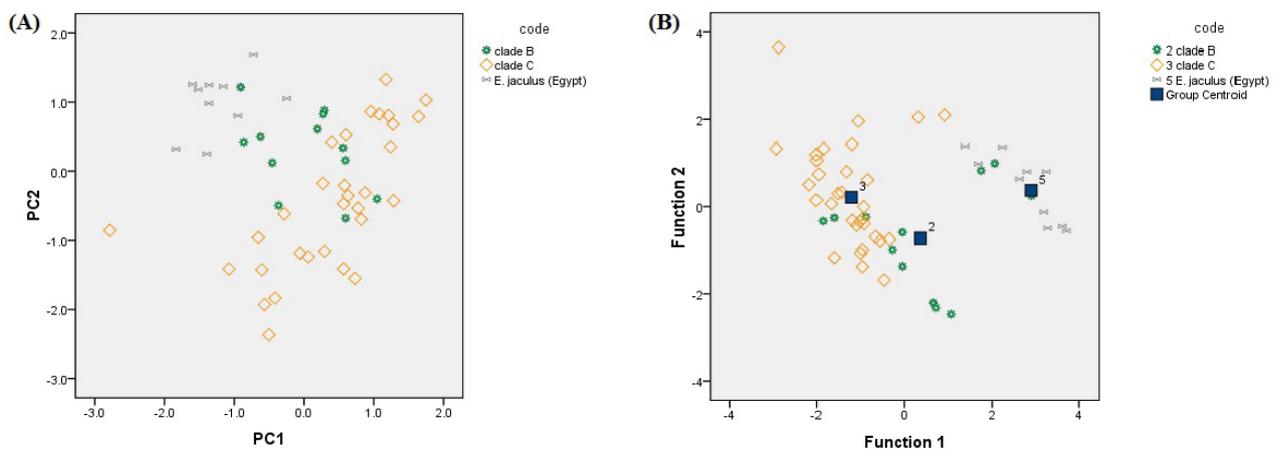
Univariate analyses revealed that all the studied characters were significantly different between major clades ( $P < 0.05$ ; Table 4). The results of LSD post hoc pairwise test indicated that the largest number of significant



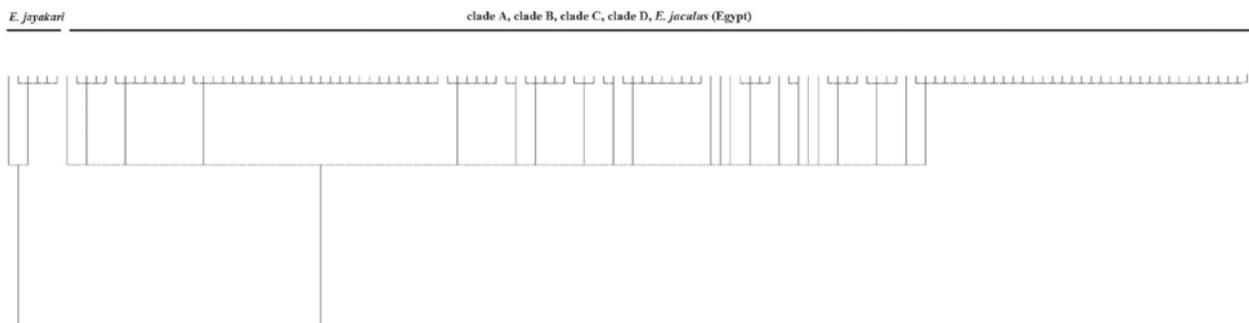
**Figure 2.** Bayesian inference phylogram based on 1263 base pairs of the concatenated cytochrome *b* and 16S sequence data set. The numbers next to the nodes indicate clade credibility in the Bayesian analysis. The specimens from the type localities are shown with an asterisk.



**Figure 3.** Plots of A: principal components and B: discriminant analyses of the populations of the genus *Eryx* for the seven studied characters (RE, LAB, BE, PIN, BEN, DS and VS).



**Figure 4.** Additional A: principal components and B: discriminant analyses among the populations of clades B, C and *E. jaculus* from Egypt.



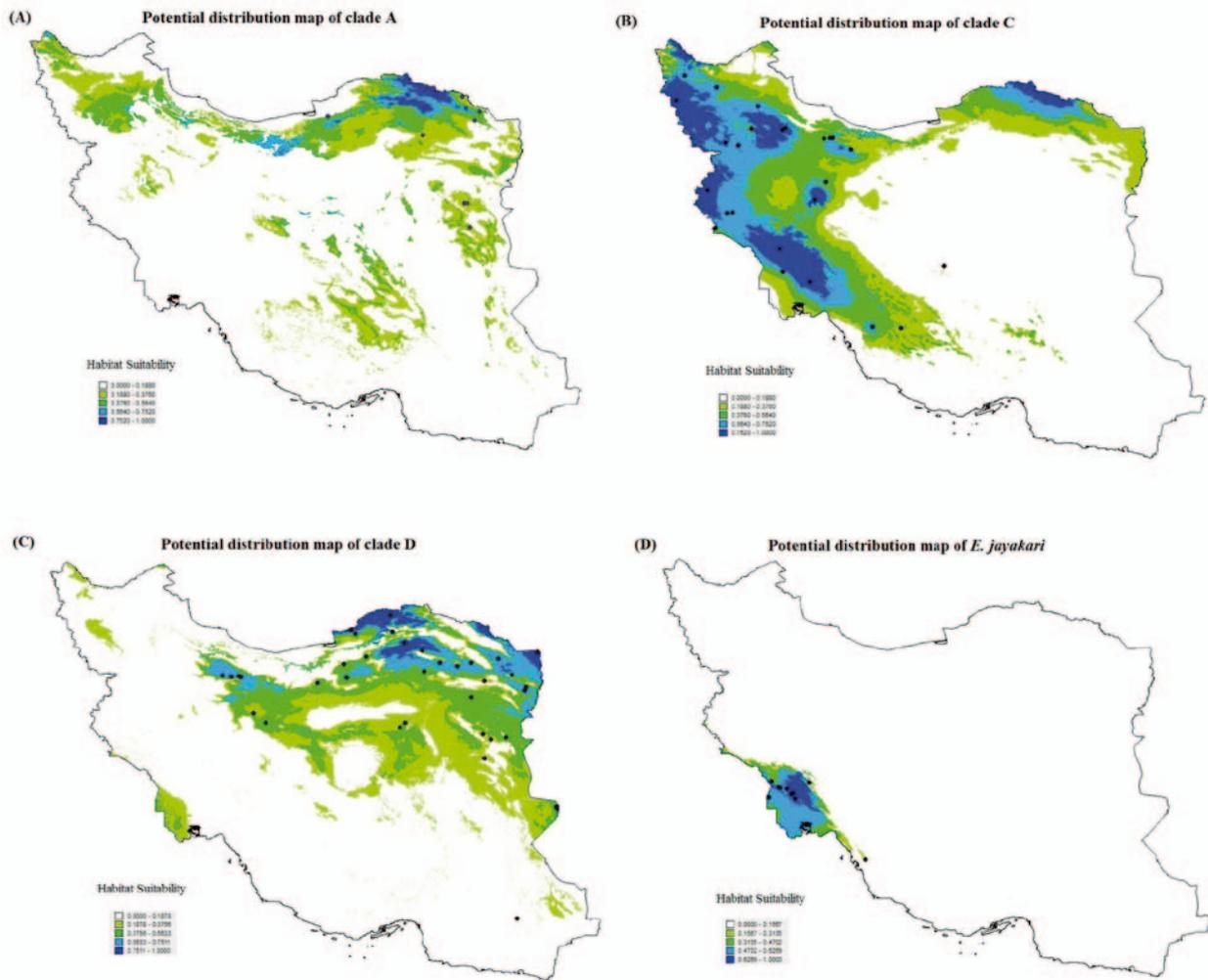
**Figure 5.** Dendrogram of cluster analysis showing morphological relationships in studied populations of the genus *Eryx*.

character differences are seen between the specimens of clades D and A and between clades D and B (Table S3).

In the PCA analysis, the first two components included 67.72 % of the total variance (Table S4, Fig. 3A). Based on classification results of DCA, 90.1 % of the original grouped cases were correctly classified. Five functions were computed for DCA and the first two functions explained 91.3 % of the total variance (Table S5, Fig. 3B). The plots of PCA and DCA indicate that the specimens of

clades B, C and the populations of *E. jaculus* from Egypt and *E. jayakari* are not distinguishable from each other. The members of clades D and A are well separated from each other and also from other populations in both plots (Figs. 3A and 3B).

Additional multivariate analyses (PCA and DCA) were also performed on the populations of the clades B, C and *E. jaculus* from Egypt to check their separation in more details. In both analyses, the population of *E. jaculus*



**Figure 6.** Potential distribution maps of Iranian clades of *Eryx*. A: the northeastern Iran clade (clade A; morphologically identified as *E. elegans* and *E. jaculus*); B: the western Iran clade (clade C; morphologically identified as *E. jaculus*); C: the *E. miliaris* and *E. tataricus* clade (clade D) and D: *E. jayakari*.

from Egypt was separated from the other two clades (B and C) (Figs. 4A and 4B). In the PCA, the first and second component respectively included 30.35 % and 24.38 % of total variance. BE, PIN and DS contribute most to the first component, and RE and VS contribute most to the second component. In the DCA, the first function with 92.9 % of total variance has the most correlation with PIN, BE, DS and RE; and the second function with 7.1 % of total variance has the most correlation with VS, BEN and LAB.

The results of the studied multistate characters are presented in Table S6. Based on the results of the cluster analysis (using multistate characters), only *E. jayakari* is well separated from other populations and no separation was detected between other clades (Fig. 5).

#### Ecological niche modeling and niche overlap

The AUC value, percent contribution, and permutation importance of the applied environmental factors are mentioned in Table S7. The niche modeling of clade A reveals that the northern parts of Iran, especially the Kopet Dagh and Alborz Mountains as the most suitable habitat for the presence of the members of this clade

(Fig. 6A) with annual mean temperature as the most effective factor influencing their distribution (Table S7). The specimens of clade C distributed from north-west to south-west of Iran along the Zagros Mountains with one specimen from Yazd Province. The western part of Iran in addition to highland areas in NE Iran are revealed as the most suitable habitats for this clade (Fig. 6B). Annual precipitation is the most effective factor in the distribution of western clade (Table S7). The members of clade D distributed in the high land area and foothills of Alborz, Kopet Dagh and Eastern Mountains in addition to the Dasht-e Kavir and more interior area. The most suitable habitat for the members of clade D is located in the highland areas of NE and N Iran (Fig. 6C) and the distribution of *E. jayakari* specimens in Iran is limited to SW Iran (Fig. 6D). Altitude has the most effect on the distribution of members of both clades D and *E. jayakari* (Table S7). The Schoener's D metric value varied from 0.009 (between the populations of clades A and *E. jayakari*) to 0.431 (between the populations of clades A and C) (Table 5).

## DISCUSSION

### Clade A: the north-eastern clade: *E. elegans*

The clade A includes specimens with two post internasal scales belonging to *E. elegans* and those that have previously mistakenly been identified as *E. jaculus* (Eskandarzadeh et al., 2013; Rastegar-Pouyani et al., 2014). Members of this clade inhabit foothills and substrates covered with gravel and rocks. As is obvious from previous studies (Table S1), the reported maximum number of dorsal scales in mid-body for *E. elegans* was 43. It is notable that the number of dorsal scales in mid-body overlaps in all the other studied species (Table 4). This number in the studied specimens from north-east of Iran and Afghanistan (including the holotype and three syntypes ZISP 8711, ZISP 8473 and ZISP 8462 of *E. elegans*) varied from 35 to 46. The minimum number of dorsal scales in the mid-body in *E. jaculus* specimens from Egypt and in the members of clade D were 43 and 39, respectively. Considering the overlap in the number of dorsal scales in the mid-body among the studied populations, this character cannot be an informative, distinctive character for separation of different erycid species.

**Table 3.** Calculated genetic divergence (*p*-distance) within each clades of the genus *Eryx* for the two mitochondrial markers *cytb* and 16S with standard error. One *cytb* sequence of *E. jayakari* included in this study.

	<i>cytb</i>	16S
Clade A	0.572%±0.16	0.086%±0.082
Clade B	0.189%±0.079	0.038%±0.036
Clade C	1.546%±0.305	0.332%±0.139
Clade D	1.854%±0.297	0.619%±0.156
<i>E. jayakari</i>	-	0.605%±0.298

**Table 4.** Descriptive statistics and univariate analysis of studied morphological (meristic and metric) characters among *Eryx* populations. See Table 1 for descriptions of character abbreviations.

		RE	LAB	BE	PIN	BEN	DS	VS	WI/DEM
Clade D	Mean ± SE	11.54±0.09	11.88±0.09	7.63±0.07	3.31±0.06	3.75±0.05	47.1±0.28	182.59±0.89	0.88±0.02
N=92	Range	10-13	10-14	6-9	2-4	3-4	39-54	167-235	0.6-1.25
Clade A	Mean ± SE	8.89±0.20	9.40±0.13	6.63±0.11	2.00±0.00	2.94±0.06	39.75±0.61	167.95±1.64	1.31±0.04
N=20	Range	7-11	8-10	6-7	2-2	2-3	35-46	156-184	1.2-1.7
Clade B	Mean ± SE	9.58±0.19	10.75±0.18	6.42±0.29	2.25±0.13	3.00±0.00	49.33±0.64	190.92±1.33	1.08±0.04
N=12	Range	9-11	10-12	5-8	2-3	3-3	45-53	185-198	0.90-1.37
Clade C	Mean ± SE	9.31±0.23	10.53±0.16	5.70±0.11	2.07±0.05	2.96±0.04	51.07±0.59	185.34±2.00	1.41±0.06
N=31	Range	7-11	9-12	5-7	2-3	2-3	44-57	166-203	1.1-1.95
<i>E. jaculus</i> (Egypt)	Mean ± SE	10.00±0.21	10.50±0.17	7.22±0.15	3.00±0.00	2.90±0.10	47.00±0.76	191.10±1.97	-
N=10	Range	9-11	10-11	7-8	3-3	2-3	43-51	181-200	-
<i>E. jayakari</i>	Mean ± SE	10.17±0.17	10.83±0.17	5.00±0.00	3.00±0.00	3.00±0.00	47.00±1.05	176.00±1.96	0.43±0.10
N=6	Range	10-11	10-11	5-5	3-3	3-3	45-50	172-181	0.25-0.6
<i>p</i> value		0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

### Clades B and C: the Iraqi and western Iran clades

In spite of the overlapping in multivariate analyses (Figs. 3A, 3B, 4A, and 4B), two characters, BE and WI/DEM, were significantly different between these two clades ( $P < 0.05$ ) (with overlapped limits) (Tables 4 and S3). The genetic distance between these two clades was considerable (Table 2). The *Eryx* population of western Iran inhabit mostly in foothills, among and under rocks and shrubs and in some regions (e.g. in Khuzistan plain in SW Iran) in desert areas with gravel substrate. The specimens of Iraq were collected from agriculture and pasture lands with a softer substrate. The number of post internasal scales in these two clades was two or three.

The post hoc analysis of morphological data of the Egyptian population (type locality of *E. jaculus*) showed that this is significantly different from populations of both Iraq and western Iran ( $P < 0.05$ ) (Table S3). Additional multivariate analyses showed that the Egyptian population was separated from Iraqi and western Iran populations (Figs. 4A and 4B). *Eryx jaculus* in north Africa generally occupies different kinds of habitats: including sandy coastal areas, and sometimes sparse forests and mountains with sparse tree cover and clayey and marly soil, as well as in the steppes and the desert limits (Ouni Ridha pers. comm.).

Considering the morphological and habitat differences between the populations of *Eryx* in western Iran and Egypt, and the considerable genetic distance between the populations from western Iran and Iraq, we suggest that the population of *Eryx* in western Iran can be considered as a distinct species from *E. jaculus*. Considering the results and lack of the tissue sample of *E. jaculus* from the type locality, here we named the population of *Eryx* in western Iran as *Eryx* sp. and those from Iraq as *Eryx* cf. *jaculus*.

**Table 5.** Calculated Schoener's D metric between major clades of *Eryx* in Iran reveals the degree of ecological niches overlap.

	Clade A	Clade C	Clade D
Clade C	0.431		
Clade D	0.414	0.359	
<i>E. jayakari</i>	0.009	0.116	0.083

#### Clade D: *E. miliaris*

The genetic variations among the members of clade D, including specimens of *E. t. tataricus*, *E. m. miliaris* and *E. m. nogaorum* from Russia, Chechnya, Kalmykia, Kazakhstan and Iran, is low (Table 3). The genetic distance between the specimens of *E. t. tataricus* and *E. m. miliaris* from their type localities reveal that these two taxa should be attributed to the same species and considering the principle of priority, *E. tataricus* is a junior synonym of *E. miliaris*. Also considering the genetic distance between *E. m. miliaris* and *E. m. nogaorum*, the subspecies rank for *E. m. nogaorum* seems to be invalid. The members of this clade dwell in various types of habitats, including agriculture fields, sandy deserts, semi-desert and desert areas with a hard substrate, foothills, among and under rocks and shrubs. In spite of the diversity in morphological characters and habitat, the genetic diversity among populations of this major clade is low. Maximum intraspecies genetic distance (except for a specimen from Bampoor in south-eastern Iran) for *cytb* is 3.4 %. The maximum calculated intraspecies genetic distance for the specimen from Bampoor is 5.9 % for *cytb*. Based on the potential distribution map of the clade D (Fig. 6C), the locality of this specimen in south-eastern Iran is out of the predicted suitable habitat. So, more specimens from the area (south-eastern Iran) are needed to shed more light on its taxonomic status. Based on the molecular and morphological results the members of this clade are well separated from other studied *Eryx* spp. The position of eyes in the members of this clade is dorsolateral, upward and/or lateral with 2, 3 or 4 post internasal scales. The morphology of this species has been well discussed in previous studies (Eskandarzadeh et al., 2013; Rastegar-Pouyani et al., 2014; Zarrintab et al., 2017).

#### *E. jayakari*:

*Eryx jayakari* is the sister taxon to the other studied species which were considered here, and well distinguished from other species in the morphological cluster analysis. The bent scale on the tail tip and absence of the oblique line from the eye to the corner of mouth are the prominent characteristics of this species in comparison to other studied *Eryx* spp. This species inhabits sand dunes of south-western Iran. Based on the findings of niche overlap, the most overlap is between *E. elegans* and *Eryx* sp. (43 %) and the least overlap is between *E. elegans* and *E. jayakari* (0.9 %). The most amount of niche segregation among the studied population of *Eryx* spp. in Iran is seen between *E. jayakari* and the other species (Table 5). As mentioned above, *E. jayakari* is a well-adapted species

for a burrowing lifestyle and is highly depended on sand dunes. The niche similarities among *E. elegans*, *E. miliaris* and *Eryx* sp. are the highest (Table 5).

## CONCLUSION

Based on the results, we confront cryptic diversity in studied erycids, which is not uncommon among serpentes (Feldman & Spicer, 2002; Rawlings & Donnellan, 2003; Marin et al., 2013; Avci et al., 2015; Fathinia et al., 2017; Toriki, 2017; Portillo et al., 2018; Ruane et al., 2018; Wüster et al., 2018). In spite of the high morphological similarities in the examined groups of erycids, the results reveal considerable genetic divergence. *Eryx jayakari* is the only species that can be morphologically distinguished by key characters from the other studied erycids in the region. The descriptions of morphological characters of four species (*E. elegans*, *E. miliaris*, *Eryx* sp. and *E. jayakari*) are revised here. More specimens, especially the molecular data of *E. jaculus* from Egypt, are needed to clarify the exact taxonomic status of the Iraqi population (*Eryx* cf. *jaculus*).

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## How does captivity affect skin colour reflectance of golden mantella frogs?

Luiza F Passos<sup>1</sup>, Gerardo Garcia<sup>2</sup> & Robert Young<sup>3</sup>

<sup>1</sup> School of Psychology and Natural Sciences, James Parsons Building, Liverpool John Moores University, Liverpool, L3 3AF, UK

<sup>2</sup> Chester Zoo, Cedar House, Caughall Road, Upton by Chester, Chester CH2 1LH, UK

<sup>3</sup> School of Environment and Life Sciences, Peel Building, University of Salford Manchester, Salford, M5 4WT, UK

Coloration is an important trait for social communication in amphibians, being used in intra- and intersexual signalling to express information about individual body condition and health state, amongst other things. The striking colour pattern exhibited by some anuran species are also used in “aposematic” signals to advertise unpalatability to predators. The aim of this study was to investigate how the captive environment affects the colour of golden mantella frogs by comparing captive reared frogs with wild conspecifics. A USB-2000 portable diode-array spectrometer and a xenon strobe light source were used to perform spectrophotometric measurements on captive and wild populations. Hue, chroma and brightness of skin colour were analysed as well as body condition using the scaled mass index. Analyses showed variation among populations, but significant differences were only found between captive and wild populations. Generalised linear mixed models were used to evaluate the effects of body condition on colour variation and showed that animals with lower body condition from one captive population had significantly different coloration than their wild counterparts. Importantly, one captive population was not greatly different in coloration from their wild counterparts – demonstrating that this problem is not inevitable in captivity. These results can have important implications for reintroduction programmes.

*Keywords:* amphibians, body condition, coloration, conservation

### INTRODUCTION

The role of coloration and colour vision in animal communication has been a fundamental question in evolutionary biology for many decades.

The current global amphibian crisis has resulted in an unprecedented rate of amphibian biodiversity loss, resulting in a growth of captive breeding as a conservation tool for amphibians (Griffiths & Pavajeau, 2008). Maintaining captive populations is important in terms of species conservation for potential reintroduction into the wild (Harding, Griffiths & Pavajeau, 2016).

One of the biggest challenges faced while keeping amphibians in captivity is mimicking their diet and nutritional needs (Livingston et al., 2014). This can directly impact many aspects of an individual’s ecology, including skin pigmentation (Brenes-Soto & Dierenfeld, 2014). It is common to observe amphibians kept in captivity displaying a faded coloration in comparison to their wild counterparts (Brenes-Soto & Dierenfeld, 2014). In amphibians, skin coloration influences courtship and mate preference, recognition of breeding partners and perception of fitness, consequently affecting breeding success, resulting in advantages for selective females and for strikingly coloured males (Brenes-Soto et al., 2017).

Skin colour may also inform about physiological conditions, such as oxidative and immune status (McGraw, 2005), and parasite load (Molnár et al., 2013). Carotenoid-based colorations can be used to assess physiological and health status of an individual while coloration associated with melanin informs about hormonal levels and social dominance (Candolin, 2003).

The remarkable colour patterns displayed by many anuran species (Hoffman & Blouin, 2000) are also used as conspicuous “aposematic” indicators; warning signals to advertise unpalatability to potential predators (Hegna et al., 2013; Maan & Cummings, 2012; Ruxton et al., 2004). By reducing the frequency of costly encounters with predators, the protection that aposematism confers can lower the costs of otherwise risky behaviours, including foraging and sexual displays (Dugas et al., 2015).

Divergent antipredator strategies such as aposematism not only require integration of physiology, morphology and behaviour; they also alter the way selection acts on other suites of traits (Stankowich & Blumstein, 2005). It is expected that, in some scenarios, an increase in toxicity should also coincide with a greater visual contrast of the warning signal, with “nastier” animals “shouting loudest” (Maan & Cummings, 2012; Speed & Ruxton, 2007). This is because the greater risk

*Correspondence:* Luiza Passos (l.figueiredopassos@ljmu.ac.uk)

of detection and attack on highly conspicuous prey can be compensated for by the stronger predator deterrence induced by high toxicity (Darst et al., 2006). A positive relationship may also emerge from physiological or energetic trade-offs between the two traits (Blount et al., 2012).

Colour refers to a sensory experience, not an objective quantity, and how animals perceive this information can vary quite considerably according to their visual system and how they process colour (Maia et al., 2013). The vertebrate retina contains two types of photoreceptor: rods that function at low light levels, and cones that function in daylight and provide the basis for colour vision. Colour vision requires at least two spectrally distinct classes of cone cells combined with a nervous system that can compare the quantum catch of one class of cone with the quantum catch of another (Bowmaker & Hunt, 2006).

Animals have evolved their visual sensitivity to match aspects of their photic environment (Bowmaker & Hunt, 2006), with modern teleosts, reptiles and birds possessing rods and four spectral classes of cones, each representing one of the five visual pigment families, giving these species the potential for tetra chromatic colour vision. In contrast, mammals, due to their nocturnal ancestry, have rod-dominated retinas with colour vision reduced to a basic dichromatic system (Bowmaker, 2015).

However, 'colour' refers to a sensory experience, not an objective quantity, and the realisation that animals can vary quite considerably in their visual system and how they process this information. The role of coloration and colour vision in animal communication has been a fundamental question in evolutionary biology for many decades (Maia et al., 2013). Colour is involved in a wide range of biological phenomena such as thermoregulation, crypsis, mimicry, communication as well as indicating health status of an individual (Endler, 1993; Forsman et al., 2002; Robertson & Rosenblum, 2009). Therefore, the quantification of animal colour variation is a crucial component of conservation and ecological studies. If captive animals are bred for conservation purposes and reintroduction is a future goal, these issues are of major concern. The aim of this study was to investigate if the captive environment is affecting the colour of golden mantella frogs and, if so, to quantify this difference.

## METHOD

### Ethical Approval

All the research reported in this study was approved by the Ethics Commission of Chester Zoo, UK, and from the Research Ethics Committee at the University of Salford, and it conforms to all regulations and laws in all relevant countries in relation to care of experimental animal subjects. To collect data from wild individuals, permission (through permits) was obtained from the government of Madagascar. Furthermore, we can confirm, from our post-experimental monitoring, that no animals suffered any injuries, became ill or had their survivorship negatively affected because of this study.

### Study subjects

The golden mantella frog (*Mantella aurantiaca*) is a critically endangered species (Vence & Raxworthy, 2009) found only in Madagascar, with a distribution restricted to a fragment of forest that is under severe threat from mining, agriculture, timber extraction and over-collecting for the pet trade (Randrianavelona et al., 2010). According to the Amphibian Ark, ex situ assistance is vital for the long-term survival of the golden mantella frog (Johnson, 2008). This is an ideal species to test the effects of captivity on coloration because the species is naturally only one consistent orange colour.

### Study sites

Mangabe area (Madagascar wild): Most breeding ponds for the golden mantella frogs are found in this area according to recent studies concerning conservation priority sites for mantella frogs. Also known as, the "blue forest", Mangabe is a site of international biodiversity importance, divided between two administrative districts, Moramanga in the north and Anosibe An'ala to the south. Data sampling (15 males and 15 females) for this study was done in a protected area of the Moramanga region.

Ambatovy Mining Site (Madagascar wild): Ambatovy's Mine is located within a species-rich region of Madagascar at the southern end of the remaining Eastern Forest Corridor at Moramanga region. As part of the Environmental Management Plan, there is a Conservation zone of native forest kept under pristine conditions by the mining company. During this study animals from the Conservation zone (15 males and 15 females) were sampled.

Mitsinjo Association Captive Breeding Centre (Madagascar captive): Mitsinjo Association is a community-run conservation organisation. This is Madagascar's first biosecure facility to safeguard amphibians from extinction, currently maintaining a genetically viable population of the golden mantella frog taken from the Ambatovy mining site (i.e., genetic founders), their offspring (F1) that are intended for reintroductions at artificially created breeding and natural ponds. Animals are kept in tanks with aquarium gravel as substrate, a potted plant, coconut shells for hiding. Animals were fed a variety of live invertebrates (fruit flies- *Drasophila* sp., isopods – *Trichorhina* sp., springtails - *Collembolas*). We sampled 8 males and 8 females founder frogs (i.e. wild caught) and the same number from their F1 frogs.

Chester Zoo (UK): Chester Zoo is actively involved in the conservation of the golden mantella frogs in Madagascar. The zoo currently maintains two ex situ groups of *M. aurantiaca*, one is on public display at the Zoo's Tropical Realm exhibit and a second group is kept off show in a biosecurity container specifically for conservation-related research. Animals have been in captivity for more than 5 generations. Animals are kept in naturalistic tanks with different live species of plants, moss for substrate, water, hiding places under rocks and UV light. Animals

are fed different live invertebrates (fruit flies- *Drosophila* sp., isopods – *Trichorhina* sp., springtails - *Collembolas*). We sampled 8 males and 8 females from the Chester Zoo off-show colony.

### Spectrophotometric measurements

We used a USB-2000 portable diode-array spectrometer and a PX-2 xenon strobe light source (both from Ocean Optics, Dunedin, USA), probe positioned at an angle of 90°, to perform spectrophotometric measurements. To exclude ambient light and standardise measuring distance, a cylindrical plastic tube was mounted on the fibre optic probe. The equipment allowed spectral analyses to be conducted in the 300 and 700 nm range. Spectral reflectance measurements were always taken of each individual from the dorsum, three consecutive measurements per frog, with only adult frogs during breeding season used during this study. Colour measurements sampled the most visible surfaces to obtain a representative sample (within an individual) of the spectral shape of the entire body. Golden mantellas do not show any sexual dichromatism, allowing the use of both male and females. Summary variables for the colour measurements were calculated. Spectralon white standard measurements were taken between each individual to account for lamp drift. This methodology was based on previous studies measuring colour variation in different species (Crothers, et al., 2011; Maan & Cummings, 2008; Siddiqi et al., 2004).

### Colour analyses

Colour may be described by three essential parameters: hue, chroma, and brightness and all three variables were analysed as they are customarily used in studies of animal coloration, thereby facilitating comparisons between studies. Brightness (Qt) may be defined as the total intensity of light (Endler, 1990). Qt was calculated by summing the percentage reflectance (R) across the entire spectrum (R300 and R700).

Hue represents the common meaning of colour, for example, violet, blue, orange, green (Endler, 1990); In general, the hue of a spectrum is a function of its shape. Hue is correlated with the wavelength of the maximum slope, as well as the sign of the slope (Endler, 1990). It is the wavelength within the visible-light spectrum at which the energy output from a source is greatest Hue (nm) was measured as the wavelength of maximum reflectance.

Chroma is a measure of the 'purity' or 'saturation' of a colour and is a function of how rapidly intensity changes with wavelength (Endler, 1990). Chroma was calculated as relative medium wavelength chroma (MC, calculated as  $(R_{max} - R_{min})/Qt$ ).

Brightness, hue and chroma differences between populations were analysed with a mixed model with origin (wild or captive) as fixed factors and populations as random factors. Data were analysed using the Pavo (Maia et al., 2013) package from R studio (R Studio Team 2015). The data from each population were plotted on the same graph to confirm standardisation of sampling, and no error from the sampling design was found. Data from

different populations were compared based on colour distance and colourimetric variables. Four different visual systems: human, a snake (Boidae - Bowmaker, 2015), a Scincidae lizard (New et al., 2012), representing potential predators, and a diurnal poison frog (*D. pumilio*, Siddiqi et al., 2004) under an ideal illumination condition, were used to calculate colour distance. The colour distance analyses in Just Noticeable Distance units (JND) (Wen, 2012) were used to infer if different visual systems would be able to notice differences between the different populations. In general, when  $JND < 1$ , the spectral pair is barely distinguishable under ideal conditions, and as JND becomes greater, discrimination can be made more rapidly and under increasingly unfavourable viewing conditions (Siddiqi et al., 2004). Due to the lack of data on golden mantella photoreceptor sensitivity, sensitivity data used in the analysis were those from a species with similar activity pattern (i.e. aposematic diurnal).

### Body Condition

Body condition is a valuable index that can be assessed using reliable, non-invasive techniques, and it can identify the health condition of a population before any deleterious effects can be observed (MacCracken & Stebbing, 2012). Body condition (BC) was assessed using the scaled mass index proposed by Peig & Green (2009). This method is independent of size and can be used for comparison between different populations; those characteristics potentially make it superior to the traditional residual indices and, reportedly have worked well in amphibian studies (MacCracken & Stebbing, 2012, Michaels et al., 2014). The scaled mass index of condition (SMI) was calculated as follows:

$$SMI = M * [SVL_i / SVL]^{bSMA}$$

Where M and SVL are the mass and snout-ventral length of the individual, SVL0 is the arithmetic mean SVL of the population, and bSMA is the standardised major axis slope from the regression of ln mass on ln SVL for the population (Peig & Green, 2009). Each individual SVL was measured ( $\pm 0.01$  mm) using a digital caliper (Lujii 150 mm, Omiky) mass was measured using a precision scale (accurate to 0.01 g, Smart Weigh ACC200 AccuStar). Body condition was calculated for each individual and groups were compared using an ANOVA test followed up by a post-hoc test.

## RESULTS

The colourimetric variables analysis showed no differences between wild and captive animals for brightness, but significant differences ( $p < 0.0001$ ) for hue and chroma were found.

The colour distance analyses (Table 1) showed that all visual systems tested would be able to detect a noticeable difference when comparing wild individuals from Mangabe and with individuals from Mitsinjo, for both founder and F1 generations. The individuals from Chester Zoo and the animals from Mangabe had a low or non-detectable difference in the colour distance analyses.

**Table 1.** Colour distance comparisons between golden mantella frog groups in colour distance, Just Noticeable Distance (JND) Units. JND unit reference values: 0-1 not detectable; 1-2 Low; 2-3 Medium; 3-4 High; 4-5 Very high (Bold); >6 Extremely high (Bold). W = wild population; C = captive population.

Groups	Colour distance (JND units) *			
	Human	Snake	Lizard	Frog
Chester <sup>c</sup> –F1 (Mitsinjo) <sup>c</sup>	<b>10.44</b>	4.95	<b>6.57</b>	4.73
Chester <sup>c</sup> –Founders (Mitsinjo) <sup>c</sup>	<b>10.00</b>	3.72	<b>5.61</b>	4.88
Chester <sup>c</sup> – Mangabe <sup>w</sup>	1.65	1.37	1.41	1.67
Chester <sup>c</sup> – Ambatovy <sup>w</sup>	3.36	3.61	1.15	2.87
F1(Mitsinjo) <sup>c</sup> – Founders (Mitsinjo) <sup>c</sup>	2.62	2.38	2.44	3.09
F1(Mitsinjo) <sup>c</sup> – Mangabe <sup>w</sup>	<b>9.32</b>	<b>6.21</b>	<b>6.33</b>	<b>5.41</b>
F1(Mitsinjo) <sup>c</sup> –Ambatovy <sup>w</sup>	<b>5.10</b>	4.75	<b>6.28</b>	<b>5.13</b>
Founders (Mitsinjo) <sup>c</sup> – Mangabe <sup>w</sup>	<b>11.91</b>	<b>5.62</b>	<b>5.76</b>	<b>5.82</b>
Founders (Mitsinjo) <sup>c</sup> –Ambatovy <sup>w</sup>	<b>7.64</b>	4.71	4.60	<b>5.27</b>
Mangabe <sup>w</sup> –Ambatovy <sup>w</sup>	1.32	2.78	2.52	3.55

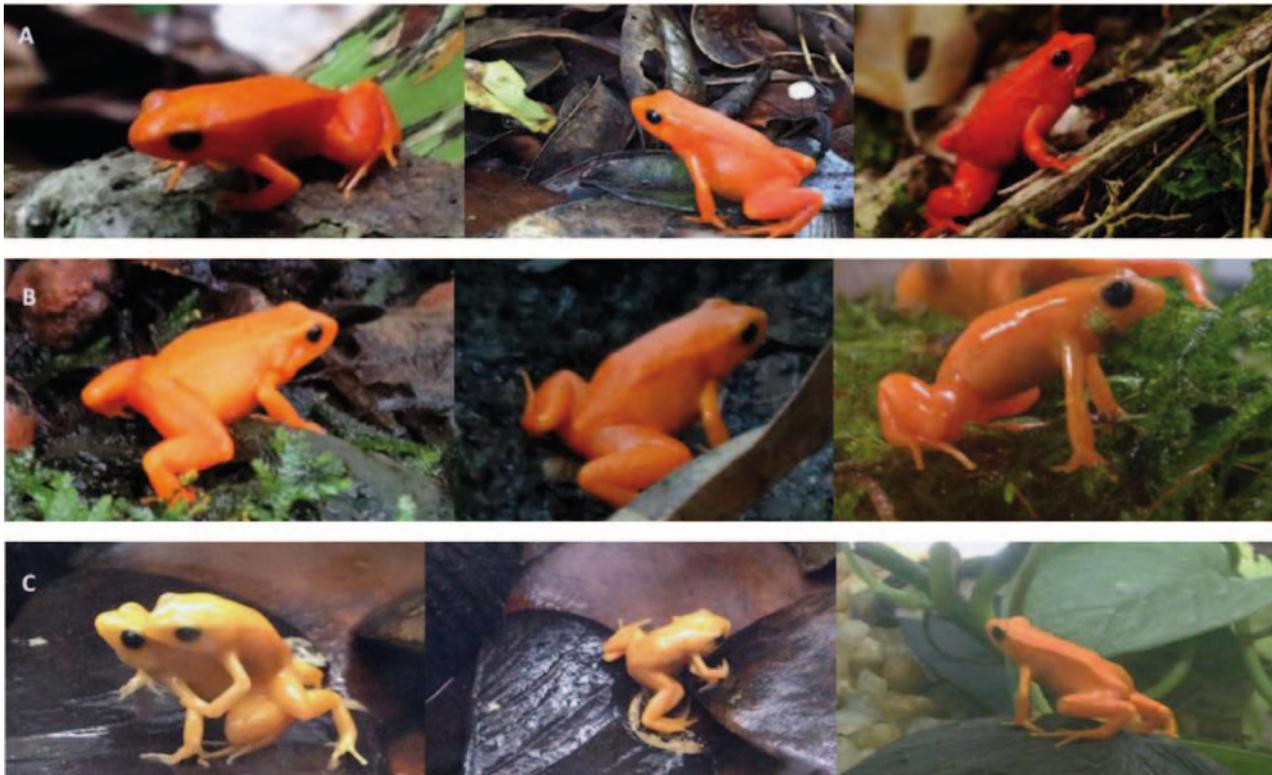
Body condition analyses showed a significant difference between the groups ( $F=7.109$ ,  $df=7$ ,  $p<0.001$ ). The *post-hoc* analyses confirmed a significant difference ( $p<0.0001$ ) between the Mitsinjo group and all other groups. Frogs kept at Mitsinjo had a significantly lower body condition, for both founder and F1 generations.

Generalised linear mixed models were used to evaluate the effects of body condition on the chroma, and hue variation. Location was included as a random factor (chroma: variance 0.38, St. Dev.  $\pm$  0.62, hue: variance 118.13, St. Dev.  $\pm$  10.86). The selected model with an Akaike information criterion (AIC) of 1195.1 for chroma and AIC of 332.80 for hue showed that body

condition had a strong impact on both chroma ( $F=7.17$ ,  $df=1$ ,  $p<0.001$ ) and hue ( $F=25.83$ ,  $df=1$ ,  $p<0.001$ ).

## DISCUSSION

In this study, we showed that different populations of golden mantella frogs vary in colour, most notably between captive and wild conditions. In general, wild frogs were brighter, more colourful and were a different shade of orange/red in comparison to captive frogs, especially those from the captive populations in Madagascar (Fig. 1). A relationship between lower body condition and duller coloration was also observed. The



**Figure 1.** Examples of skin coloration from the three groups of golden mantellas. **A)** Wild individuals from Mangabe; **B)** Chester Zoo off-show individuals; **C)** Mitsinjo F1 individuals.

implication of the differences observed could be negative survival or lower reproductive success if captive frogs were to be released to the wild (Rojas, 2016).

The hue comparison results showed that the golden mantella frogs' skin coloration has been affected by captivity with a significant difference when compared to wild conspecifics. However, during the colour distance analysis, the visual systems used would only have the ability to distinguish between animals kept at Mitsinjo and the other populations. The results showed that, even though there were significant differences between all populations, most of these would be low or non-detectable by the visual systems tested, with the exception of the Mitsinjo colony. Both founders and F1 frogs at Mitsinjo presented a coloration that differed significantly from their wild counterparts. This shows that the change in the skin coloration is not a generalised effect of captivity, since frogs kept at Chester Zoo did not display such a dramatic change.

Results showed that the individuals kept at the Mitsinjo breeding centre had a much lower body condition than any other group. Body condition is a result of many variables including nutritional status, stress levels, and abiotic variables (MacCracken & Stebbings, 2012). Replicating diverse diets in captivity creates a range of challenges including issues of environment, economics and practicality of insect husbandry (the main food item - Livingston et al., 2014). Animals tend to have nutritional imbalances, due to deficiencies of one or several nutrients; for example, low quantities of carotenoids (which are known to affect frog coloration) are common in commercially-reared insects (Finke, 2015).

A relationship between body condition and loss of skin coloration was also detected; animals with lower body condition also had a greater difference in skin coloration according to the colour distance analyses. Animal coloration is a product of different variables, including pigments obtained from the diet, such as carotenoids. Dietary carotenoids are associated with yellow, orange, and red coloration and increased levels can lead to brighter coloration and changes in hue (Umbers et al., 2016). The consequences of limited carotenoid availability on ornamental coloration have been shown in both field and captive conditions (Hill, 1999; Brenes-Soto & Dierenfeld, 2014). Healthier animals in good body condition, after meeting their physiological needs of pigments for immune and antioxidant response, can accumulate enough pigments to increase coloration, maximising sexual display (Hill, 1999). The preference of females for males with brighter coloration shows the choice for phenotypic quality connected with direct or indirect genetic benefits (Brenes-Soto et al., 2017). Striking coloration reveals an individual animal's ability to provide material advantages, such as fertility, high quality territory, nutrition and the maintenance of the genetic variation (Andersson & Simmons, 2006, Zamora-Camacho & Comas, 2019).

Previous studies have shown a clear link between skin coloration, body condition and health status in amphibians (Brenes-Soto et al., 2017), with animals with

higher levels of glucose and protein concentration in the blood showing a darker yellow and orange coloration (Brenes-Soto et al., 2017). The faded coloration and lower body condition observed on animals kept at Mitsinjo breeding centre could be interpreted as a warning sign of animals' health conditions.

Alteration of pigmentation could hypothetically affect potential recognition of breeding partners, perception of fitness, and could thus have an indirect effect on health and reproductive output (Crothers et al., 2011, Brenes-Soto & Dierenfeld, 2014, Ogilvy et al., 2012). All these factors contribute to the complexity of maintaining the frogs' wellbeing in captivity (Speed & Ruxton, 2007).

Species recognition is a fundamental problem for animals in social contexts (Kraaijeveld-Smit et al., 2006); skin coloration is also involved in the sexual behaviour of many species. The colour distance analyses using the spectral sensitivity of a diurnal poison frog have shown that frogs would be able to detect coloration differences. Diurnal species of amphibians, such as the golden mantella frogs, use visual signals as an important part of their courtship and mate selection (Maan et al., 2004), and bright coloration is an important one (Bowmaker, 2015). For example, females of different taxa prefer to mate with more colourful or brighter individuals (Maan & Cummings, 2008, Ogilvy et al., 2012). Releasing animals with different skin coloration could, potentially, compromise their breeding opportunities and, for a reintroduction to be successful, individuals released for conservation purposes must not only survive but also must breed (Giligan & Frankham, 2003; Mathews et al., 2005). If reintroduced animals survived, there is a chance that due to this phenotypic difference, captive animals may be more likely to mate with other captive-born animals, which could lead to producing two morphologically separate populations of animals (Slade et al., 2014). A low frequency of breeding between captive-bred and wild animals also means that no improvement of the wild population's genetic diversity and any possible negative genetic changes acquired in captivity would continue to be expressed in their offspring, limiting their fitness in the wild (Slade et al., 2014).

Aposematism is an important anti-predator strategy, which signals unprofitability of prey to a predator via conspicuous traits associated with unpalatability (Dreher et al., 2015). Captive-raised frogs have no alkaloids detectable in skin extracts, these mantelline frogs are dependent on dietary sources for their skin alkaloids (Daly et al., 1997) making them unpalatable. Potential predators for the golden mantella frogs would be reptile species such as *Zonosaurus madagascariensis* and *Tamnosophis lateralis* (Jovanic et al., 2009). The colour distance analysis using a model of snake and a lizard visual system showed significant and highly detectable differences in the skin coloration of frogs from Mitsinjo breeding centre in comparison to wild populations, suggesting that predators would be able to perceive these different colorations.

The colour distance analyses done using the human visual system (Bowmaker, 2015) demonstrated that keepers would be able to detect the different coloration

in the animals they manage from wild conspecifics. This could be used as a measurement to select animals with greater colour similarities to the wild populations for reintroduction purposes. Colour charts are commonly used to evaluate colour scores of animals in zoos (Brenes-Soto & Dierenfeld, 2014); although this is a qualitative measurement, a species-specific coloration chart, could be produced and used as a health/management parameter. Frogs kept under optimal condition would have a more similar coloration to their wild counterparts; that is, a health check with less need to handle animals.

*Mantella aurantiaca* is a critically endangered frog with reintroduction as part of its Species' Action Plan to help mitigate the environmental impacts on the species' natural distribution (Edmonds et al., 2015). It is important to consider the present results when thinking about releasing *M. aurantiaca* back to the wild. The aposematic coloration plays an important role on the behaviour and ecology of many species, making the results presented here important to consider when planning reintroductions. Pre-release assessment should also take into account the physical condition, and include a colour assessment as part of the process.

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# Temporal trends in *Podarcis muralis* and *Lacerta bilineata* populations in a fragmented landscape in western France: Results from a 14 year time series

Roger Meek

Rue George Clemenceau, Chasnais, France

Identifying population changes is a prerequisite for any conservation efforts, but to evaluate trends requires long-term data sets. In this paper, changes in population numbers in two species of European lizards, *Lacerta bilineata* and *Podarcis muralis*, are described. The results are based on counts of mortalities and live lizard presence on roads collected over a 14 year period, which indicated wide annual fluctuations in numbers in both species, with inter-specific annual trends strongly correlated. Snout to vent lengths (SVL) in *L. bilineata* were generally longer when annual numbers were higher but not in *P. muralis*. Regression analysis of the logarithmic transforms of annual lizard numbers as dependent variables and year as the independent variable indicated that despite population fluctuations, numbers of both species were stable or increased during the period of observation. Jackknife analysis identified unusually high numbers of *L. bilineata* in 2012 and *P. muralis* in 2010, but data from these years had minimal influence on the general trends with the pseudo-regression coefficients generated from the Jackknife analysis in agreement with the true regressions. The results were therefore congruent, indicating annual fluctuations in both species were underpinned by long-term population stability.

*Keywords:* lizards, *Lacerta bilineata*, *Podarcis muralis*, population changes, road ecology

## INTRODUCTION

Habitat fragmentation and potential impact of climate change are just two of the frequently cited threats that potentially impact on reptiles and amphibians (e.g. Alford & Richards, 1999; Gibbons et al., 2000; Chamaille – Jamme et al., 2006; Araujo et al., 2006; Luiselli et al., 2018). Perhaps due to their perceived role as indicators of environmental health, studies of amphibians appear more frequently in the scientific literature (e.g. Alford & Richards, 1999; Beebee & Griffiths 2005). However, reptiles may also be particularly sensitive to climatic effects and documenting major changes in populations, especially in the face of potential climatic effects, is central for any conservation effort. This requires long-term data sets that will enable sufficient statistical power to evaluate trends (Gibbons et al., 2000). Within the European/Mediterranean reptilia, long-term studies have mostly concerned snakes (e.g. Lourdais et al., 2002; Reading et al., 2010; Capula et al., 2014; Rugiero et al., 2014; Luiselli et al., 2018; Graitson et al., 2018; Bauwens & Claus, 2018). Fewer studies have examined the more numerous lizards (e.g. Barbault & Mou, 1988; Saint Girons et al., 1989; Capula et al., 1993; Roitberg & Smirna, 2006; Leão et al., 2018), which is perhaps surprising given that lizards are now considered as model organisms in studies of life-history and demography (Pianka & Vitt, 2003). For

example, in a recent overview on density dependence, a 10-year minimum time series criteria for inclusion resulted in only four species of lizard in the analysis (<6 % of the reptile total: Leão et al., 2018).

The aim of the present study is to examine long-term population changes in two species of European lizards, *Podarcis muralis* and *Lacerta bilineata*, both of which are common over large parts of western Europe. In the study locality they occupy a fragmented landscape where *L. bilineata* typically operates as a sentinel predator and is associated mostly with hedgerows and light urban areas. The smaller *P. muralis* operates mainly as an active forager and is more commonly found in human-dominated environments and along woodland edge (Meek, 2014). The results are based on a 14-year time series derived from two sources; road mortalities and sighting frequencies of live lizards that have entered roads. Lizards may utilise roads for several reasons including thermal opportunities, predator and prey detection, and as ecological pathways (e.g. Koenig et al., 2002; Delgado Garcia et al., 2007; Lebboroni & Corti, 2006; Meek, 2009, 2014).

## METHODS

The study area (46°27'N; 1°53'W) was established in 2005 and composed of a fragmented landscape dominated by

agriculture but with wooded areas, small villages, and isolated houses and gardens. Apart from some new housing construction, mostly in the villages, the area had otherwise experienced little or no major changes in land use other than agricultural activities in the same fields during the 14-year study period. The locality is approximately 10 km from the Bay of Biscay with a mild oceanic climate; June, July and August monthly mean air temperature is 26 °C, and November through to February monthly mean is 10.2 °C. Highest precipitation is usually between October and January (monthly mean = 85.7 mm) with June, July, and August the lowest rainfall, with the monthly mean of 51.3 mm.

The use of road mortalities to estimate animal numbers assumes that carcasses on roads represent approximations of animal numbers in adjacent habitat (Fitch, 1987). This approach has been usefully employed as a proxy to estimate population changes in amphibians (e.g. Meyer et al., 1998; Meek, 2018) and snakes (e.g. Capula et al., 2014; Rugiero et al., 2018) and while representing only an index of abundance, if carcasses are removed after detection, they are independent of double counting and autocorrelation. The presence of live lizards on roads provides an additional data source since the absence of visual barriers facilitates ease of detection and species identification (Meek, 2014). VEF (visual encounter frequency) is less robust to double counts but if made over a long time period and large geographical area, risk of double counts should be low.

Data on road mortalities and live counts were collected over a total distance ≈16 km between a wetland area close to the village of St Denis du Payre and the wetland on the edge of the village of Chasnais from 2005 to 2018. The distance between the two villages is approximately 6 km (see Meek, 2012 for a schematic view). Surveying for road mortalities began in January 2005 and was undertaken between four and six times every month throughout each year until December 31, 2018. A single observer on a bicycle travelling at 5–10 km/hour carried out surveys. This method enables good detection of lizards especially in the more brightly coloured *L. bilineata*, and hence observer error is believed to be low in this species. Possible observer error in *P. muralis* is a little higher due to its smaller size and less vivid colouring (see Meek, 2012). Road traffic volume increased slightly during surveying – see Meek (2012). Data are derived from lizards that were either present on roads or found as road-kill. When a road-killed lizard was found, snout to vent length (SVL) in mm, its location, proximate roadside habitat and distance from the road edge were recorded. Measurements of road-killed lizard SVL had a maximum estimated error of <5 mm depending on body condition. Measurements of live lizards were approximate (±10 mm) and based on photographic records, which were then compared with some object in the immediate vicinity. A number of live lizards were caught, including five injured individuals on roads, but in total this was less than 10 (see text).

#### Statistical analysis.

Before analysis, tests for normality were evaluated

using the Anderson-Darling  $a^2$  test. Differences in mean SVL were determined by ANOVA with a post hoc Tukey HSD test for unequal sample sizes (Tukey, 1949; Kramer, 1956). This evaluates all possible pair-wise comparisons of means at  $\alpha = 0.05$ , with the null hypothesis that means are in statistical agreement. Comparison of inter-specific differences in the extent of population fluctuations were made using F-tests for equality of variances. The null hypothesis is that variances do not differ (i.e.,  $H_0: s_1^2 / s_2^2 = 1$ ). Tests for departures for equality of annual lizard counts were done using G-test Goodness-of-Fit at  $n-1$  d.f. with the expected annual counts;  $1/n$ , where  $n$  is the year count; that is the combined road mortalities and live counts across all years, 23.7 for *L. bilineata* and 25.1 for *P. muralis*.

Long-term population trends were evaluated using regression analysis of the logarithmic transforms of road mortalities and live counts as dependent variables, with corresponding years as the independent variables. This gave an equation of the form:

$$\log_e N = b + m \text{ year}$$

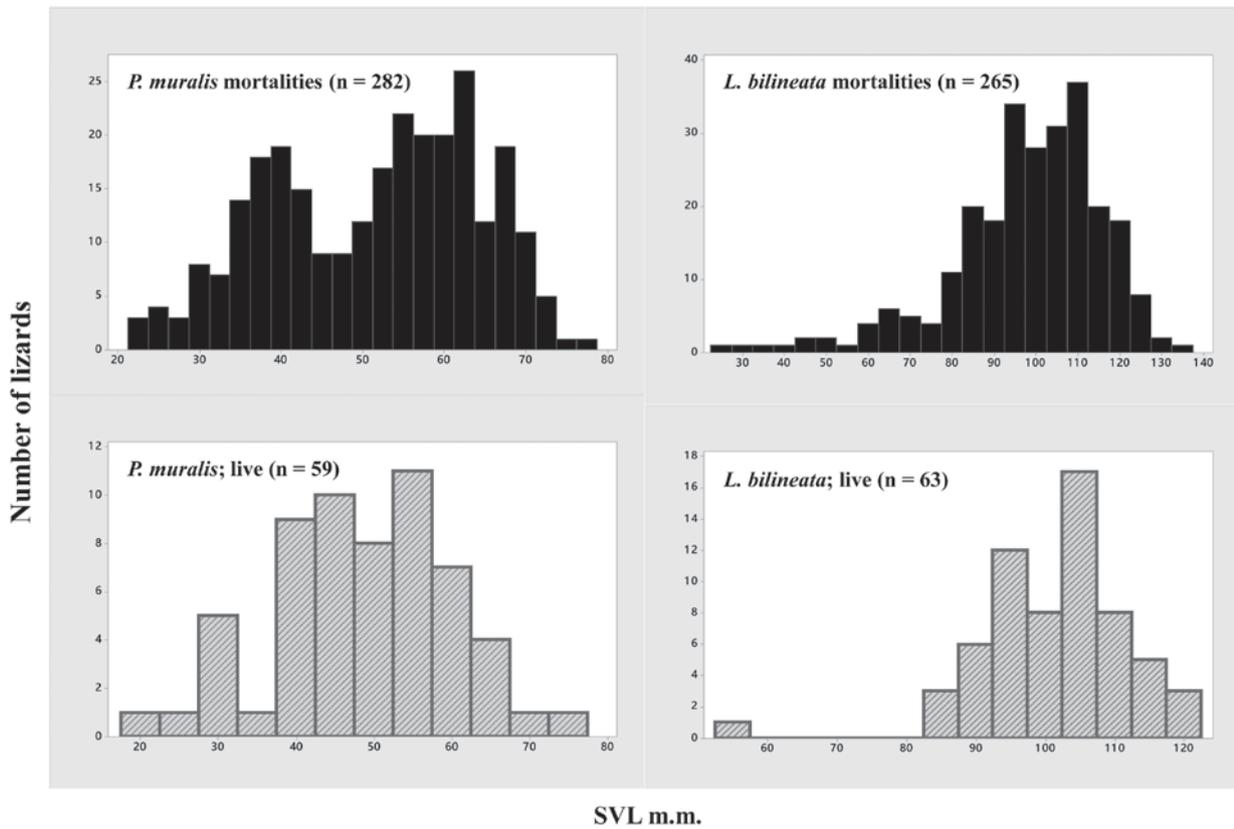
where  $\log_e N$ , represents either road mortalities, live counts or the pooled data sets,  $m$  the regression coefficient, and  $b$  the y-intercept. The null hypothesis is that  $\log_e N$  is stable when  $m = 0$ ; significant departures from 0 are indicative of population change, which was evaluated using a  $t$ -test at  $n-2$  d.f. (Bailey, 1995).

Since unusually high or low year counts may have an inordinate effect on  $m$ , a test for influence function (Gotelli & Ellison, 2004) to estimate the sampling errors of the regression coefficients was made using jackknifing (Sahinler & Topuz, 2007). Jackknifing has the advantage of giving exact repeatable results by systematically removing one-year data sets from the sample and then re-calculating regression coefficients for these restricted samples. The true regression coefficients were then compared to the distribution of the pseudo- $m$  values. Long term climatic data, rainfall and temperatures, were sourced from the nearest weather station at La Rochelle-Le Bout Blanc (around 25 km from the study locality).

## RESULTS

A total of 332 *L. bilineata* (265 road mortalities + 67 live individuals) and 354 *P. muralis* (282 road mortalities + 72 live individuals) were recorded on roads. Figure 1 shows SVL distributions of mortalities and SVL estimates of live lizards between 2005 and 2018. In both species, all distributions were negatively skewed toward larger individuals; *L. bilineata* live = -1.15 & mortalities = -1.23; *P. muralis* live = -1.26 with mortalities only moderately skewed = -0.27.

*Lacerta bilineata* were found as road mortalities between maximum dates of April 8 and October 25 and live individuals between maximum dates of April 9 and September 22. Peak mortalities were in August and peak live sightings in April. Mortalities of *P. muralis* were found between March 24 and November 12 with live sightings between April 4 and October 18 of each year. Live counts



**Figure 1.** SVL's of lizards measured from 2005–2018. Live SVL's are approximate values derived from lizards that were sighted on roads and estimates considered possible. Note scale differences on the x-axis.

**Table 1.** Basic statistics of annual numbers of lizards found as mortalities or as live counts on roads over the 14 year period. See text for statistical comparisons.

	Mortalities Mean all years	Std Dev	Max	Min	Live Mean all years	Std Dev	Max	Min
<i>P. muralis</i>	20.1	14.7	46	3	5.1	4.5	16	1
<i>L. bilineata</i>	18.9	8.6	33	8	4.8	4.8	17	1

and mortalities peaked during September. These data are partly derived from Meek (2014) based on data collected between 2005 and 2013, with additional data from 2013 to 2018. Table 1 shows the basic statistics of the pooled annual data.

#### Long term trends.

Both species showed wide fluctuations in annual mortalities and live road presence. The  $G$ -tests for equality of annual counts indicated fluctuations were significant both in *L. bilineata*; mortalities,  $G = 49.48$ ; live,  $G = 51.95$ ; pooled,  $G = 65.49$ , all  $P < 0.0001$  and *P. muralis*; mortalities,  $G = 145.6$ ; live,  $G = 43.8$ ; pooled,  $G = 154.8$  all  $P < 0.0001$ . Anderson Darling tests on annual numbers of lizards (mortalities + live) indicated normal distributions for both species (*L. bilineata*  $a^2 = 0.48$ ,  $P = 0.2$ ; *P. muralis*  $a^2 = 0.46$ ,  $P = 0.22$ ) with the  $F$ -test indicating that the variances of the annual counts for both species were in good agreement ( $F = 1.54$ ,  $P = 0.44$ ) as were the long term trends in annual fluctuations of both species (Pearson correlation,  $r = 0.68$ ,  $P = 0.008$ ). Figure 2 shows

the trends along with the overall means across all years.

The regressions of annual road mortalities + live counts against year produced coefficients (Table 2) indicating either population stability or a significant increase in numbers during the study period (Fig 1). In *L. bilineata*, jackknifing identified 2012 with unusually high numbers and a potential influence function. However, re-analysis with the 2012 data jackknifed gave a pseudo-regression coefficient in agreement with the true coefficient (jackknifed 2012  $m = 0.07 \pm 0.02$ , true  $m = 0.07 \pm 0.01$ ). Jackknifing indicated unusually high numbers of *P. muralis* in 2010 but removal and re-analysis gave a pseudo-regression coefficient ( $0.15 \pm 0.04$ ) in close agreement with the true coefficient ( $0.14 \pm 0.04$ ). In neither species did Jackknife analysis support long-term population decline (Table 2).

Comparison of annual SVL was applied only to mortalities due to potentially greater error in the measurement of live lizards. There was no significant difference in annual mean SVL of road mortalities in *P. muralis* ( $F_{(13, 266)} = 1.12$ ,  $P = 0.35$ ). However, significant

**Table 2.** Regression analysis of temporal changes in annual road mortalities and live counts of *L. bilineata* and *P. muralis*. The regression coefficients  $m$  are shown with standard errors along with values of  $b$  the y-intercepts. The  $t$ -tests and P-values represent the results of tests of the estimated regression coefficients against a hypothetical regression of 0, indicative of long-term population stability. The tests of M (mortalities) versus L (live counts) are intra-specific variance comparisons between annual counts. See text for further details.

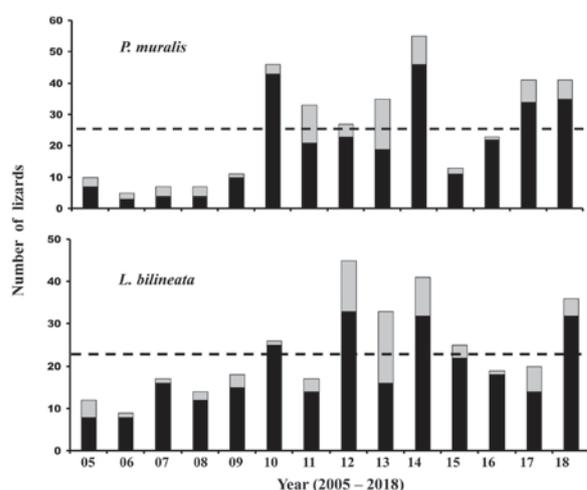
<i>L. bilineata</i>	$m$	Std error	$b$	$t$	$p$	tests M v L
mortalities	0.07	0.02	-142	2.89	0.01	
live	0.08	0.06	-167	1.37	0.19	L=3.0, P=0.09
pooled	0.07	0.02	-147	3.03	0.01	
<i>P. muralis</i>						
mortalities	0.17	0.04	-350	3.87	0.002	
live	0.05	0.06	-109	0.98	0.35	L=14.1, P=0.001
pooled	0.14	0.04	-288	3.61	0.004	

differences were identified in *L. bilineata* ( $F_{(13, 249)} = 3.94$ ,  $P = 0.0001$ ). Post hoc tests showed SVL's in 2012 were significantly longer and during 2005 and 2009 significantly smaller. *Lacerta bilineata* SVL's were generally longer when annual numbers were higher (Pearson correlation  $r = 0.56$ ,  $P = 0.04$ ) but not in *P. muralis* (Pearson correlation  $r = 0.31$ ,  $P = 0.29$ ). Figure 3 shows the general trends.

Data from the La Rochelle-Le Bout Blanc weather station indicated higher than average annual rainfall from 2009 – 2011 (mean increase 11.1 cm), the period preceding several years of high lizard numbers, but no longer-term correlation between lizard numbers and rainfall patterns could be found in either species ( $P = 0.43$  &  $0.10$ ). Annual temperature changes are an additional possibility, but were moderately correlated only in *L. bilineata* (Pearson  $r = 0.57$ ,  $P = 0.04$ ).

## DISCUSSION

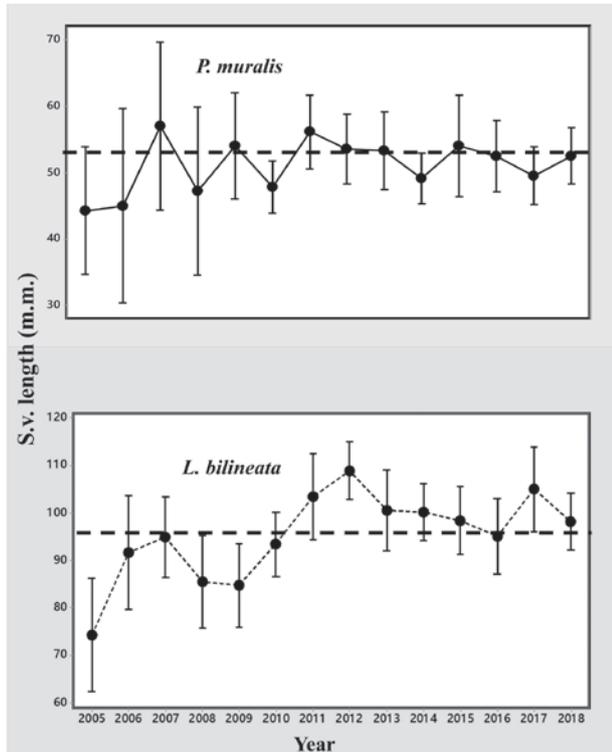
The results indicate that in a fragmented landscape, despite wide annual fluctuations, numbers of both species were relatively stable over the 14 year time period, with no indications of population decline. A potential to recover from very low numbers is fast generation times (Barbault & Mou, 1988; Bauwens & Díaz-Uriarte, 1997) which must be a key factor in enabling long term population stability. Support for this notion is the capacity of introduced populations of *P. muralis* and *L. bilineata* to rapidly attain high numbers from small founding introductions (e.g. Hedeem 1984; Allan et al., 2006; Mole, 2010; Heym et al., 2013). This capability is apparently due to rapid growth and short time to maturity, which have been cited as key contributory factors in Canada where introduced *P. muralis* reached maturity in their second full summer. Where both species have been monitored as invasive species, rates of population increase were similar (Mole, 2010). This agrees well with populations in their native range (e.g. Bauwens & Díaz-Uriarte, 1997; Allan et al., 2006). In the present locality female *P. muralis* produce two clutches of eggs annually, that must also contribute to enabling rapid population increases. The high numbers in both species between 2010 and 2014 included a significant increase in SVL's in



**Figure 2.** Histograms showing annual numbers of *P. muralis* and *L. bilineata* mortalities or live count presence on roads. Black bars represent road mortalities and grey bars live counts. The broken line indicates the overall mean of year counts for the pooled data of live lizards and road mortalities and represents the expected number under a null hypothesis of equality of annual counts. See text for further details.

*L. bilineata* (Fig. 2). This could reflect a longer life span. For example, in western France potential maximums of 8 – 9 years have been observed (Saint Girons, et al., 1989). In general, larger lacertids live longer adult lives with higher annual fecundity than smaller species (e.g. Strijbosch, & Creemers, 1988; Bauwens & Díaz-Uriarte, 1997), but age at maturity is apparently similar in *L. bilineata* and *P. muralis* (Bauwens & Díaz-Uriarte, 1997) and may explain comparable inter-specific population trends over the 14-year time period.

Life spans and growth in lacertids are strongly influenced by climate (e.g. Roitberg & Smirna, 2006) and hence climate is probably the first area to examine for population drivers but little correlation was found in this study. However, rainfall has been shown to influence lizard populations in other regions (e.g. Tinkle, 1993; Spiller & Schoener, 1995; Gibbons et al., 2000) but weather effects on lizards are likely complex and must also involve additional variables such as food availability. It is clearly no simple task to identify climatic effects on



**Figure 3.** Annual changes in mean SVL (in mm) of *P. muralis* and *L. bilineata* road mortalities. Circles represent mean values and vertical bars 95 % confidence intervals. The broken lines show the degrees of departure of the annual means from the long-term means (mean of means). See text for further details.

population fluctuation, especially from simple time series (Krebs & Berteaux, 2006; Knape & de Valpine, 2012; Flesch et al., 2017). Of interest is that increases in lizard numbers commencing 2010 mirrored similar increases in sympatric amphibians (Meek, 2018 and unpublished data) suggesting that a more informative approach would be to simultaneously examine the ecology of several sympatric species.

In non-lacertid lizards, population fluctuations have been linked to population density by influencing growth rates and survivorship in yearling lizards (Dunham, 1981; Tinkle et al., 1993). Population density impacts on the lacertid *Z. vivipara* numbers due to male aggression towards adult females reducing survivorship and fecundity, leading to population decline (Le Galliard et al., 2005). In the present study, potential examples of intra-specific aggression in *L. bilineata* were found during years of high numbers in four males and one female. These lizards were found (April - May) on roads in an apparent state of semi-consciousness displaying no flight behaviour when approached with no apparent injuries (2), recent tail loss (2) and one example of head injuries (Meek, 2011). Except for one lizard, all recovered after treatment. Examples are shown in Fig. 4. *Podarcis muralis* and *L. bilineata* are known for intense territorial behaviour and intra-sexual conflict (Arnold, 2002) and density dependence aggressive encounters may serve to operate as a regulatory mechanism. Aggressive encounters that spilled onto roads resulting in mortalities



**Figure 4.** Examples of road-injured *L. bilineata* that appear unrelated to road traffic collisions. **A)** female 2012, SVL 97, **B)** male SVL 106. See text for details.

from road vehicles have been observed in male *L. agilis* (Bird, personal communication).

Density dependence is now recognised as a common mechanism of population regulation (e.g. Brook & Bradshaw, 2006; Knape & de Valpine, 2012) but detection is problematic (Solow & Steele, 1990). Although analytic methods for detecting density dependence have improved in recent years, the required long-term time series studies are limited but crucial (Leão et al., 2018). In part this is due to various mathematical approaches giving conflicting results, and the quality of long-term time series (e.g. Knape, 2008; Knape & de Valpine, 2012), especially in lizards. Discussing amphibians Pechmann et al. (1991) cautioned that if population fluctuations are large relative to the length of the data set, certain trends could be misinterpreted as declines – this can be expected on other groups including lizards where fluctuations are a normal dynamic. The long-term studies that are required may have funding issues in addition to the logistic difficulties involved, however if used in conjunction with other approaches (e.g. skeletochronology; Smirina et al., 1984) may provide essential insight into lacertid population dynamics and are a fundamental requirement for our understanding of effects of habitat loss and climate change on animal populations (Araújo et al., 2006).

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## Anuran species in Brazil's protected areas network

Alessandro Ribeiro Morais<sup>1</sup>, Tainã Lucas Andreani<sup>2,6</sup>, Rhayane Alves<sup>1,7</sup>, Carolina Emília dos Santos<sup>3</sup>, Jhonatan Barros<sup>1</sup>, Wadson Rodrigues Rezende<sup>4</sup> & Priscila Lemes<sup>5</sup>

<sup>1</sup>Laboratório de Biologia Animal, Instituto Federal Goiano, Campus Rio Verde, Rio Verde, GO, Brazil

<sup>2</sup>Programa de Pós-Graduação em Biodiversidade Animal, Instituto de Ciências Biológicas, Universidade Federal de Goiás, Goiânia, GO, Brazil

<sup>3</sup>Programa de Pós-Graduação em Ecologia e Evolução, Instituto de Ciências Biológicas, Universidade Federal de Goiás, Goiânia, GO, Brazil

<sup>4</sup>Programa de Pós-Graduação em Biologia Animal, Universidade Estadual Paulista "Júlio de Mesquita" (UNESP), Campus São José do Rio Preto, São José do Rio Preto, SP, Brazil

<sup>5</sup>Departamento de Botânica e Ecologia, Instituto de Biociências, Universidade Federal do Mato Grosso, Cuiabá, MT, Brazil

<sup>6</sup>Programa de Pós-Graduação em Ecologia e Conservação, Universidade Federal de Mato Grosso do Sul, Campo Grande, MS, Brazil

<sup>7</sup>Programa de Pós-Graduação em Biodiversidade e Conservação, Instituto Federal Goiano, Campus Rio Verde, Rio Verde, GO, Brazil

Amphibians are one of the most threatened among the vertebrates species and urgently require conservation protection and planning. The establishment of protected areas (PAs) is one of the most important strategies in protecting biodiversity, as they offer a partial solution to habitat modification. Our main objective was to analyse the temporal and spatial trends in the anuran species inventories of PAs in Brazil, therefore providing an extensive list of anuran species in the Brazilian PAs network for the first time. We considered a combination of keywords while using the data on "Scopus", "Scielo", and "Web of Science". We found 115 papers that published anuran species lists for 101 Brazilian protected areas. Overall, we registered 605 species distributed in 20 families. Only seven out of the 605 anuran species registered in the present study are threatened by extinction and 40 are listed as Data Deficient. The number of anuran species inventories in Brazilian protected areas has increased over time ( $r = 0.17$ ;  $r^2 = 0.267$ ;  $p < 0.01$ ), with its peak in 2011 ( $n = 15$  inventories). The majority of the species inventories were conducted in protected areas located in the Atlantic Forest (55.45%). The number of anuran species per protected area varied from seven to 80; however, we did not find any relationship between the species richness and size of the protected areas ( $r^2 = 0.027$ ;  $r = 0.165$ ;  $p = 0.092$ ). Our results can be useful to fill the gaps and integrate knowledge; and this reinforces the importance of the present study in contributing to the knowledge and conservation of anuran species in Brazil.

*Keywords:* Brazilian biomes, species list, inventory, temporal and spatial trends

### INTRODUCTION

Financial resources and time are running out in the race to protect biodiversity around the world (Brooks et al., 2006). Effective conservation actions need to be quickly implemented in order to avoid the loss of species. In this context, the establishment of protected areas (PAs) is one of the most important local, regional, and global strategies to protect all biodiversity components (Rodrigues et al., 2004; Nori et al., 2015). PAs have been created and utilised since the nineteenth-century (Watson et al., 2014). Currently, the IUCN (Dudley & Stolton, 2008) defines PAs as "a clearly defined geographical space, recognised, dedicated and managed, through legal or other effective means, to achieve the long term conservation of nature with associated ecosystem services and cultural values". Since 2010, the Convention on Biological Diversity (CBD, 2010) recommends that 17% of the territory of all countries should be legally protected through of PAs. As a consequence, the coverage of these areas has considerably expanded in the last decade (e.g., Jenkins & Joppa, 2009; Juffe-Bignoli et al., 2014; WDPA,

2016). However, the nature protection in situ is still far from ideal and only 13% of terrestrial land is protected (Bertzky et al., 2012).

Amphibians comprise over 8,000 species of frogs, salamanders, newts, and caecilians (Frost, 2019). Roughly 31.4% (~2,100 spp.) of the amphibian species evaluated by the IUCN are classified within one of the threat categories, while another 22.3% (~1490 spp.) are classified as Data Deficient (IUCN, 2019). Even though it is the most threatened group among the vertebrates (Stuart et al., 2004; IUCN 2019), amphibians are still underrepresented in the global network of PAs (Jenkins et al., 2013; Nori et al., 2015). Several factors have negatively affected the amphibians; however, the habitat loss and fragmentation have been considered the main threats to these animals, since their effects have caused population declines in different amphibian species around in the world (Stuart et al., 2004; Becker et al., 2010). Amphibian species should be a conservation priority (Stuart et al., 2004), since sufficient evidence points towards the danger of them disappearing (Pyron, 2018), without proper protection (Nori et al., 2015).

*Correspondence:* A.R. Morais (alessandro.morais@ifgoiano.edu.br)

Recently, Brazil has previously been recognised as a global leader in the creation of protected areas (Jenkins & Joppa, 2009; Loyola, 2014), a key conservation strategy considering in protecting the large number of amphibians species (~1,140 amphibian species; Segalla et al., 2019). In Brazil, the first protected area was established in 1937 (Cabral & Brito, 2013) and ever since the United Nations Conference on Environment and Development in 1992 (the Rio "Earth Summit") more than two thousand have been created (Vieira et al., 2019). The late expansion of the Brazilian protected areas network can be explained by the National System of Conservation Units – NSCU (Brazilian Law nº 9,985/2000 - Sistema Nacional de Unidades de Conservação - SNUC) which established criterion to create and manage the PAs (Brasil, 2000; Silva, 2005). According to the NSCU, the Brazilian PAs are categorised into two major groups: (1) strictly protected areas (National Parks, Biological Reserves, Ecological Stations, Natural Monuments, and Wildlife Refuges) of which only one indirect use of nature resource is allowed; and (2) sustainable use reserves (Extractive Reserves, Sustainable Development Reserves, Wildlife Reserves, National Forests, Environmental Protection Areas, and Areas of Relevant Ecological Interest) that aims to harmonise the nature conservancy and a sustainable use of natural resources including different forms of management.

By recognising the importance of the PAs network, the present study provided a list of anuran species occurring in the Brazilian PAs network based on an intensive search in the literature. Herein, our main objective was to analyse the temporal and spatial trends in the anuran species inventories in Brazilian PAs. Specifically, we addressed the following questions: (1) How many and which anuran species effectively occur in Brazilian protected areas? (2) How did the number of anuran species inventories in PAs increase over time? (3) Does the number of species inventories differ among the Brazilian biomes? (4) How does the size of the protected areas influence the anuran species richness?

## METHODS

We chose to focus on anurans for this study, as 96 % of amphibians in Brazil are anuran species (Segalla et al., 2019); of which 41 species are currently threatened by extinction and 167 species are Data Deficient (according to the Brazilian Red List; MMA, 2018). We searched for studies that provided list of anuran species in Brazilian protected areas. Firstly, we accessed the sources of the data on "Scopus", "Scielo", and "Web of Science" and used the combination of the following keywords: anuran\*, species list, AND inventory AND Brazil\* AND protected area\* OR Conservation Unit OR Park OR Biological Reserve OR Ecological Station OR Extractive Reserve OR Sustainable Development Reserve OR Wildlife Reserve OR Natural monument OR Forest OR Environmental Protection Area OR Area of Relevant Ecological Interest and Private Reserve of Natural Heritage. We did not consider grey literature (i.e. conference proceedings, dissertation, theses and technical reports). All possible redundant studies were removed and articles published

after June 2019 were not included in our sample.

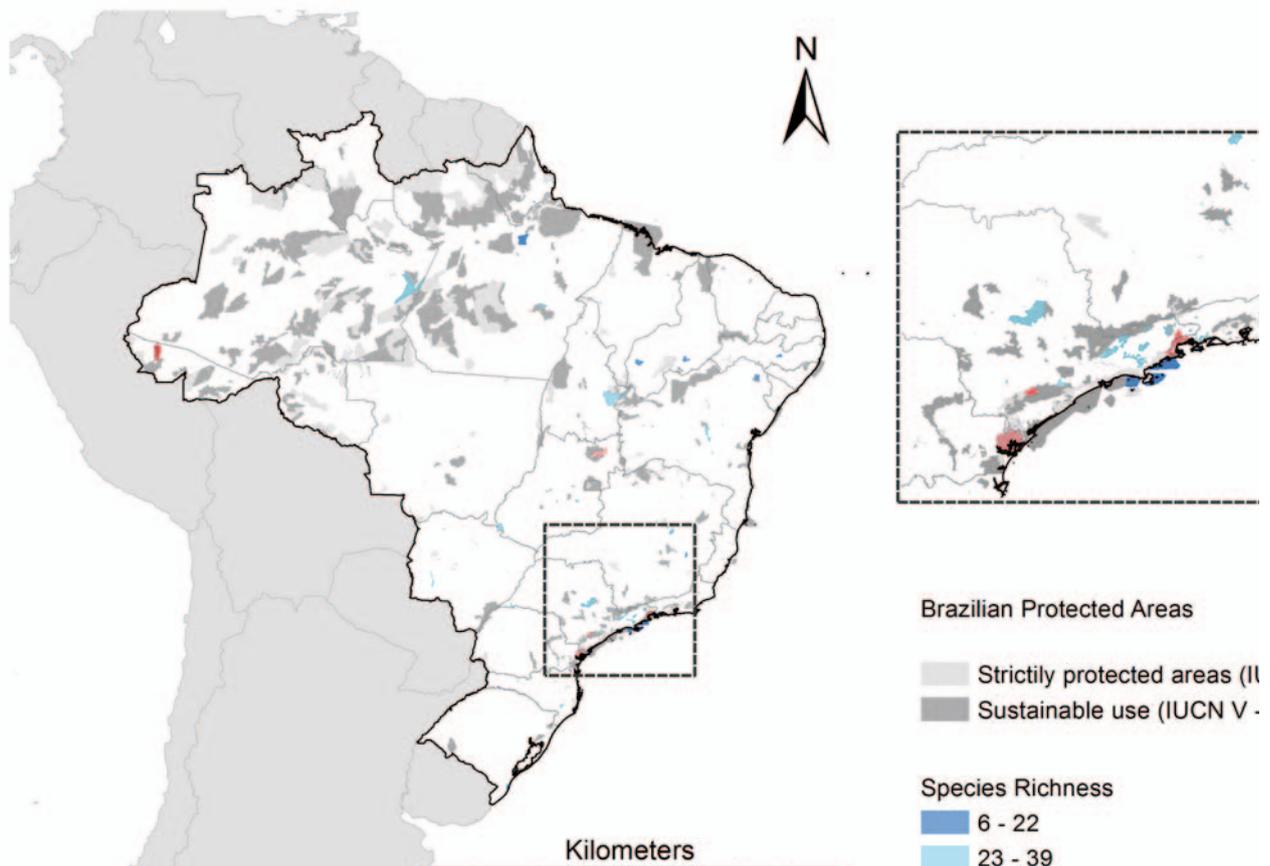
For each study, we obtained the following variables: (i) year of publication; (ii) title; (iii) number of anurans species; (iv) species list (species composition); (v) PAs name; (vi) PAs size (in hectare); (vii) geographic coordinates; (viii) biome, as cited in the study; and (ix) federal state name. Undetermined species such as "sp.", "cf.", "gr." and "aff" were not included in our analysis. The taxonomy and nomenclature used follow Frost (2019). To verify whether the number of lists of anuran species in protected areas from Brazil increased throughout the years, we used a simple linear regression analysis. Furthermore, we used a simple linear regression to test the influence of the size of the Brazilian PAs had on anuran species richness in each anuran species inventory. This statistical procedure describes a linear relationship between one response variable (e.g. number of anuran species inventory or anuran species richness) and another independent variables (e.g. years or size of the protected areas). For this, we tested the assumptions (e.g. homogeneity of variance and normal distribution) of the test prior to analysis and considered our significance level to be 5 %.

## RESULTS

Our database includes 115 papers of which each one contain, at least, an anuran species list for 101 PA in Brazil (Fig. 1; Supplementary Materials Table 1). In general, these studies include inventories from a singular PA, but three studies simultaneously considered more than one protected area. Fourteen protected areas were systematically studied more than once, because they were considered in more than one study. All 115 studies considered, we identified 605 species distributed in 20 families (Fig. 2; Supplementary Materials Table 2). According to the Brazilian Red List of Threatened Species categories (MMA 2014), there are one Critically Endangered species, one Endangered, and five Vulnerable (Table 1), while nine species are classified as Near Threatened, 40 Data Deficient, and 549 Least Concern species found in Brazilian PAs.

Some families are represented in more PAs than others. In total, about 37.2 % of species belong to the family Hylidae, while the families Allophryniidae and Alsodidae were represented by one species of each family. In the present study, *Boana albomarginata*, *B. albopunctata*, *B. faber*, *Dendropsophus elegans*, *D. minutus*, *D. nanus*, *Elachistocleis cesarii*, *Haddadus binotatus*, *Leptodactylus fuscus*, *L. labyrinthicus*, *L. latrans*, *L. mystaceus*, *L. mystacinus*, *Physalaemus cuvieri*, *Rhinella diptycha*, *R. icterica*, *R. ornata*, *Scinax fuscomarginatus*, and *S. fuscovarius* were the species more representative within protected areas, since they have been identified in more than 20 PAs (Supplementary Materials Table 2). Approximately 33 % of species were identified in only one PA.

The anuran species list from Estação Ecológica do Taim was the first inventory of a Brazilian PA made in 1988 (Supplementary Material Table 1). Hereafter, the number of anuran species inventories in Brazilian protected areas has increased over time ( $r = 0.517$ ;  $r^2 = 0.267$ ;  $p < 0.01$ ;



**Figure 1.** Brazilian PAs with anuran species inventories. Zoom illustrates PAs of the Atlantic Forest

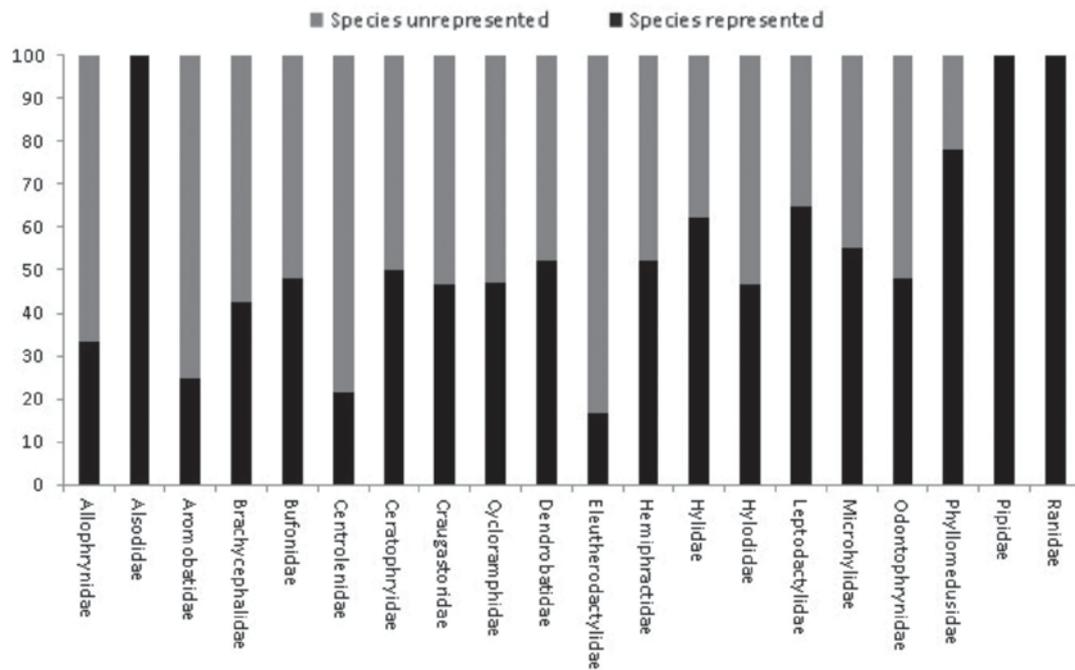
**Table 1.** Threatened anuran species found in the inventories conducted in Brazilian protected areas network.

Species	Brazilian red list category (MMA 2014)	Protected area where the species occurs	Reference
<i>Allobates goianus</i>	Endangered	Floresta Nacional de Silvânia / Parque Nacional da Chapada dos Veadeiros	Morais et al. (2012) / Santoro & Brandão (2014)
<i>Allobates ofersiodes</i>	Vulnerable	Área de Proteção Ambiental de Lagoa Encantada e Rio Almada / Reserva Sapiranga / Reserva Ecológica da Michelin / Reserva Particular do Patrimônio Natural Serra Bonita	Dias et al. (2014a) / Bastazini et al. (2007) / Camurugi et al. (2010) / Dias et al. (2014b)
<i>Boana cymbalum</i>	Critically Endangered	Parque Natural Municipal Nascentes de Paranapiacaba	Trevine et al. (2014)
<i>Boana curupi</i>	Vulnerable	Parque Estadual do Turvo / Parque Estadual Frtiz Plaumann / Parque Nacional das Araucárias / Parque Natural Municipal de Sertão	Iop et al. (2011) / De Bastiani & Lucas (2013) / Lucas & Marocco (2011) / Zanella et al. (2014)
<i>Melanophryniscus dorsalis</i>	Vulnerable	Parque Estadual de Itapeva	Colombo et al. (2008)
<i>Physalaemus maximus</i>	Vulnerable	Parque Estadual da Serra do Brigadeiro	Moura et al. (2012)
<i>Scinax duartei</i>	Vulnerable	Reserva Particular do Patrimônio Santuário do Caraça	Canelas & Bertoluci (2007)

Fig. 3), peaking in 2011 ( $n = 15$  inventories). The number of species inventories and the anuran species richness per protected area in each Brazilian biome are described in Table 3. The majority of the species inventories were conducted in PAs located in the Atlantic Forest ( $n = 56$  PAs; 55.45 %), followed by the Cerrado ( $n = 18$  PAs studies; 17.82 %), Amazon ( $n = 13$  PAs; 12.87 %), and the Caatinga ( $n = 7$  PAs; 6.93 %) (Table 2). Although some PAs in ecotone regions were also studied (5 %, Table 3). There are not available any anuran species inventory of PAs in the Pantanal biome. In general, more studies were realised in strictly PAs ( $n = 76$ ) than sustainable use

protected areas ( $n = 25$ ) (Table 2).

The Brazilian PAs of which contains an anuran species inventory cover an area greater than 8 million of hectares. The RPPN Campo Escoteiro Geraldo Hugo Nunes (Atlantic Forest biome), with 45.2 hectares, is the smallest protected area with a list of anuran species, while the Reserva de Desenvolvimento Sustentável Piagaçu-Purus (Amazon biome), with 834,245 hectares, is the largest one (Supplementary Material Table 1). The number of anuran species per protected area range from seven to 80 ( $33.7 \pm 16.27$  species;  $n = 101$  protected areas). Two PAs contain the greatest anuran species richness



**Figure 2.** Representativeness of families of anuran species within Brazilian protected areas network

**Table 2.** Number of protected areas and anuran species richness per protected area for each Brazilian biome

Biome	Number of protected areas		Species richness	
	Strictly protected areas	Sustainable use protected areas	Strictly protected areas	Sustainable use protected areas
Atlantic Forest	46	10	34.26±17.84 (7-80)	39.5±17.61 (19-79)
Cerrado	14	4	32.14±8.69 (19-54)	28±11.74 (16-42)
Amazon	5	8	35.4±14.92 (13-49)	43.62±21.25 (21-80)
Caatinga	7	--	19±7.18 (7-31)	--
Atlantic Forest-Cerrado ecotone	2	2	35±15.55 (24-46)	37±1.41 (36-38)
Caatinga-Cerrado ecotone	1	--	18	--
Coastal Marine	1	--	32	--
Pampa	--	1	--	18
Total	76	25	32.13±15.76 (7-80)	38.48±17.17 (16-80)

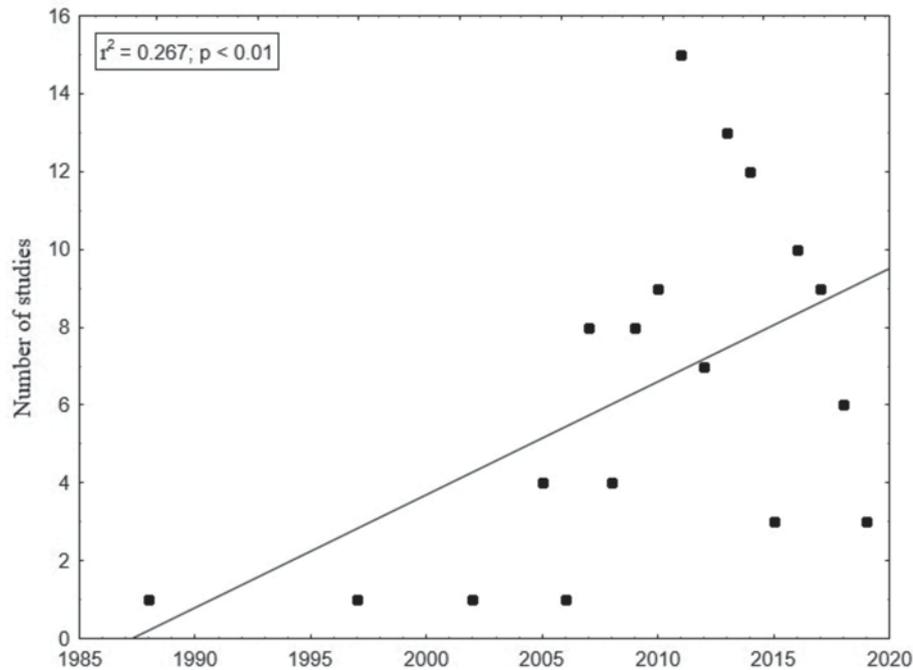
(n = 80) namely Parque Natural Municipal Nascentes de Paranapiacaba (Atlantic Forest biome) and Reserva Extrativista Riozinho da Liberdade (Amazon biome). Surprisingly, there is not a relationship between the species richness and size of the PAs ( $r^2 = 0.027$ ;  $r = 0.165$ ;  $p = 0.092$ ).

## DISCUSSION

PAs are a key strategy for protecting biological resources (Oliveira et al., 2019) presupposing the species extinctions prevention in situ. PAs inventories are important to know which species are currently protected (Bruner et al., 2001); therefore, they are a first step for conservation management options and strategy analysis in order to increase PA effectiveness (Bruner et al., 2001). In Brazil,

there is a knowledge gap in PAs inventories, since less than 5 % of all Brazilian PAs (see Vieira et al., 2019) have a list of anuran species available in literature. The lack of financial resources or qualified personnel (e.g., Taxonomists) can explain such knowledge gap; however, we suggest that the logistical difficulties of accessing many Brazilian protected areas as well as the bureaucracy to obtain collection permits within PAs (especially at state and municipal levels) also contribute to this gap.

Approximately half of all anuran species inhabiting in Brazil (Segalla et al., 2019) were included into these inventory studies, and those one occur in at least one of the 101 protected areas. Recently, Nori et al. (2015) illustrated that only 63 % of all currently extant amphibian species (~8,050 spp.) are estimated to occur in the world's global protected area network. Previous studies have



**Figure 3.** Temporal variation in the number of inventories of anuran species in Brazil's protected areas network

demonstrated a positive relationship between amphibian species diversity and species knowledge (Bini et al., 2006; Diniz-Filho et al., 2009) where sampling efforts are not affected by accessibility difficulties (i.e., Oliveira et al., 2016). Here, we emphasised that the Atlantic Forest is the most inventoried biome (Fig. 1; Table 3). Recently, this region has been identified as a global priority area for anuran research, mainly due to the highest anuran extant diversity, but also due to ongoing human pressure (Nori et al., 2018). However, the current state of anuran diversity in the Atlantic Forest may be an outcome of increased species knowledge due to the presence of major universities, dense populations and improved infrastructure in the region.

When implementing conservation strategies, the first step should be to verify the species representativeness and the site's irreplaceability degree (Le Saout et al., 2013). Such management efforts are advisable even where there are currently establishments PAs. Beyond this, the inclusion of Endangered and Data Deficient species (Polak et al., 2016) can be an important tool for management the conservation options (Nori & Loyola, 2015). According to some global analyses, both Endangered and Data Deficient anuran species have typically been poorly represented in PAs (Nori et al., 2015; Nori & Loyola, 2015) even when distribution maps (minimum convex polygon; IUCN 2019) were used. Gap analyses are meaningful for conservation actions (Rodrigues et al., 2004; Venter et al., 2014; Watson et al., 2014; Nori et al., 2015), but they may include some commission errors which can occur when falsely assuming species presence in PAs; therefore, this reinforces the importance of the present study to subsidize the management and conservation actions for amphibians species.

In general, the anurans inventories might be used as a guide for the conservation management of PAs since they offer a detailed species list from exhaustive hours of

fieldworks. Here, we gathered and reported all literature pertinent to the anuran inventory of Brazil. The first Brazilian anuran species inventory in a PA was conducted in 1988 (Estação Ecológica do Taim); and since 2005, the number of inventories has considerably increased. Many other anuran inventories were performed according as the expansion of the Brazilian PA network increased, mainly in the Atlantic Forest biome. Considering that most conservation actions can occur at regional and local levels (Possingham et al., 2002) it has become urgent to focus in research efforts regarding those poorly sampled areas (Nori et al., 2018). For example, many environmental agencies, NGOs, and research fund may act at regional and local levels, and this can be useful to reduce the knowledge gap between Brazilian biomes. Additionally, we have observed an increase in research regarding the Caatinga biome (e.g., Garda et al., 2013; Cavalcanti et al., 2014; Pedrosa et al., 2014; Magalhães et al., 2015; Caldas et al., 2016; Costa et al., 2018) which may have filled the knowledge gap about anuran species in previously data deficient Brazilian PAs.

We provided an extensive list of anuran species present in the Brazilian PAs network. Initiatives that objective to collect and compile data on biodiversity allowing to fill gaps and also the integration of knowledge must be supported (Jenkins et al., 2015). Then, our study is important to include into conservation management options for all biomes, including those less represented in our anuran inventories dataset. In this sense, we suggest that inventories must be conducted in more areas, especially those in which there are rapid destruction of habitats and sampling gaps (e.g., Amazon and Cerrado Biomes). For this, we suggest that it would be ideal to create an integrated network of researchers focused on conducting anuran species inventories in protected areas across Brazil.

Finally, for a long time now, Brazilian researchers have

been subject to insufficient funding (or simply no funding) and discouraged by actual and recent past governments which hinders necessary herpetological expeditions far from large urban centres. Also, there is a movement to discredit and impede current environmental research and conservation management of biological resources in Brazil which diverge from economic plans for the development of natural resources (e.g., Ferrante & Fearnside, 2019). We really hope that science in Brazil can continue to progress hand in hand with development in order to be intelligent drive conservation plans decided upon by Brazilian decision-makers.

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## Multiscale endemism analysis for amphibians of Paraguay

Hugo Cabral<sup>1,2</sup>, M. Dolores Casagrande<sup>3</sup>, Francisco Brusquetti<sup>2</sup>, Flavia Netto<sup>2,5</sup>,  
Vanda Ferreira<sup>4</sup> & Esteban Lavilla<sup>3,6</sup>

<sup>1</sup>Asociación Guyra Paraguay, Avenida Coronel Carlos Bóveda, Parque Asunción Verde, Vías Cué, Paraguay

<sup>2</sup>Instituto de Investigación Biológica del Paraguay, Del Escudo 1607, Asunción, Paraguay

<sup>3</sup>Unidad Ejecutora Lillo – Consejo Nacional de Investigaciones Científicas y Técnicas, Tucumán-Argentina

<sup>4</sup>Instituto de Biociências, Universidade Federal de Mato Grosso do Sul, Brazil

<sup>5</sup>Itaipu Binacional División de Áreas Protegidas, Dirección de Coordinación Ejecutiva, Hernandarias, Paraguay

<sup>6</sup>Fundación Miguel Lillo, Tucumán-Argentina

Although there are many studies that analyse and describe the distribution patterns of diverse organisms in South America at different scales, Paraguay has been poorly assessed from a biogeographic point of view. Some of the available contributions on the biogeography of Paraguay are based on different taxonomic groups, such as mammals, birds, reptiles, and plants, describing relationships between species and their habitats by using indices of similarity and cluster analysis. The main objective of this contribution is to identify areas of endemism based on the distribution of the 87 amphibian species known from Paraguay, and to compare the results with the three schemes of ecoregion proposed for the country. Eight areas of endemism were identified at different size of grids/scales, congruent with Dry Chaco, Atlantic Forest, Cerrado, Grasslands of Mesopotamia, Ñeembucú, and the Great American Chaco ecoregions.

*Keywords:* Anura, Areas of Endemism, Biogeography, Distribution data, NDM/VNDM, South America

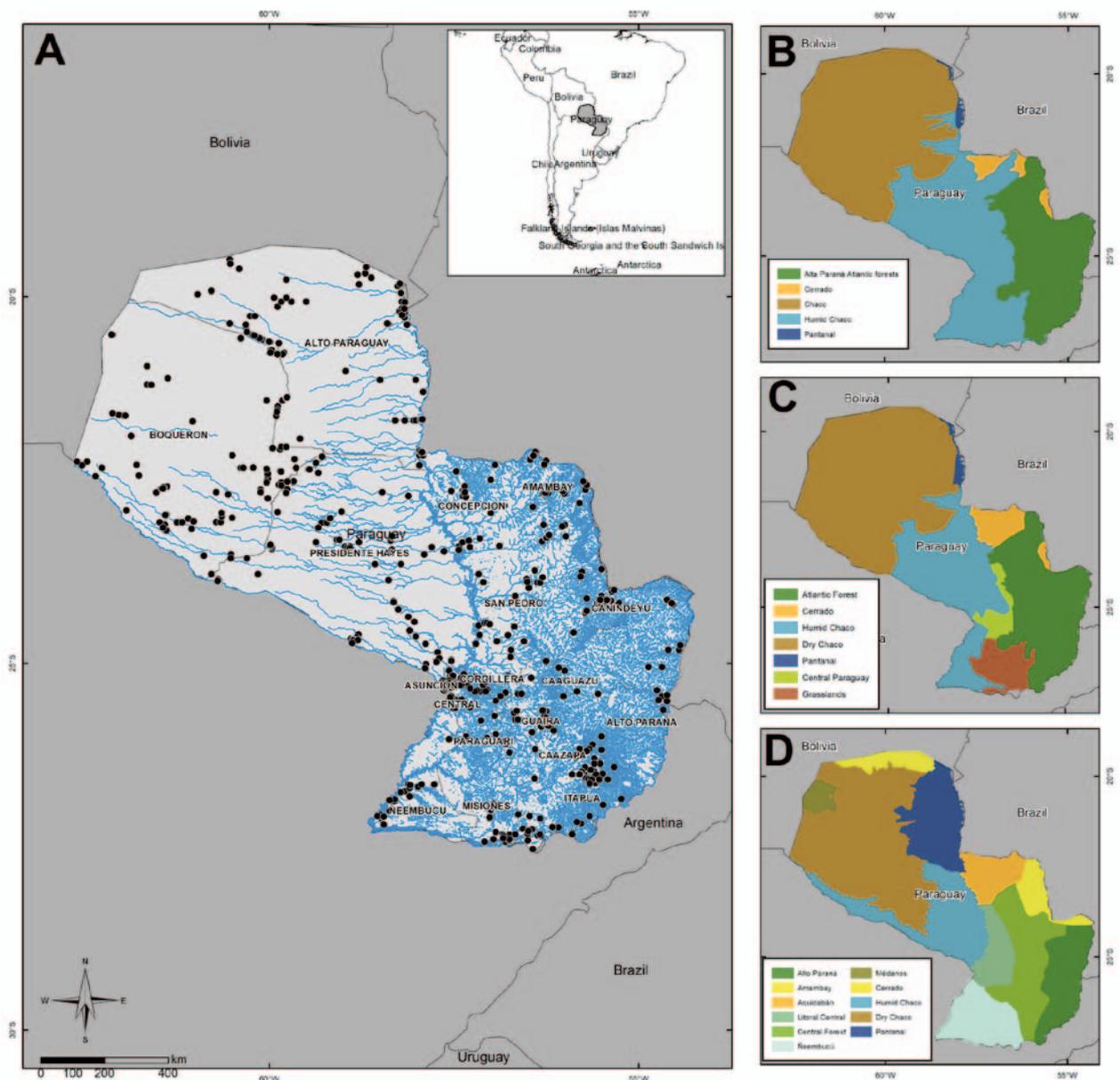
### INTRODUCTION

There are several studies that analyse and describe the distribution of different organisms at different scales in South America (e.g. Cabrera & Yepes, 1960; Cabrera & Willink, 1973; Morrone, 2001; Diniz-Filho et al., 2006; Guedes et al., 2014; Xavier et al., 2014; Azevedo et al., 2016; Hoffmeister & Ferrari, 2016), however, two information gaps are still evident. Despite the known worldwide population declines in amphibians (Blaustein et al., 1994; Corns, 1994; Stuart et al., 2008), detailed distribution analyses of this group are scarce; and the distributional biodiversity patterns of Paraguay are still poorly known. In general, available biogeographical information for Paraguay is based on either studies that include the country as a part of a wider area (i.e. continental or regional analyses, for example; Lundberg et al., 1998; Leynaud & Bucher, 1999; Oakley et al., 2005; Cáceres, 2007; Werneck, 2011; Nascimento et al., 2013; Giarla & Jansa, 2014; Silva et al., 2014; Arzamendia & Giraud, 2015; Nori et al., 2015; Hoffmeister & Ferrari, 2016), or local and fragmentary studies based on different taxonomic groups (mammals: López-González, 2004; 2005; Stevens et al., 2007; Rumbo, 2010; birds: Hayes, 1995; reptiles: Bauer, 2014; Cacciali & Ubilla, 2016; and plants: Keel et al., 1993; Spichiger et al., 1995; Chernoff et al., 2004). These contributions are mostly focused on describing the relationships between species and

their habitats using indices of similarity, cluster analysis and predefined areas. Despite the contribution on the distribution of amphibians' species in Paraguay provide by Weiler et al. (2013), no progress has been made in the direction of formal analyses of distributions. The need for detailed studies on the distribution of amphibians in Paraguay is urgent in order to develop efficient conservation policies, especially in the biomes affected by the advance of agricultural frontiers, as in the Chaco region.

The concept of "areas of endemism" is used in biogeography to refer to those geographic areas delimited by the congruence in the distributions of at least two taxa (Platnick, 1991). These areas describe particular characteristics of biodiversity (Grehan, 1993; Carvalho, 2011) and their identification constitutes an important tool for conservation and a fundamental step in the understanding of the evolutionary history of taxa (Casagrande & Grosso, 2013; Warren et al., 2014). Several methodologies have been proposed in the last years for the identification of areas of endemism (Morrone, 1994; 2014; Hausdorf, 2002; Dos Santos et al., 2008; Veech, 2014; Da Silva et al., 2015; Guerin et al., 2015; Oliveira et al., 2015; Vilhena & Antonelli, 2015), however Parsimony Analysis of Endemicity (PAE; Morrone, 1994) and Endemicity Analysis (EA; Szumik et al., 2002 and Szumik & Goloboff, 2004) are the most used (Da Silva & Oren, 1996; García-Barros et al., 2002; Nori et al., 2011; Agesen et al.

*Correspondence:* María Dolores Casagrande (dolores.casagrande@gmail.com)



**Figure 1.** Reference Maps. **A)** Amphibian records used mapped on natural watercourses with political boundaries of Paraguay; **A-B-C)** Ecoregion schemes proposed for Paraguay by Dinerstein et al.(1995) **(B)**; del Castillo & Clay (2005) **(C)**; Secretaría del Ambiente (2013) **(D)**.

2012; Escalante, 2015; Cacciali & Ubilla, 2016; Andrade-Díaz et al., 2017). Different to other methods, EA has been exclusively developed for the identification of areas of endemism and shows advantages over other methods due to inclusion of spatial information in the searches (Casagrande et al., 2012).

Multiple definitions of “endemic” and “endemism” can be found in the literature, generating confusion and misunderstanding around the term (see Anderson, 1994). In the present study, we adopt the definition of Platnick (1991), considering an area of endemism as a geographic area defined by the congruent distribution of two or more taxa. Following this definition, a species will be considered as endemic when, together with other(s) species, it participates in the delimitation of an area of endemism. Since any species can contribute to the delimitation of areas of endemism at some geographic scale, no species

were discarded from the analyses in the present study, even if its distributional range exceeded the area of study.

**Table 1.** Parameters used in NDM/VNDM during the search of areas of endemism and consensus areas

	Grid Sizes		
	0.5°x0.5°	0.7°x0.7°	1°x1°
Fill	70	30	10
Assumed	100	50	30
Minimum species score	0.5		
Sets with	2 or more endemic species		
Sets with score above	2		
Random seed	1		
Repeat search	20		
Loose consensus rule	40 %		

The main goals of this papers are: 1) to identify areas of endemism based on the distribution of the 87 amphibian species known from Paraguay; and 2) to compare them with three ecoregion schemes proposed for Paraguay: (a) Dinerstein et al. (1995); (b) del Castillo & Clay (2005) and (c) Secretaría del Ambiente (2013) (Fig. 1B–D) and; 3) to provide updated and complete information on the distributional range of the amphibian species in the country, covering spatial gaps initially observed in the data.

## METHODS

### Study site

Paraguay is located in the centre of South America (Fig. 1A), occupying an area of 406,752 km<sup>2</sup>. The Paraguay River divides the country in two main regions: the Oriental region and the Occidental region or Chaco, which covers more than the 60 % of the national territory. The Oriental region presents an average temperature of 23°C and 1200–800 mm of annual precipitation (ENPAB, 2016), while the Chaco shows a similar average temperature (25°C) but an annual precipitation of approximately 400–200 mm. Paraguay does not contain large orographic chains or high elevations, with the greatest altitude at the Cerro Peró (840 m.a.s.l.).

### Data

Our database included 4744 records of 87 anuran species (32 genera, 10 families) distributed across Paraguay (Supplementary Table). These data were obtained from the main museum collections in the country: the Museo Nacional de Historia Natural del Paraguay (MNHNP) and the Instituto de Investigación Biológica del Paraguay (IIBP), as well as data from recent publications (i.e. Brusquetti & Lavilla, 2006; Weiler et al., 2013; Caballero et al., 2014; Brouard et al., 2015; Lavilla et al., 2016). The distribution records were revised and corrected, with taxonomy updated following Pyron & Wiens (2011), Duellman et al. (2016) and Dubois (2017). Records of doubtful taxonomic identity and imprecise localities were discarded. Records including the description of collection localities, but lacking geographic coordinates, were georeferenced with the help of Google Earth and ArcGis 10.1. Finally, records of the same species for the same collection locality were deleted in order to obtain a matrix of unique records. Our final database included 2560 unique localities for 87 anuran species distributed in Paraguay (Fig. 1A).

### Areas of Endemism

In order to identify areas of endemism, the distributional dataset was analysed with the software NDM/VNDM ver. 3 (Goloboff, 2004), which applies the optimality criteria described by Szumik et al. (2002) and Szumik & Goloboff (2004). Since geographic scale (grid size) influences pattern recognition (Casagrande et al., 2009; Szumik et al., 2012; Ocampo et al., 2019), in the present paper we analysed the data under three different grid sizes: 0.5° x 0.5°, 0.7° x 0.7° and 1° x 1°.

The information gaps in the distributions of species are mostly due to incomplete inventories (the Wallacean shortfall). To deal with data gaps in our matrix, we used the

fill option available in VNDM. This function infers potential presences of a species in cells that are surrounded — within a certain radius— by cells where that species is observed. Values used for the fill function are detailed in Table 1. In the case of species where automatic fill function was not enough to cover important data gaps, a hand-made fill was made, guided by the distribution maps of amphibians published by the International Union for the Conservation of Nature (IUCN, 2016).

During the search of areas of endemism, VNDM calculates an Endemicity Index for each species (EIs) distributed within the set of the cells evaluated (areas). The IEs measures the congruence among the distribution of a species and the given area, and varies from 0 to 1; where the maximum value of 1 is assigned to a species distributed uniformly and exclusively in the evaluated area, that is, a perfect fit. The IEs value decreases as the distribution of the taxon increases outside of the area and/or its distribution inside the area is scattered. The Endemicity Index of an area of endemism (EIa) is equal to the sum of the IEs of the endemic species it contains. The search parameters used in the analyses are detailed in Table 1 (parameters keeping their default values are not included in the table).

Areas of endemism similar in spatial structure and/or species composition (fide Casagrande et al., 2012) were grouped in consensus areas (CAs, see Aagesen et al., 2013) to summarise the results obtained. Two rules have been proposed for the construction of CAs: the tight and loose consensus rules (more details in Aagesen et al., 2013). Both rules group the areas according to a percentage of shared species defined by the user; in this work we used a loose consensus rule of 40 % of similarity. The general patterns described here are based on CAs and are compared with three ecoregion schemes proposed for Paraguay: (a) Dinerstein et al. (1995); (b) del Castillo & Clay (2005); (c) Secretaria del Ambiente (2013) (Fig. 1B–D).

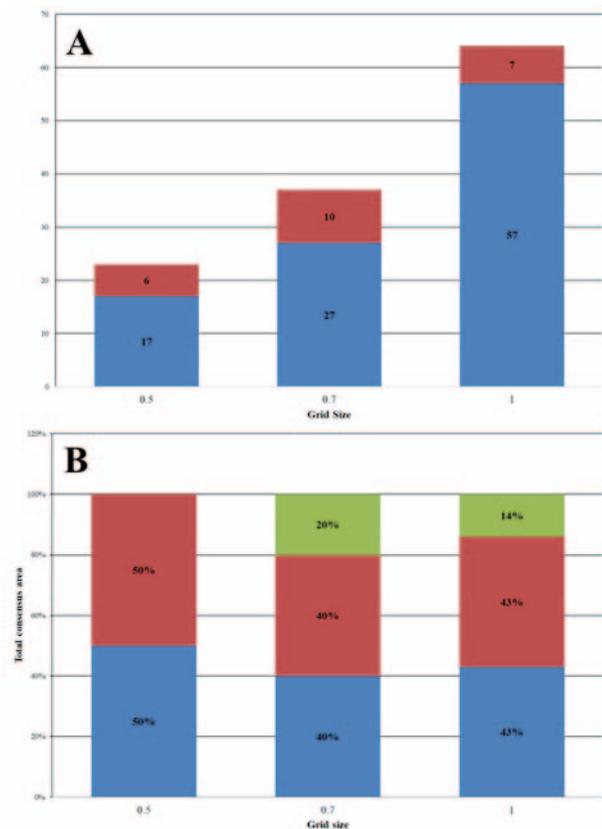
## RESULTS

### Identified Areas of Endemism

The searches resulted in 17, 27 and 57 individual areas of endemism (IA) for the 0.5°, 0.7° and 1° grid sizes, respectively; that were grouped in 6, 10, and 17 CAs. The CAs obtained under different grid sizes are mostly congruent among them (Fig. 2). The size of grid in which each CA was identified is indicated by a subscript number. The EI values for each area, the endemic species and consensus values are detailed in Table 2.

### Grid 0.5° x 0.5°

17 IAs were identified under this grid size and grouped into 6 CAs (Fig. 3A–B). The CA<sub>0.5</sub> covers all the country (Fig. 3B), while CA<sub>1.0.5</sub> is located in the south-eastern Oriental region, (Fig. 3A). CA<sub>2.0.5</sub> and CA<sub>4.0.5</sub> are found in the southern part of the country, defined by species characteristic of forested and open areas, respectively (Fig. 3A–B). CA<sub>3.0.5</sub> covers the western part of the Occidental region. Finally, CA<sub>5.0.5</sub> is located in the northern Oriental region (Fig. 3A).



**Figure 2.** **A)** Total number of individual areas (IA, blue) and consensus areas (CA, red) recognised at different scales (0.5°x0.5°, 0.7°x0.7°, 1°x1°). **B)** Total number of CAs recognised at different scale of analyses (0.5°x0.5°, 0.7°x0.7°, 1°x1°). Different colours in the bars represent the percentage of CAs exclusively recognised under each scale (green); percentage of CAs recognised under two scales (red); and percentage of CA recognised under all scales of analyses (blue).

#### Grid 0.7° x 0.7°

With the grid size of 0.7° x 0.7°, 27 IAs were identified and grouped in 10 CAs (Fig. 3C-F). CA<sub>0.7</sub> is defined by species associated with forest and is located in the south-eastern part of the Oriental region (Fig. 3E). CA<sub>1.0.7</sub> and CA<sub>4.0.7</sub> contain species widely distributed in the country (Fig. 3F). The limits of CA<sub>2.0.7</sub> coincide with the Oriental region and part of the Occidental region, with species related to open areas (Fig. 3C). CA<sub>5.0.7</sub>, located in the Occidental region (Fig. 3E); CA<sub>6.0.7</sub> covers the entire Occidental and part of the Oriental region (Fig. 3D). CA<sub>7.0.7</sub> is located in the northern Oriental region, with species characteristic of both forested and open areas (Fig. 3E). Finally, CA<sub>8.0.7</sub> and CA<sub>9.0.7</sub> are located in the south of the Oriental region, with species typical of forested and open areas (Fig. 3D-E).

#### Grid 1° x 1°

In this grid size, 57 IAs were identified and grouped in 7 CAs (Fig. 3G-I). CA<sub>1</sub> covers all the country, with species of wide distribution that are associated with many different types of habitats (Fig. 3G); while the CA<sub>1</sub> and CA<sub>2</sub> are located in the centre of the country, with species associated mostly with open areas; however, some species related to

forested areas are also present (Fig. 3H). CA<sub>3</sub> is located in the north of the Oriental region, defined by both open and forested area species, while CA<sub>4</sub> covers the entire Occidental region. CA<sub>5</sub> is located in the south of the Oriental region, with species characteristic of forested and open areas (Fig. 3I). CA<sub>6</sub> covers the entire Oriental region and the northern Occidental region (Fig. 3G).

#### CAs compared to ecoregions

Several CAs found are congruent with different ecoregions proposed for Paraguay. The Dry Chaco (sensu Dinerstein et al., 1995) was recovered by CA<sub>3.0.5</sub>, CA<sub>5.0.7</sub> and CA<sub>4</sub> (Fig. 4A); the Atlantic Forest (sensu Dinerstein et al., 1995) was recovered with CA<sub>1.0.5</sub> and CA<sub>0.7</sub> (Fig. 4B); and the Cerrado (sensu Dinerstein et al., 1995) was recovered by CA<sub>5.0.5</sub>, CA<sub>7.0.7</sub> and CA<sub>3</sub> (Fig. 4C). The Mesopotamian Grasslands (del Castillo & Clay, 2005) was recovered by CA<sub>2.0.5</sub> and CA<sub>9.0.7</sub> (Fig. 4G), while the Ñeembucú ecoregion (Secretaria del Ambiente, 2013) was recovered with CA<sub>4.0.5</sub> and CA<sub>8.0.7</sub> (Fig. 4F). Also, the Oriental region was recovered as an area of endemism by CA<sub>2.0.7</sub> (Fig. 4E) and the Dry Chaco + Humid Chaco (sensu Dinerstein et al., 1995) was recovered as a single area in the CA<sub>6.0.7</sub> (Fig. 4D). The species scores for each CA and the corresponding values are found in Table 2.

#### Dry Chaco

The Dry Chaco ecoregion (sensu Dinerstein et al., 1995) (Fig. 4A) is defined by taxa traditionally recognised as endemic to this ecoregion (Table 2), such as *Leptodactylus laticeps* (De Sá et al., 2014), *Chacophrys pierottii*, *Lepidobatrachus laevis* and *Lepidobatrachus llanensis*, and *Ceratophrys cranwelli* (Brusquetti & Lavilla, 2006; Faivovich et al., 2014). Also inhabiting part of this ecoregion are the species *Dermatonotus muelleri*, *Elachistocleis haroi*, *Leptodactylus bufonius*, *Rhinella major*, and *Odontophrynus lavillai*.

#### Atlantic Forest

Our results recover this ecoregion by the congruent distributions of *Melanophryniscus atroluteus*, *Melanophryniscuskrauczuki*, *Chthonerpetonindistinctum*, *Boana pulchella*, *Boana curupi*, *Crossodactylus schmidti*, *Itapotihyla langsdorffii*, *Argenteohyla siemersi*, *Phyllomedusa tetraploidea*, *Proceratophrys avelinoi*, and *Luetkenotyphlus brasiliensis* (Table 2). Almost all these species are associated with the Atlantic Forest (Fig. 4B) in Argentina, Brazil and Paraguay (Brusquetti & Lavilla, 2006; Brusquetti & Lavilla, 2008; Caldart et al., 2010; Motte et al., 2011), except for *Melanophryniscus devincenzii* that has a disjunct distribution, with populations in Uruguay separated from those in north-eastern Argentina and southern Paraguay (Maneyro & Kwet, 2008; Airaldi et al., 2008; Boeris et al., 2010), and *A. siemersi* with a unique record known from Paraguay (Villarrica, Department of Guairá; see Brusquetti & Lavilla, 2006), in the transition area among Atlantic Forest and Humid Chaco (sensu Dinerstein et al., 1995).

#### Cerrado

The Cerrado ecoregion (sensu Dinerstein et al., 1995) (Fig. 4C) is characterised in our analyses by *Physalaemus*

**Table 2.** Consensus areas in congruence with the ecoregions proposed for Paraguay. By column: Grid size of analysis (Grid), Consensus Areas ID (CA), Endemic Species, ID of the Individual Areas of Endemism included in the CA; Consensus Endemicity Value (CEV).

Grid	CA	Species	AEI	CEV
<b>Dry Chaco ecoregion</b> (Dinerstein et al., 1995)				
0.5	3	<i>Ceratophrys cranwelli</i> , <i>Chacophrys pierottii</i> , <i>Lepidobatrachus laevis</i> , <i>Leptodactylus laticeps</i>	10	3.04-3.29
0.7	5	<i>Ceratophrys cranwelli</i> , <i>Chacophrys pierottii</i> , <i>Lepidobatrachus laevis</i> , <i>Lepidobatrachus llanensis</i> , <i>Leptodactylus laticeps</i> , <i>Odontophrynus lavillai</i>	8, 10, 15	2.82-3.32
1	4	<i>Ceratophrys cranwelli</i> , <i>Chacophrys pierottii</i> , <i>Lepidobatrachus laevis</i> , <i>Lepidobatrachus llanensis</i> , <i>Leptodactylus laticeps</i> , <i>Leptodactylus bufonius</i> , <i>Odontophrynus lavillai</i> , <i>Elachistocleis haroi</i> , <i>Dermatonotus muelleri</i> , <i>Rhinella major</i> , <i>Phyllomedusa sauvagii</i>	6, 13, 37, 39, 56	3.42-4.92
<b>Atlantic Forest ecoregion</b> (Dinerstein et al., 1995)				
0.5		<i>Melanophryniscus atroluteus</i> , <i>Melanophryniscus krauczuki</i> , <i>Chthonerpeton indistinctum</i> , <i>Boana pulchella</i> , <i>Crossodactylus schmidtii</i> , <i>Phyllomedusa tetraploidea</i>	3, 8, 15, 17	2.95-3.45
0.7		<i>Melanophryniscus atroluteus</i> , <i>Melanophryniscus krauczuki</i> , <i>Melanophryniscus devincenzii</i> , <i>Rhinella ornata</i> , <i>Proceratophrys avelinoi</i> , <i>Luetkenotyphlus brasiliensis</i> , <i>Boana curupi</i> , <i>Itapotihyla langsdorffii</i> , <i>Crossodactylus schmidtii</i> , <i>Argenteohyla siemersi</i> , <i>Phyllomedusa tetraploidea</i>	0, 3, 11, 12, 21, 25	2.02-3.88
<b>Cerrado ecoregion</b> (Dinerstein et al., 1995)				
0.5	5	<i>Physalaemus centralis</i> , <i>Physalaemus marmoratus</i> , <i>Leptodactylus furnarius</i> , <i>Rhinella icterica</i> , <i>Dendropsophus jimi</i>	14	3.71-3.96
0.7	7	<i>Physalaemus centralis</i> , <i>Physalaemus marmoratus</i> , <i>Leptodactylus furnarius</i> , <i>Rhinella icterica</i> , <i>Rhinella scitula</i> , <i>Dendropsophus jimi</i>	18	4.64-4.89
1	3	<i>Physalaemus centralis</i> , <i>Physalaemus marmoratus</i> , <i>Leptodactylus furnarius</i> , <i>Rhinella icterica</i> , <i>Rhinella scitula</i> , <i>Dendropsophus jimi</i> , <i>Dendropsophus elianae</i> , <i>Elachistocleis matogrosso</i> , <i>Siphonops paulensis</i>	3, 25, 51, 52	2.93-5.24
<b>Mesopotamian grasslands ecoregion</b> (Del Castillo & Clay, 2005)				
0.5	2	<i>Melanophryniscus atroluteus</i> , <i>Melanophryniscus krauczuki</i> , <i>Chthonerpeton indistinctum</i> , <i>Boana pulchella</i> , <i>Crossodactylus schmidtii</i> , <i>Phyllomedusa tetraploidea</i>	7, 12, 13	2.25-3
0.7	9	<i>Melanophryniscus atroluteus</i> , <i>Melanophryniscus krauczuki</i> , <i>Chthonerpeton indistinctum</i> , <i>Boana pulchella</i> , <i>Crossodactylus schmidtii</i>	22	4.29-4.54
<b>Ñeembucú ecoregion</b> (Mereles et al., 2013; Secretaría del Ambiente, 2013)				
0.5	4	<i>Pseudopaludicola mystacalis</i> , <i>Physalaemus santafecinus</i> , <i>Scinax similis</i>	11	2.39-2.64
0.7	8	<i>Pseudopaludicola mystacalis</i> , <i>Physalaemus santafecinus</i> , <i>Scinax similis</i>	19	2.43-2.68
<b>Oriental region</b>				
0.7	2	<i>Boana albopunctata</i> , <i>Boana caingua</i> , <i>Boana faber</i> , <i>Dendropsophus minutus</i> , <i>Ollolygon berthae</i> , <i>Proceratophrys avelinoi</i> , <i>Leptodactylus labyrinthicus</i> , <i>Rhinella ornata</i>	2, 4, 9, 13, 17	2.21-3.71
<b>Great American Chaco</b> (TNC et al., 2005)				
0.7	6	<i>Physalaemus biligonigerus</i> , <i>Leptodactylus bufonius</i> , <i>Dermatonotus muelleri</i> , <i>Elachistocleis haroi</i> , <i>Rhinella major</i>	16	4.05-4.30

*centralis*, *Physalaemus marmoratus*, *Lepodactylus furnarius*, *Rhinella scitula*, *Dendropsophus elianae*, and *Dendropsophus jimi* all formerly described as endemic to the Cerrado (Table 2) (Napoli & Caramashi, 1999; Caramashi & Niemeyer, 2003; Nascimento et al., 2006; Baldo et al., 2008; Vasconcelos et al., 2014; Loebmann et al., 2017). Other species occurring in this area are *Elachistocleis matogrosso*, associated with the Pantanal and the Cerrado (Caramashi, 2010; Brouard et al., 2015), and *Rhinella icterica*, which occurs both in Cerrado and Atlantic Forest (Brusquetti & Lavilla, 2006; Valdujo et al., 2012).

### Mesopotamian grasslands

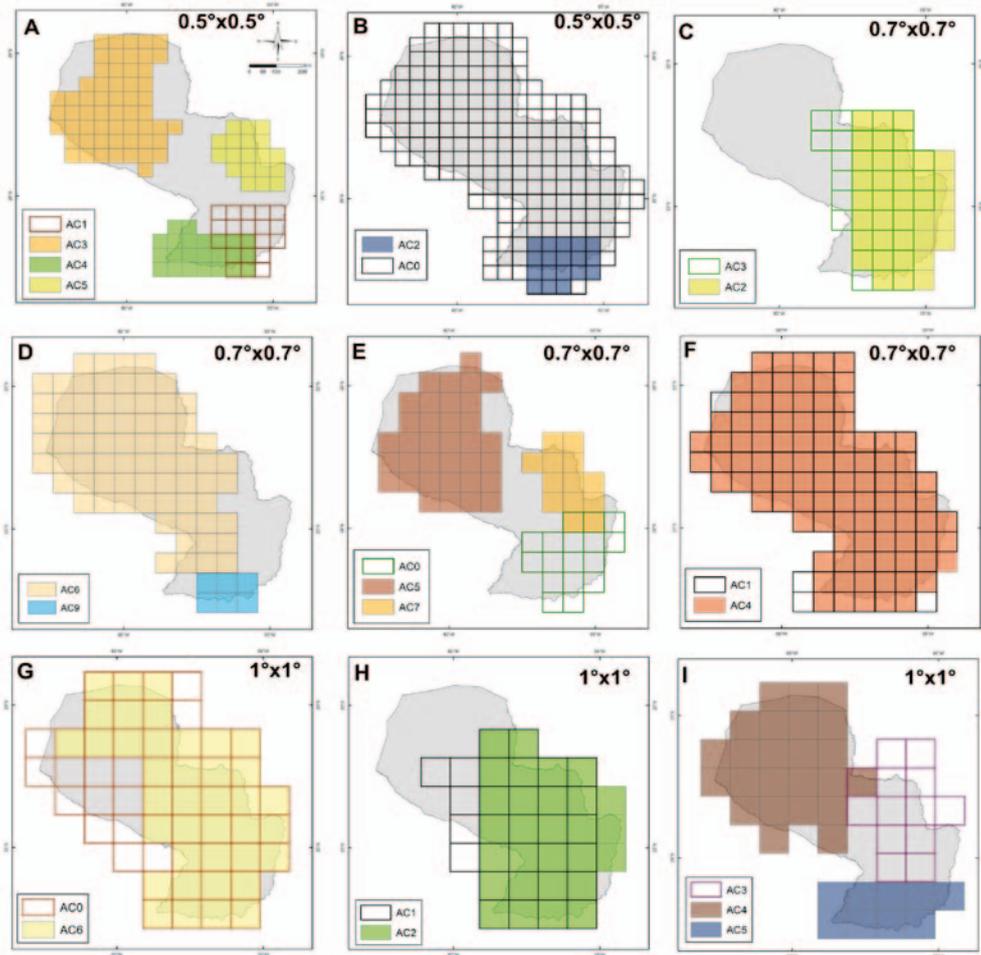
The Mesopotamian grasslands ecoregion is defined in our results by the congruent distributions of *C. schmidtii*, *P. tetraploidea* and *M. krauczuki*, species with distributions related to the Atlantic Forest (Table 2; Fig. 4G; Baldo & Basso, 2004; Brusquetti et al., 2007; Caldart et al., 2013). The fact that these species, predominantly distributed in

the Atlantic Forest, score for an open formation ecoregion could be a consequence of poor sampling. In Paraguay, these three species are only known from only a single locality each, and all these localities are on the geographic boundaries of the Mesopotamian Grasslands (see Fig. 1C). Species of wider distribution such as *C. indistinctum* —an aquatic species associated with the Parana River system — (Brusquetti & Lavilla, 2006; Cajade, 2012), *B. pulchella* and *M. atroluteus* — which are associated with open areas in Brazil, Argentina and Paraguay— (Ceí, 1980; Brusquetti & Lavilla, 2006) also contribute to define this ecoregion.

### Ñeembucú

*Scinax similis*, *Physalaemus santafecinus* and *Pseudopaludicola mystacalis* (Table 2) defined the Ñeembucú ecoregion (Secretaría del Ambiente, 2013; Fig. 4F), all three are species associated to open areas (Brusquetti & Lavilla, 2006; Brusquetti et al., 2009; Ingaramo et al., 2011).

### Oriental region



**Figure 3.** Consensus areas identified under different scales: **(A-B)** CAs identified when using grids of  $0.5^{\circ} \times 0.5^{\circ}$ ; **(C-F)** CAs identified when using grids of  $0.7^{\circ} \times 0.7^{\circ}$ ; **(G-I)** CAs identified when using grids of  $1^{\circ} \times 1^{\circ}$

The Oriental region is extended from the eastern margin of the Paraguay River to the Rio Parana (Fig. 4E) and was defined by widely distributed species such as *Dendropsophus minutus*, *Boana albopunctata* and *Leptodactylus labyrinthicus* (Ceï, 1980; Brusquetti & Lavilla, 2006; de Sá et al., 2014; Gehara et al., 2014) and species associated with the Atlantic Forest, such as *Boana caingua*, *B. faber*, *P. avelinoi* and *Rhinella ornata* (Table 2, Ceï, 1980; Kwet & Faivovich, 2001; Baldissera et al., 2004; Brusquetti & Lavilla, 2006; Lavilla & Brusquetti, 2010). *Oligolygon berthae* is also included as endemic to this area, a species characteristic of open areas and the Atlantic Forest in Argentina, Brazil, Uruguay and Paraguay (Lopez et al., 1999; Brusquetti & Lavilla, 2006; Duellman et al., 2016). The distributions of all these species reach the Paraguay River in the Oriental region, but none of them has been found in the Occidental region.

#### Great American Chaco (Dry Chaco + Humid Chaco)

The Dry Chaco + Humid Chaco (Great American Chaco sensu Dinerstein et al., 1995; Fig. 4D) is identified by typical Chacoan species like *E. haroi* and *L. bufonius* (Table 2) (Narvaes & Rodrigues, 2009; Caballero et al., 2014; de Sá et al., 2014; Pereyra et al., 2016). *Dermatonotus muelleri*, which has a wide distribution in Argentina, Bolivia, Brazil and Paraguay (Brusquetti & Lavilla, 2006) and is associated with the dry diagonal of open formations (Prado & Gibbs,

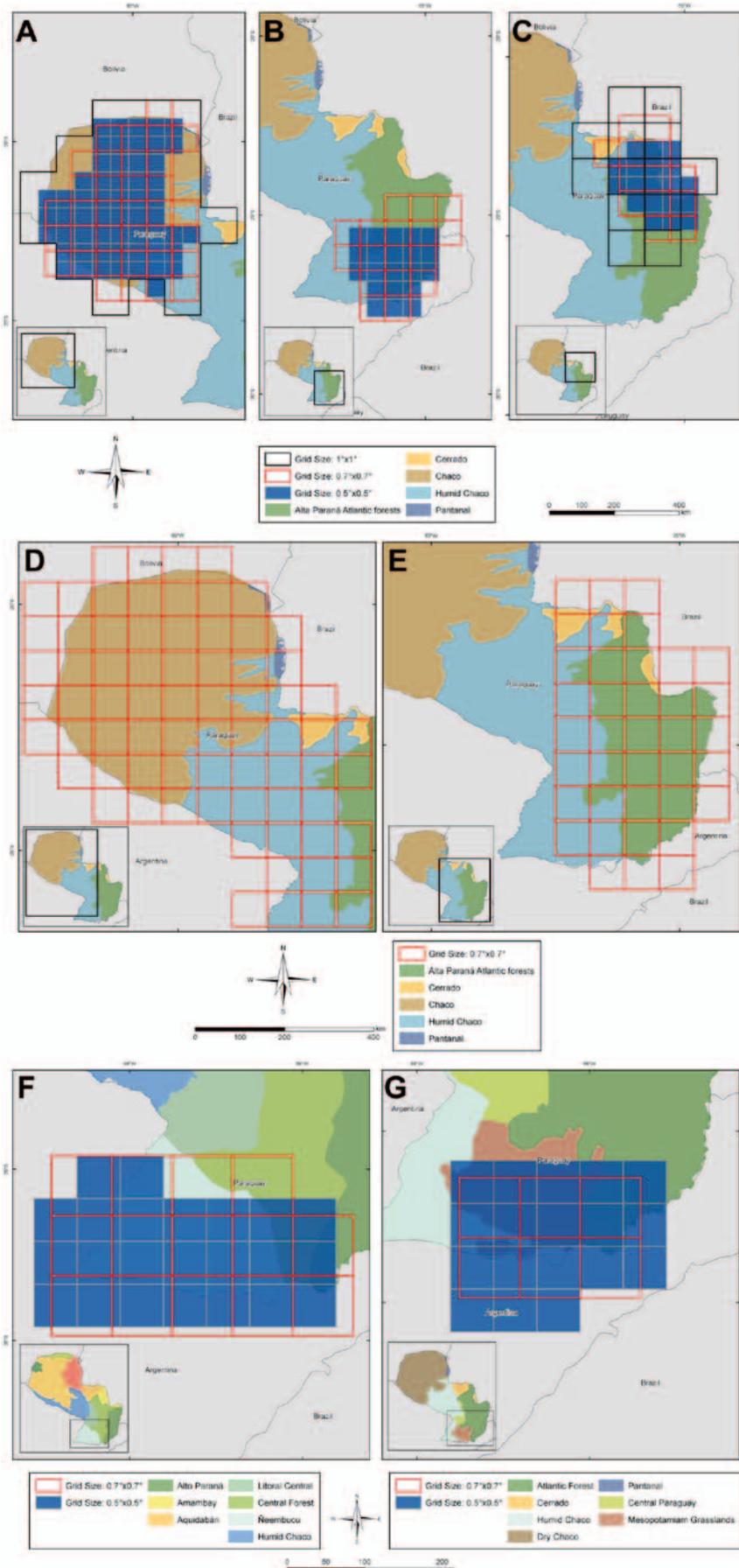
1993), *R. major* and *Physalaemus biligonigerus*, widely distributed in Argentina, Bolivia, Brazil and Paraguay (Brusquetti & Lavilla, 2006; De La Riva et al., 2000; Narvaes & Rodrigues, 2009), also contribute to define this CA.

## DISCUSSION

#### Areas of endemism and ecoregions

Our results indicate the existence of two large areas of endemism in Paraguay: the Dry Chaco ( $AC_{3_{0.5}}$ ,  $AC_{5_{0.7}}$  and  $AC_{4_1}$ ) and the Oriental region ( $AC_{2_{0.7}}$ ) (Figs 4A, E). The Dry Chaco was identified under all the grid sizes used ( $0.5^{\circ}$ ,  $0.7^{\circ}$ ,  $1^{\circ}$ ) and matches with the definitions of several authors (Dinerstein et al., 1995; Mereles et al., 2013). This area is characterised by species strongly linked with Chacoan environments such as *C. cranwelli*, *Ch. pierottii*, *L. laevis*, *L. llanensis*, *Le. laticeps* and *O. lavillai* (De la Riva et al., 2000; Brusquetti & Lavilla, 2006; Faivovich et al. 2014).

In the Oriental region five CAs, related to previously defined ecoregions, were identified for amphibians (Atlantic Forest, Cerrado, Ñeembucú, Mesopotamian grasslands and Oriental region), in contrast with previous studies that identified only two areas of endemism for birds: Campos Cerrados to the north and Paraná to the east (Cracraft, 1985; Hayes, 1995). The Campo Cerrado and Parana (Cracraft, 1985; Hayes, 1995) were also recovered in our analysis and are congruent with the Cerrado and



**Figure 4.** Consensus area identified in congruence with the ecoregions proposed by different authors. **(A-E)** Dinerstein et al. (1995). **A)** Dry Chaco; **B)** Atlantic Forest; and **C)** Cerrado; **D)** Great American Chaco (Dry Chaco + Humid Chaco); **E)** Oriental region no formal proposal as an ecoregion; **F)** Ñeembucú according to Secretaría del Ambiente (2013); and **G)** Mesopotamian grasslands according to del Castillo & Clay (2005).

Atlantic Forest ecoregions (Figs. 4C and B). The Cerrado is an ecoregion related to xerophyte environments of South America (Prado & Gibbs, 1993; Cacciali & Ubilla, 2016). In this work, we identify the Cerrado as an area independent from the Atlantic Forest, each one identified by unique and characteristic species, thus sustaining the identity of each area. This differs from what was found by Cacciali & Ubilla (2016) for reptiles, where the Atlantic Forest was recognised as an area of endemism with the Cerrado nested inside. However, these authors suggested that some sampling problems could have influenced on their results, like sampling concentration in specific localities and total absence of records in large areas.

The Paraguay River dates from the early Miocene (Potter, 1997) and it has been proposed as a physical barrier to the distribution of the species of *Thylamys* (Mammalia) (Giara & Jansa, 2014). This was also observed by Piatti (2017) for different species of the genus *Xenodon*, which can be found on opposite sides of the river in natural areas with different biotic characteristics (e.g. *Xenodon pulcher* and *X. semicinctus* on the western side, and *X. histricus* and *X. dorbignyi* in the east). As stated by Myers (1982) the differences between both sides of the Paraguay River are attributed to distinct biotic characteristic, which determine different habitats on each side: forested humid habitats in the east and xerophytic and arid habitats in the west. In line with the observed for mammals and reptiles, major differences in the general composition of amphibian species can be observed between the eastern and western sides of the river. However, large areas along the river with similar habitats in both margins, present certain species characteristic of the Great American Chaco (Dry Chaco + Humid Chaco) occurring on both sides (see Souza et al., 2010; Sugai et al., 2013; Weiler et al., 2013; Brusquetti et al., 2018). This fact indicates that, although the river acts as a barrier for several taxa, its effectiveness differs among groups and among species. More studies are needed to better understand the role of the river as a barrier in the distribution of amphibians and to determine which factors make it more or less effective in limiting dispersal of different groups.

Our results also recover the Great American Chaco (Dry Chaco + Humid Chaco) as a single area of endemism, in concordance with those described by Szumik et al. (2012). Unlike the Dry Chaco, this area is defined by widespread species, which besides the Chaco also occurs in the Yungas (e.g., *E. haroi*), Cerrado and Caatinga (e.g., *D. muelleri*), and Amazonia (e.g., *R. major*). A similar area of endemism was also identified for birds by Hayes (1995) and for reptiles by Cacciali & Ubilla (2016).

#### Different scales in the identification of areas of endemism

As discussed by several authors, the use of different scales/grid sizes influences the search and identification of areas of endemism (Aagesen et al., 2009; Casagrande et al., 2009; Szumik et al., 2012). Our results show an increase in the number of areas of endemism identified when increasing the grid size (Fig. 2), furthermore, some areas of endemism are only identified when using a specific grid size (Table 2). The effects of the grid size on the identification of areas of endemism is especially relevant when analysing datasets with sampling gaps

(Casagrande et al., 2009; Szumik et al., 2012), and species with discontinuous distributions, as is the case of some amphibians in Paraguay. The filling tools offered by NDM/VNDM helped to deal effectively with this problem, diminishing the impact of data incompleteness.

In their identification of areas of endemism for reptiles of Paraguay, Cacciali & Ubilla (2016) found only three areas of endemism when applying the Parsimony Analysis of Endemicity (PAE). These authors suggested that poor sampling efforts in some areas, together with intensive collection in urban centres and along access roads, would be the cause for the poor pattern recognition and questioned the validity of the results. Although similar sampling problems were observed in our amphibian dataset, the use of different grid sizes together with the manual filling tool allowed us to ameliorate the gap information problem, identifying more areas of endemism and characterising these better. A possible cause of the limited number of AEs identified by Cacciali & Ubilla (2016) - not explored by the authors - is their methodological choice. As discussed by several authors, PAE has shown to be very sensitive to incomplete sampling (a common problem in distributional databases; Arias et al., 2010), as well as a to have a poor performance when dealing with the identification of overlapping and disjunctive patterns, relatively common in nature (Casagrande et al., 2012; Szumik et al., 2018).

#### Final considerations

Our paper presents the first delimitation of areas of endemism in Paraguay based on amphibians. Most ecoregions previously proposed for Paraguay (such as Pantanal and Humid Chaco; Dinerstein et al., 1995; Mereles et al., 2013) show a high congruence with the CAs recovered in our analyses, however, the CA<sub>2,0.7</sub> does not show correspondence with the ecoregions but represent an original distributional pattern, related to the transition zone between the Atlantic Forest and the Cerrado. This CA indicates a biotic cline area characterised for a mixture of species: from species adapted to more humid regions (e.g. *B. caingua*, *B. faber*, *O. berthae*, *P. avelinoi*, *R. ornata*) to species widely distributed that mainly inhabit much drier environments (e.g. *Le. labyrinthicus*, *B. albopunctata*).

Our results corroborate that classic ecoregions, qualitative defined on the base of flora, also represent natural patterns for amphibians. The application of a quantitative method delivered hypothesis of endemism feasible to be tested, as well as made available a list of endemic amphibian species for each area of endemism, facilitating future discussion of results. Quantitative studies, like the present, allow the replication of analyses, facilitating the discussion of hypotheses under the light of new evidence. In this sense, much is still to be done in biogeography of Paraguay and we hope this contribution will be a first step in this direction.

The description of areas of endemism for amphibians provides fundamental information to discuss the evolution of these taxa across time and space, and opens new questions about the incidence of ecological and historical factors on their distributional ranges. Understanding the processes involved in shaping the distribution of amphibians are important in a time where their existence

seems to be threatened from multiple fronts (Scheele et al., 2019). Furthermore, about 50 % of the amphibian species of Paraguay are distributed in the Chaco, one of the most diverse biomes in South America (WWF, 2015), hosting a wide diversity among which about a quarter are endemic (Redford et al., 1990; Nori et al., 2016) and subjected to strong environmental pressures. In the last 10 years, the great American Chaco has reached the highest rate of deforestation in the world, with more than 1500 hectares of habitat destroyed every day (Hansen et al., 2013; Caballero et al., 2014). This ecoregion has been set as a priority for conservation of Neotropical terrestrial vertebrates (Loyola et al., 2009), that is why studies that resume and formalise the knowledge on the geographic distribution of the species inhabiting the Chaco become urgent as a first step to preserve these.

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## A review of mono- and bispecific genera of Amphibians worldwide

Giovanni Amori<sup>1</sup>, Marco A. Bologna<sup>2</sup> & Luca Luiselli<sup>3</sup>

<sup>1</sup>National Research Council (CNR) Institute of Research on Terrestrial Ecosystems, viale dell'Università 32, I-00185 Rome, Italy

<sup>2</sup>Dipartimento di Scienze, Università Roma Tre, Viale G. Marconi, 446, 00146 Rome, Italy

<sup>3</sup>Institute for Development, Ecology, Conservation and Cooperation, via G. Tomasi di Lampedusa 33, I-00144 Rome, Italy and Department of Applied and Environmental Biology, Rivers State University of Science and Technology, P.M.B. 5080 Nkpolu, Port Harcourt, Rivers State, Nigeria

Monospecific and bispecific genera are of particular interest in studies of taxonomic diversity and speciation evolution. Here, the distribution patterns of mono- and bispecific amphibians worldwide are investigated, with some discussion of on the conservation implications also presented. Based on an online database search (available from the American Museum of Natural History, New York), we found that the mean number of mono- and bispecific genera was similar among zoogeographic regions, with a total of 120 mono- (95 Anura, 17 Caudata, 8 Gymnophiona) and 65 bispecific (48 Anura, 10 Caudata, 7 Gymnophiona) genera. Out of 73 known amphibian families worldwide, only 35.6 % of them do not contain any mono- or bispecific genera. The frequency of mono- or bispecific genera by family was not significantly different among Anura, Caudata and Gymnophiona. There was a general tendency for the number of mono- and bispecific genera of amphibians to be positively correlated with the total number of genera in that family. In Anura, there was a preponderance of mono-specific genera in Afrotropical and Neotropical regions. Concerning bispecific genera, there was a clear preponderance in the Neotropical region for anurans. There was a positive correlation between the number of threatened genera (according to the IUCN Red List) in both the mono- and bispecific groups and the relative number of species in each taxon, thus showing that taxonomical speciosity clearly influences the frequency of occurrence of mono- and bispecific taxa in each family and order. In this regard, Anura dominated in both the number of worldwide described mono- and bispecific taxa as well as in that of the threatened ones according to IUCN Red List.

*Keywords:* Anura, Caudata, Gymnophiona, monospecific genera, bispecific genera, biogeography, conservation

In studies of taxonomic diversity, monospecific and bispecific genera are noteworthy compared to multispecies genera, as they may be considered either evolutionarily older (Ridley, 1993) or not-yet-diversified young lineages (Alroy et al., 2008). Furthermore, in many cases, mono- and bispecific genera are endemic to narrow areas (Ridley, 1993). Mono- and bispecific genera may be susceptible to higher extinction risk than multispecies genera, with the eventual extinction of such genera being particularly negative in evolutionary terms because it would represent the extinction of an evolutionary lineage (Cotgreave & Pagel, 1997; Purvis et al., 2000).

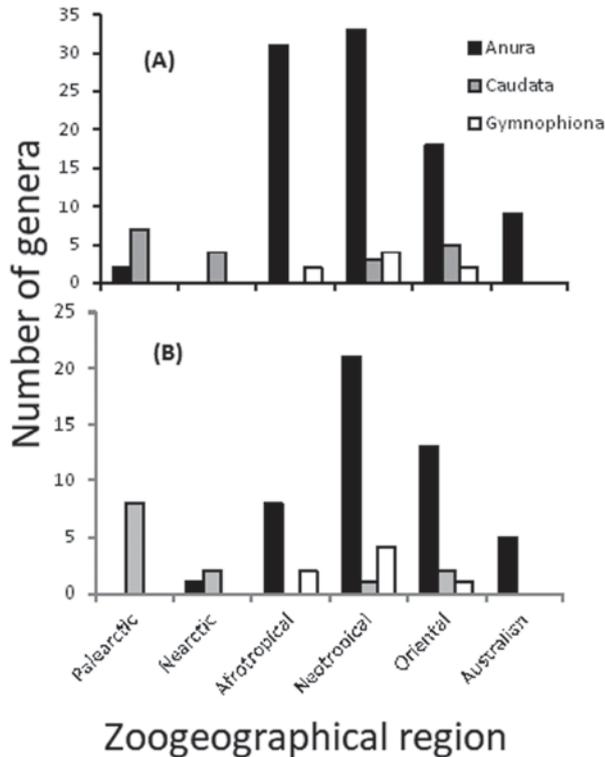
Recent studies have focused on the patterns of occurrence of mono- and bispecific genera of rodents, soricomorphs and chelonians, and their conservation implications (e.g. Amori et al., 2008, 2017; Amori & Luiselli, 2018). These studies revealed that the frequency of mono- and bispecific genera was significantly uneven across geographic regions, with the highest fraction of these genera associated with the Orient, followed by the Neotropical and Afrotropical regions for the chelonians (Amori & Luiselli, 2018), whereas the peaks of mono- and bispecific genera richness were observed in Neotropical, Oriental and Afrotropical regions for rodents, and in the Palearctic region for soricomorphs (Amori et al., 2008, 2017).

Despite amphibians being among the most threatened groups of animals (with 43 % of species threatened; e.g. Beebe & Griffiths, 2005; Stuart, 2008; Ceballos et al., 2010; IUCN, 2018), no previous studies have focused on their patterns of occurrence and conservation implications of their mono- and bispecific genera. Amphibians may represent ideal subjects of study because of their ancient history (Duellman & Trueb, 1994; Roelantz et al., 2007; Vitt & Caldwell, 2013), limited dispersal abilities (e.g. Smith & Green, 2005; Cushman, 2006; Semlitsch, 2008), and high rates of speciation in islands (e.g., Vences & Wake, 2007; Bell et al., 2015; Schluter & Pennell, 2017). In this paper, we explore the distribution patterns of the mono- and bispecific genera of amphibians worldwide, and also present some conservation considerations.

*Correspondence:* Giovanni Amori ([giovanni.amori@uniroma1.it](mailto:giovanni.amori@uniroma1.it))

**Table 1.** Synopsis of the number of mono- and bispecific genera of selected vertebrate groups, in relation to the total number of genera known from that specific taxon. Data for the number of mono- and bispecific genera were drawn from the present study (Amphibians), Amori & Luiselli, 2018 (chelonians), Amori et al., 2017 (rodents and soricomorphs). Data on the total number of genera per group were drawn from Halliday & Adler, 2002 (amphibians), Rhodin et al., 2017 (chelonians), and Wilson & Reeder, 2005 (mammals).

	Amphibians	Chelonians	Rodents	Soricomorphs
No. of monospecific genera (%)	118 (27.2 %)	28 (29.8 %)	200 (41.6 %)	18 (40 %)
No. of bispecific genera (%)	66 (15.2 %)	18 (19.1 %)	90 (18.7 %)	2 (4.4 %)
Total No. of genera	434	94	481	45



**Figure 1.** Frequency distribution of monospecific (A) and bispecific (B) genera of amphibians among zoogeographical regions

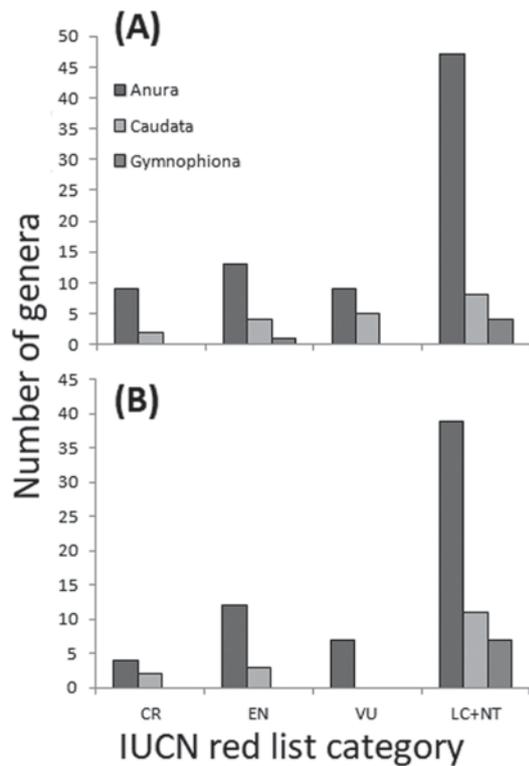
Data concerning the mono- and bispecific genera of amphibians were extracted from Frost (2018), and their distribution from both Frost (2018) and IUCN (2018). Their conservation status was assessed on the basis of the IUCN (2018) Red List criteria. For all analyses, fossil species were excluded. The zoogeographical region of each taxon was classified according to Wallace (1894), revised in Rueda et al. (2013). In this regard, we used the term 'Afrotropical' instead of 'Ethiopian', which was used originally by Wallace (1894). Data were obtained through the American Museum of Natural History, New York database (available at <<http://research.amnh.org/vz/herpetology/amphibia/>>) on the 9th of March 2017. For all analyses, we considered taxa occurring in more than one zoogeographical region as 'cosmopolitan'. Non-normally distributed variables were log-transformed prior to applying any statistical analyses. Non-normality of a given variable was assessed by Shapiro-Wilk W (assuming a non-normal distribution when  $p < 0.05$ ).

We analysed the differences in the mean number of species per genus across zoogeographical regions by one-way ANOVA. For mono- and bispecific taxa, in the analyses of the mean number of species per genus across zoogeographical regions, we pooled all groups of amphibians (Anura, Caudata and Gymnophiona) given that the number of cases was too low for some groups in some zoogeographical regions (for instance: in Anura, only three families occurred in the Palearctic region). We assessed the correlation between the number of mono- or bispecific genera and the total number of genera in a given family by Pearson's correlation coefficient. We performed observed-versus-expected  $\chi^2$  test in order to compare the frequencies of; (i) mono- or bispecific genera among amphibian orders, (ii) mono- and bispecific genera among zoogeographical regions, and (iii) mono- versus bispecific genera by zoogeographical region. In order to evaluate whether the various zoogeographical regions differed in terms of relative IUCN threat level, we first determined the expected frequency of each IUCN (2018) category (Critically Endangered (CR), Endangered (EN), Vulnerable (VU), and the non-threatened Least Concern (LC) + Near Threatened (NT)) by dividing the total number of monospecific taxa listed under each category by the total number of Red List assessed monospecific taxa (% AST). Then, we multiplied the % AST of each category for the total number of taxa of the same IUCN Red List category by the number of taxa of each category in each zoogeographical region (observed; OBS) and obtained the expected (EXP) values. Then, we compared EXP and OBS using a  $\chi^2$  test. All analyses were performed by PAST 3.0 statistical software, with alpha = 0.05 and all tests were two-tailed.

The distribution of the number of species per genus is presented in Appendix 1. The mean number of species per genus did not vary significantly across zoogeographical regions (one-way ANOVA:  $F_{5,63} = 0.784$ ,  $p = 0.565$ ).

In total, 120 known monospecific (95 Anura, 17 Caudata, 8 Gymnophiona) (Online Appendix 2) and 65 bispecific (48 Anura, 10 Caudata, 7 Gymnophiona) (Online Appendix 3) amphibian genera are analysed.

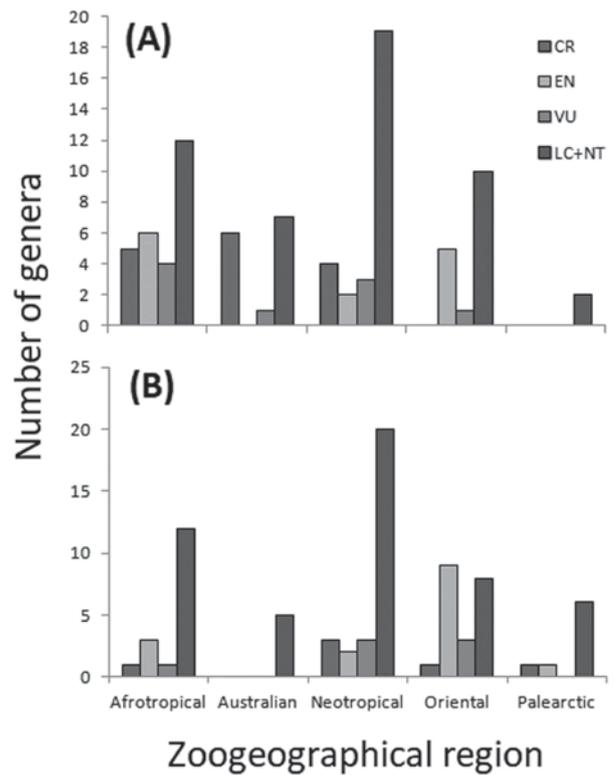
Overall, there were 73 amphibian families, with 35.6% without any mono- or bispecific genera. The percentage of families with no mono- or bi-specific genera was 33.3% in Anura ( $n = 54$  families in total), 44.4% in Caudata ( $n = 9$ ), and 40% in Gymnophiona ( $n = 10$ ). The frequency of mono- or bispecific genera was not significantly different among Anura, Caudata and Gymnophiona ( $\chi^2 = 4.711$ ,  $df = 2$ ,  $P > 0.050$ ). In Gymnophiona, the number of monospecific genera was positively correlated with



**Figure 2.** Frequency distribution of monospecific (A) and bispecific (B) genera of amphibians among IUCN Red List categories

the total number of genera in that family ( $r = 0.873$ ,  $P < 0.001$ ), whereas the same relationship was not statistically significant for the bispecific genera ( $r = 0.541$ ,  $P = 0.106$ ). The same relationships were found in Caudata (for monospecific taxa –  $r = 0.948$ ,  $P < 0.001$ ; for bispecific taxa –  $r = 0.513$ ,  $P = 0.158$ ), whereas in Anura there was a significant relationship for both the number of mono- and of bispecific genera (respectively,  $r = 0.912$  and  $r = 0.920$ , all cases  $P < 0.001$ ).

The geographical distribution of the number of species per genus is presented in Online Appendix 1. The mean number of species per genus did not vary significantly across zoogeographical regions (one-way ANOVA:  $F_{5,63} = 0.784$ ,  $p = 0.565$ ). In Anura, the frequency distribution of monospecific genera among zoogeographical regions differed significantly from equality ( $\chi^2 = 67.210$ ,  $df = 5$ ,  $P < 0.001$ ), with a preponderance of genera in Afrotropical and Neotropical regions (Fig. 1A). In Caudata and Gymnophiona, the number of monospecific genera was too low for any statistical analyses, but the absence of the former taxon from Afrotropical region and of the latter taxon from temperate regions (Nearctic and Palearctic) are linked to the absence of the whole group from the respective zoogeographical regions. The frequency distribution of bispecific genera among zoogeographical regions was also statistically different ( $\chi^2 = 29.300$ ,  $df = 5$ ,  $P < 0.001$ ), with a clear preponderance for bispecific genera in the Neotropical region (Fig. 1B). The frequency of distribution of monospecific genera did not differ significantly from that of bispecific genera across the



**Figure 3.** Frequency distribution of monospecific (A) and bispecific (B) genera of amphibians among IUCN Red List categories, by zoogeographical region

various zoogeographical regions ( $\chi^2 = 4.424$ ,  $df = 5$ ,  $P = 0.505$ ).

The summary of the IUCN Red List status for the mono- and bispecific amphibian genera worldwide is presented in Figure 2. Anura clearly dominated the number of threatened mono- and bispecific genera, but this likely reflects a sampling effect as Anura also dominated the number of worldwide amphibian genera (Fig. 2). Overall, a large portion of the mono- and bispecific genera were not threatened according to the IUCN criteria. In addition, there was no significant difference between mono- and bispecific genera in terms of frequency of the various threatened categories ( $\chi^2 = 2.591$ ,  $df = 3$ ,  $P = 0.469$ ). The distribution of the mono- and bispecific genera of amphibians in relation to the IUCN status by zoogeographical region is presented in Figure 3. Concerning the monospecific genera, the highest frequency of threatened taxa (especially CR) was found in the Afrotropical region (Fig. 3A); conversely, for the bispecific genera, the Oriental region supported the highest frequency of threatened taxa (Fig. 3B).

Amphibian taxonomy has undergone substantial changes over the past 20 years and still remains fluid, which is likely to also affect the number of mono- and bispecific genera. Despite this, here we show that (i) the mean number of mono- and bispecific genera was similar among zoogeographical regions, with only about 35 % of the amphibian families do not containing any mono- or bispecific genera; (ii) the number of mono- and bispecific genera depended on the total number of

genera in that family; (iii) in Anura, mono-specific genera occurred especially in Afrotropical and Neotropical regions, and bispecific genera in the Neotropical region. Whilst patterns (i) and (ii) depended merely on statistical reasons, pattern (iii) is worth of discussion in the frame of the general ecology and evolution of amphibians. Indeed, the excess of mono- and bispecific anuran genera in Afrotropical and Neotropical regions is linked to the availability of multiple niches in the tropical forest habitat, that have likely favoured speciation mechanisms for exploiting new resources and minimising interspecific competition (Duellmann, 1989; Slatyer et al., 2007; Losos, 2008). For instance, many mono- and bispecific genera are included in the family Microhylidae, which houses several ecologically highly specialised taxa with their eggs hatching into forest tree-holes or leaf axils, or with eggs developing into froglets in underground chambers (Halliday & Adler, 2002). Thus, we hypothesise that the occurrence of mono- and bispecific genera in amphibians is primarily linked to the exploitation of new micro-niches in tropical forest habitats.

Previous studies have shown that a higher diversity of mono- and bispecific genera occurred in the Oriental region in turtles (Amori & Luiselli, 2018), whereas peaks of mono- and bispecific genera richness were observed in Neotropical, Oriental and Afrotropical regions in Rodentia and in the Palearctic region in Soricomorpha (Amori et al., 2017) (Table 1). Therefore, the comparative evidence among taxonomic groups is that there is no consistent geographic pattern in these types of genera, with the frequency of mono- and bispecific genera differing remarkably by taxonomic groups in terms of “biogeographic hotspots”. We think that inter-taxa differences may reflect the evolutionary history of the various taxa rather than the relative dispersal abilities. Indeed, turtles, amphibians and small mammals are generally small to medium sized vertebrates, with relatively low dispersal potential (at least in comparison with other vertebrate groups) (Cagle, 1944; Gaines & Johnson, 1982; Ousterhout & Liebgold, 2010; Slavenko et al., 2016).

Concerning the IUCN threatened taxa, Amori et al. (2017) found that most mono- and bispecific threatened genera of rodents occurred in the Neotropical region, with no statistical pattern emerging for Soricomorpha.

For turtles, the “diversity hotspot” for threatened mono- and bispecific genera was the Oriental region (Amori & Luiselli, 2018; Rhodin et al., 2018), whereas we found an even more complicated pattern for amphibians, with Afrotropical region being the most important region for threatened monospecific genera (especially due to the contribution of Madagascar) and the Oriental region for the bispecific genera. Thus, once more our data reveal no inter-taxa consistency in the observed patterns. Also in this case, the frequency of IUCN’s (2018) threatened mono- and bispecific genera by zoogeographical region was correlated positively with the relative richness of mono- and bispecific genera in each region. The same correlation between frequency of threatened mono- and bispecific genera and of total genera richness by zoogeographical region was also observed in turtles (Amori & Luiselli, 2018) and in rodents (Amori et al.,

2017).

Pooling the mono- and bispecific genera, there was a similar percentage of occurrence among taxa (44 % in both Amphibians and Soricomorpha, and 48 % in turtles), with only rodents showing a considerably higher percentage (60 %). In addition, in all four taxa studied so far, bispecific genera were always substantially lesser than monospecific genera (representing 60-68 % in Amphibians, Chelonians and Rodents, and 90 % in Soricomorphs). Unfortunately, it is presently unknown whether this pattern was generated by chance or whether monospecific genera are really less rare than bispecific genera in the natural world. In this regard, it would be interesting to extend our approach to further taxa in order to evaluate at least the generality of the observed patterns.

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