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Front cover: Agamura persica from Central Iran.
See article on opposite page. Photographed by Saeed Hosseinian.

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FULL PAPER

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Taxonomic revision of the spider geckos of the genus *Agamura* senso lato Blanford, 1874 (Sauria: Gekkonidae) in the Iranian Plateau

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In this study, we present an integrative systematic revision of the spider gecko, *Agamura* senso lato, in Iran. We sampled 56 geckos of this complex from its distributional range in Iran and western Pakistan and sequenced these for two mitochondrial markers, cytochrome b and 12S ribosomal RNA, and one nuclear marker, melano-cortin 1 receptor. We combined our molecular data with species distribution modelling and morphological examinations to clarify *Agamura persica* systematics and biogeography. Due to a lack of published data, we used only our data to investigate the spatial and temporal origin of spider geckos within a complete geographic and phylogenetic context. The phylogenetic analyses confirm the monophyly of *Agamura*. Among spider geckos, *Rhinogekko* diverged around the early-mid Miocene (17 Mya) from the Lut Block, and then *Cyrtopodion* diverged from the *Agamura* clade about 15 Mya in the mid-Miocene as a result of the uplifting of the Zagros Mountains. Subsequent radiation across the Iranian Plateau took place during the mid-Pliocene. *Agamura kermanensis* exhibits deep divergence from two other species of *Agamura* (*A. persica* and *A. cruralis*), whereas no geographical substructure was observed on the Iranian Plateau for *A. persica* and *A. cruralis*. Our findings reveal that diversification is consistent with a biogeographical model explained by different dispersal waves and vicariant events on the Iranian Plateau during the last 18 Mya. The divergence times between clades are compatible with orogenic events in southern Iran that resulted from the collision with Arabia. According to the genetic differentiation of both mtDNA genes (12S and cytochrome b), the systematic status of *A. cruralis* is confirmed, the new clade was distinguished from the genus *Agamura*, monophyly of Rhinogekko was confirmed and the allocation of *Cyrtopodion gastrophole* to the *Cyrtopodion* clade was confirmed.

Key words: Agamura, *agamuroides* group, divergence time, Iranian Plateau, *Rhinogekko*, spider geckos, vicariance

INTRODUCTION

The genus *Agamura* has a wide distribution range in the Iranian Plateau and occupies different habitat types (Anderson, 1999). However, due to the different habitat types, a high degree of geographic variation and genetic divergence exist. The Iranian Plateau is located in south-western Asia and is comprised of various ecoregions (e.g., both mountains and dry deserts), surrounded by several mountain ranges such as the Zagros in the west, Alborz in the north, Kopet Dagh in the north-east and Hindokush and Soleiman in the east and the south-east (Macey et al., 1998). These mountain ranges create a rain shadow, preventing high precipitation on the plateau, creating hot dry deserts in this central region (Ahmadzadeh et al., 2012). The Iranian Plateau has a high number of endemic lizards, especially within the arid clades of geckos (Šmid et al., 2014). This high endemicity may have been facilitated by the various habitat types in the region such as mesic, alpine and xeric, which are restricted by mountain chains (Farahmand & Nazari, 2015). The formation of the plateau started ca.40 million years ago (Mya) as a result of the merger/collision among Arabian, Eurasian and Indian plates (Mouthereau, 2011). The formation and the geological history of the Iranian Plateau has had a fundamental influence in shaping the distribution patterns of the reptilian biota and the speciation processes by both dispersal and vicariance events (Macey et al., 2000).

Iran is an interesting region to study biogeographical patterns because it is located near the junction of the Eurasian, Arabian and Indian continental plates (Macey et al., 1998). Collisions between these plates created different mountain chains that affected the herpetofaunal biodiversity in the region (Van Hinsbergen et al., 2012; Ahmadzadeh et al., 2012). In south-western Asia, several phylogenetic studies have been conducted to gain insights on the biogeographical patterns and speciation events among geckos (Bauer et al., 2013; Šmíd

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et al., 2013; de Pous et al., 2015). One such example is the study of Šmíd et al. (2013) on the genus *Hemidactylus* and the following descriptions of several new species. Bauer et al. (2013) presented the phylogeny of naked-toed geckos suggesting that most speciation events in the western Palearctic took placed during the Miocene by vicariance due to different uplift events in the Iranian Plateau. There are more than 15 genera of the naked-toed gecko and one of them is *Cyrtopodion* that was later divided into several genera (Szczerbak & Golubev, 1996; Bauer et al., 2013).

Within Gekkonidae, the spider gecko, *Agamura*, is a monotypic genus, phylogenetically close to *Cyrtopodion*, *Rhinogekko*, *Bunopus* and *Crossobamon* (Bauer et al., 2013), which ranges across Iran, Pakistan and Afghanistan. In Iran, it is known throughout most of the Iranian Plateau, east of the Zagros Mountains and south of the Alborz and Kopet Dagh mountain ranges, but is absent from the Kavir and Lut central deserts. *Agamura* occupies stony and rocky habitats, including hillsides and barren plains, with sparse shrubby vegetation (Anderson, 1999, Sindaco & Jeremčenko, 2008). To date, neither morphological investigations nor molecular studies on this monotypic genus have been carried out across its distribution range. *Agamura* has previously been used as an outgroup in other studies (Červenka et al., 2010; Bauer et al., 2013; de Pous et al., 2015); however, these studies did not investigate the phylogenetic relationships within the genus, or its population structure and diversity. *Agamura* previously included four taxa (i.e., *A. persica*, *A. gastropholis*, *A. misonnei* and *A. femoralis*; (Szczerbak & Golubev, 1996), all of which were endemic to the Iranian Plateau. Later studies examined the genus and excluded the three latter species from *Agamura*: *A. misonnei* and *A. femoralis* were allocated to the genus *Rhinogekko*, and *A. gastropholis* to the genus *Cyrtopodion* (Anderson, 1999; Krysko et al., 2007). Thus, currently the genus *Agamura* includes a single species, *A. persica*, which exhibits differences among populations in colour pattern and morphological characteristics (Szczerbak & Golubev, 1996; Anderson, 1999). The subspecies *A. p. persica* and *A. p. cruralis* represent western and eastern populations, respectively (Anderson, 1999; Szczerbak & Golubev, 1996). Differences between these two subspecies are apparent in the shape of the rostral scale, the number of the dorsal scales, and the sharper shape of the tubercles in the eastern populations (Anderson, 1999). *Rhinogekko misonnei* is another species that is endemic to Iran and only distributed around the Lut Desert and its phylogenetic status among other gekkonid species requires verification, as there has been no study on the species (Šmid et al., 2014).

In this study, we conduct an integrative revision of the monotypic genus *Agamura* senso lato in Iran. We provide the first comprehensive morphological and molecular analyses throughout its wide range in the Iranian Plateau with the aim of elucidating the evolutionary and biogeographic history of this enigmatic genus. For this purpose, we sampled different taxa throughout the Iranian Plateau including *A. persica* (different populations of the species from whole distribution range in Iranian Plateau) and its close relatives, *Rhinogekko misonnei* (providing the first genetic data for this genus), *C. gastrophole*, *C. persepolense*, and *C. agamuroides*. We used nuclear and mitochondrial markers to revise the systematics of *Agamura*. We used species distribution modelling and phylogeographic analyses to explore the phylogeographic structure of *Agamura persica* on the Iranian Plateau, and climate suitability that can affect the divergence between species in Iranian Plateau.

MATERIALS AND METHODS

We used two criteria to assess the species limits; first, the identification of lineages based on mitochondrial and nuclear markers and, second, the presence of diagnostic morphological characters.

Sampling, DNA extraction and amplification

A total of 56 individuals were collected from the Iranian Plateau during field trips from 2014 to 2016. We used two recently described species of the genus *Cyrtopodion* that is clearly situated in the main clade of *Cyrtopodion* (Nazarov et al., 2009; Nazarov et al., 2012). The dataset contains seven recognised species: *Agamura persica*, *A. cruralis*, *A. kermanensis*, *Rhinogekko misonnei*, *Cyrtopodion gastrophole*, *C. persepolense*, *C. sistanense* and *C. agamuroides* (Hosseinian Yousefkhani et al., 2018). Localities and coordinates for each sample are presented in the Supplementary Materials, Table S1. Specimens were deposited in the Sabzevar University Herpetological Collection (SUHC). Sequences of *Hemidactylus turcicus* were retrieved from GenBank and used as outgroup (Table S1).

DNA was extracted from tissue samples using the salt method (Kabir et al., 2006). The quality of extracted DNA was measured using 1% agarose gels stained by 0.5 µl GreenViewer 6X and visualised under ultraviolet light. We amplified two mitochondrial genes 12S rRNA (12S) using the primers 12SL (5′-AAACTGGGATTAGATACCCCACTAT-3′) and 12SH (5′-GAGGGTGACGGGCGGTGTGT-3′) (Kocher et al., 1989), and Cytochrome b (Cytb) with the primers L14724 (5′-GACCTGCGGTCCGAAAAACCA-3′) and H16064 (5′-CTTTGGTTTACAAGAACAATGCTTTA-3′) (Burbrink et al., 2000) and L14919 (5′-AACCACCGTTGTTATTCAACT-3′) and Ei700r (5′-GGGGTGAAA GGGGATTTTRTC-3′) (Rastegar-Pouyani et al., 2010) and one nuclear gene Melano-cortin 1 receptor (MC1R) with the primers MC1R-F (5′-AGGCNGCCATYGTCAAGAACCGGAACC-3′) and MC1R-R (5′-CTCCGRAAGGCRTAAATGATGGGGTCCAC-3′) (Eskandani et al., 2010).

Phylogenetic analyses and haplotype network construction

We used Clustal W as implemented in Bioedit alignment editor v. 7.0 (Hall, 1999) to align sequences with default parameters. Protein coding sequences (Cytb and MC1R) were translated into amino acids with Mega v.6.0 (Tamura et al., 2013) and no stop codons were observed. Uncorrected genetic distances (*p*-distance) were calculated using Mega v.6.0 and ExcaliBAR (Aliabadian et al., 2014) for 12S and Cytb gene fragments independently.

The best-fit models of nucleotide evolution were assessed using ModelTest 3.7 (Posada & Crandall, 1998) and the best fit models of evolution according to the Akaike Information Criterion (AIC) were: 12S-GTR+I+G; cyt b-TIM+I+G; mc1r-TrN+I+G.

The phylogenetic analyses were performed using Maximum Likelihood (ML) and Bayesian Inference (BI) methods and for this purpose, all gene alignments were combined into a single alignment totalling 1670 bp (389 bp of 12S; 625 bp of cytb; 656 bp of mc1r). We also considered *Hemidactylus* as outgroup in the analyses (Pyron et al., 2013). We used 50 cytb sequences from Genbank to clarify the phylogenetic structure of the genera.

Maximum Likelihood analyses were conducted with RaxML 7.4.2 (Stamatakis, 2006) as implemented in RaxmlGUI 1.3 (Silvestro & Michalak, 2012) with a GTR+I+G model. The analyses were run in heuristic search and the nodal support was obtained by bootstrap analysis with 1000 replicates (Felsenstein, 1985). MrBayes 3.2.1 (Ronquist et al., 2012) was used for the BI analyses and the best-fit models were specified above for concatenated dataset. The analyses were run for 10^7 generations with a sample frequency of every 1000 generations. Some parameters, like the number of runs and the number of chains, were kept as default and a sufficient number of generations were evaluated by the log likelihood value (lnL) and split frequency lower than 0.01. We conservatively discarded the first 25% of trees as burn-in (Condamine et al., 2015). To reconstruct the ancestral area Bayesian Binary MCMC (BBM; Ali et al., 2012), we employed Reconstruct Ancestral State in Phylogenies (RASP) (Yu et al., 2012) using all spider gecko sequences. MrBayes was used to prepare the input tree file. Six areas were designated based on zoogeographical regions for reconstruction as: Central Plateau, East Iran, South Iran, South-west Pakistan, South-west Iran, and Zagros Mountains. We chose these areas to identify the direction of dispersal within the Iranian Plateau spider geckos.

Relationships among lineages and species were assessed with allele network of the *mc1r* nuclear marker. The nuclear alignments were imported into TCS 1.21 (Clement et al., 2000) using a parsimony method to obtain the haplotype network.

Estimation of divergence time

Because of the absence of internal calibration points for spider geckos and their relatives, we applied direct estimations obtained from other groups of lizards. The substitution rate of the same mitochondrial genes (12S and Cyt*b*) that were calculated for three lizard families from the Canary Islands: *Tarentola* (Phyllodactylidae) (Carranza et al., 2000), *Gallotia* (Lacertidae) (Cox et al., 2010) and *Chalcides* (Scincidae) (Brown & Pestano, 1998) were used to estimate the divergence time. These substitution rates have already been used for divergence time estimates for different taxa including *Hemidactylus*, *Bunopus*, *Asaccus* and etc. (Carranza & Arnold, 2012; Sindaco et al., 2012; Šmíd et al., 2013). BEAST 1.8 (Heled & Drummond, 2010) was used to estimate divergence time among the spider geckos and the models and priors were applied as follows (otherwise by default): evolutionary models were set for each gene separately; random starting tree; clock models were set as lognormal relaxed clock with unlinked status; tree priors were set as coalescent and constant size. Finally, divergence times were assessed by the mean rate of molecular evolution for the ucld. priors for 12S (mean: 0.00755, stdev: 0.00247) and Cyt*b* (mean: 0.0228, stdev: 0.00806) gene fragments (Carranza & Arnold, 2012) independently.

Species distribution modelling

A total of 189 presence records from the examined species and clades were obtained from the literature, museum records and our direct filed surveys (Supplementary Materials, Table S2). Climatic layers were downloaded from the worldclim website (www.worldclim.org) in 30 arc second (Hijmans et al., 2005) and extracted using ArcGIS 10.3 (ESRI) only for Iran (Table S3).

Correlations between climatic layers were calculated using ENMTools 1.3 (Warren et al., 2010) and the correlative layers (>0.7) were removed from the analyses. Maxent 3.3.3e (Phillips et al., 2006) was employed to predict the potential distribution area using only presence records. The final set of variables with lower correlation than 0.7 used for all species distribution modelling consisted of 12 bioclimatic variables (Table 2). All models were run for 10 replicates under a crossvalidate model and 10000 background points, with a convergence threshold of 0.00001, and maximum number of iterations as 500. The model accuracy was evaluated using area under the curve (AUC) criterion that ranges between 0 and 1 (Fielding & Bell, 1997). Model visualisations were done by ArcGIS 10.3 (ESRI) and we exported relevant maps as the species distribution prediction.

Morphological analyses

Populations of *Agamura* were examined using 27 morphological characters (12 metric and 15 meristic characters; Supplementary Materials, Table S4) on the samples that were sequenced for phylogenetic analyses. Metric characters were measured using digital callipers (rounding to nearest 0.1 mm) and meristic characters were examined using an Olympus loupe. Operational taxonomic units (OTUs) were classified according to the cluster analysis and zoogeographic regions on the Iranian plateau (Anderson, 1999). Three OTUs were defined as western, eastern and southern clades. Analysis of variance (ANOVA) performed on the OTUs and the significant variables ($P< 0.05$) were excluded to run principal component analysis (PCA) and canonical variate analysis (CVA) and to visualise the morphological variation by these analyses.

RESULTS

Taxon sampling and sequence data

Our dataset included mitochondrial fragments of 12S (389bp; *V* = 182; *Pi* = 136) and Cyt*b* (625 bp; *V* = 297; *Pi* = 239) and a nuclear gene fragment MC1R (656 bp; V = 94; Pi = 63) totalling 1670 bp. Thirty-six unique

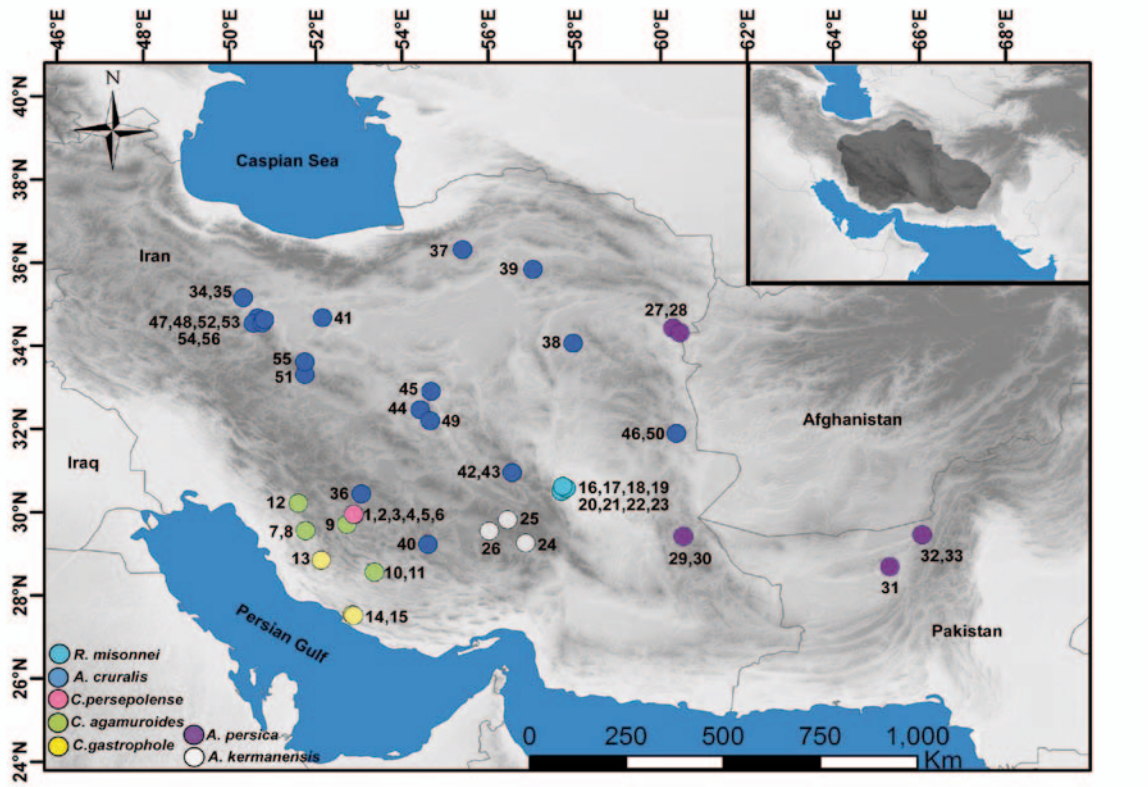

Figure 1. Sampling localities of spider geckos including all species. Numbers correspond to specimens listed in Table S1 and colours to specimens in Fig. 2 and 3. The black region in the right-top map indicates the sampling region in the Iranian Plateau.

Table 1. Uncorrected genetic variation (*p*-distance) among different species of angular-toed geckos in the Iranian Plateau. Above the diagonal represents the variation in 12S and below the diagonal refers to Cyt*b* diversity.

	(1)	(2)	(3)	(4)	(5)	(6)	(7)
(1) *A. cruralis*		3.6	3.7	14.7	16.7	14.0	19.5
(2) *A. persica*	8.3		4.2	14.4	15.8	13.2	19.1
(3) *A. kermanensis*	13.2	11.7		14.0	15.7	14.2	18.5
(4) *C. agamuroides*	20.1	18.8	19.6		4.4	9.3	20.1
(5) *C. persepolense*	19.4	18.1	19.3	4.0		9.6	21.7
(6) *C. gastrophole*	17.9	17.4	19.3	14.1	13.7		21.1
(7) *R. misonnei*	19.1	18.5	21.5	18.5	18.5	18.7	

Table 2. Percentage contribution of climate variables under the Maxent modelling conducted in the present study. Definitions of variables are presented in Supplementary Materials, Table S3. Bold values refer to the most contributed variable in each species distribution modelling.

Variable	*A. cruralis*	*A. persica*	*A. kermanensis*	*C. agamuroides*	*C. gastrophole*	*C. persepolense*	*R. misonnei*
BIO2	5.9				8.7	15.7	
BIO4	8.5	**39.4**	**44.5**				
BIO6		34.3		**21.3**	**51.2**	16.4	**46**
BIO9	19			5.5			
BIO11	**23.6**	28	40.6	9.8		9.6	
BIO12	20.3		5.2			**19.9**	42.9
BIO13					12.2	15.7	
BIO14	6.6					5.4	
BIO15				13.8	6.1		
BIO16				16.6			
BIO17				15.9	6.4		
Slope			7.6				2.8

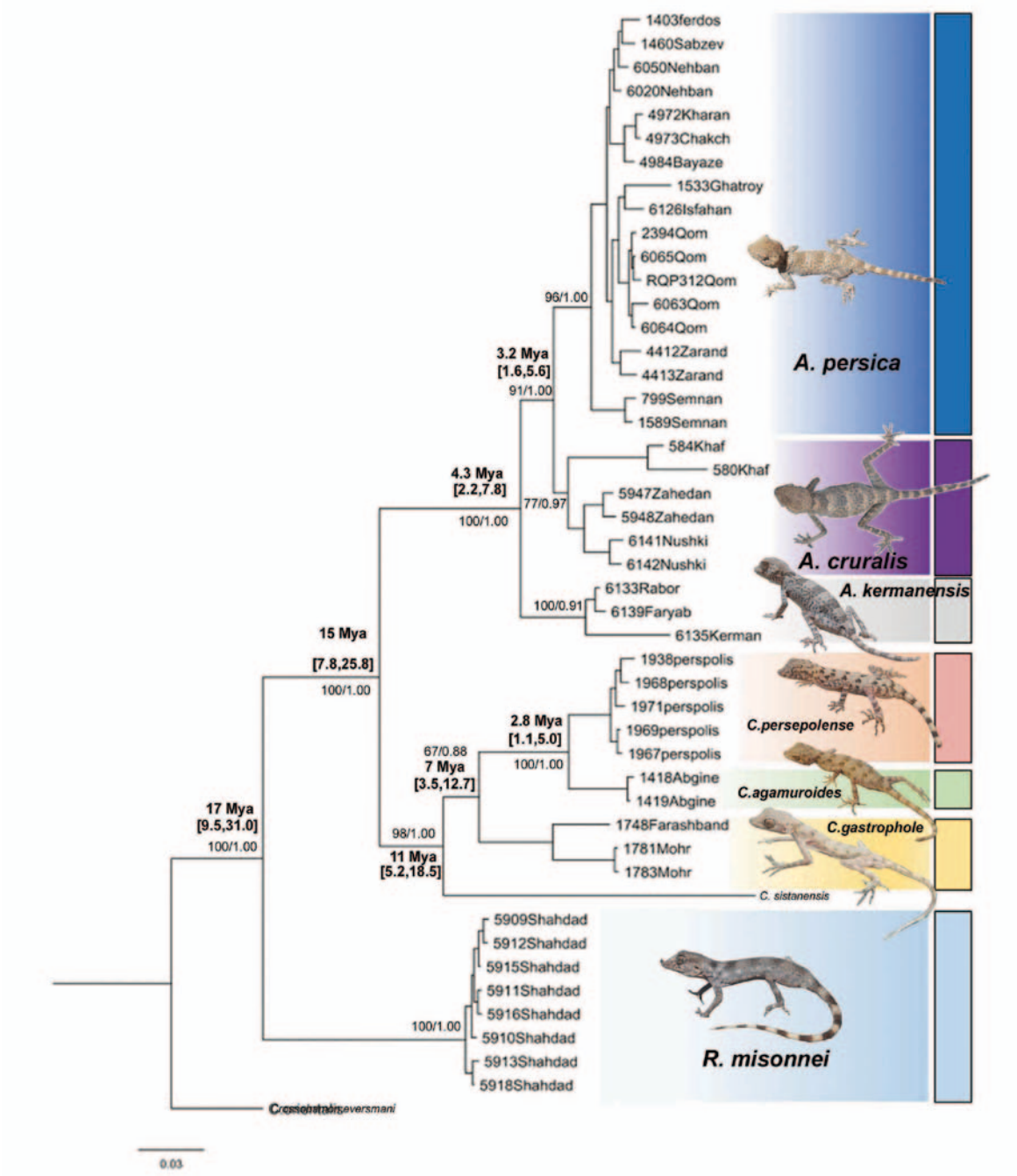

Figure 2. Bayesian Inference (BI) gene tree of spider geckos inferred from 1670 bp of mitochondrial (12S and Cyt*b*) and nuclear (MC1R) gene fragments. ML bootstrap support and posterior probability of Bayesian analyses are presented next to the nodes, respectively. Age estimated based on the substitution rates are denoted near the relevant nodes and include the mean and, between brackets, the HPD 95% confidence interval.

Table 3. Mean ± SD and range for significant characters of seven metric, meristic and ratio characters measured in *Agamura* population from Iranian Plateau. The right column refers to the significant values among populations.

Character	*A. cruralis* (n = 18)		*A. persica* (n = 5)		*A. kermanensis* (n = 7)		*P* value
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range	
HH	12.65±1.44	9.31-17.97	8.86±0.39	7.20-12.89	9.35±0.48	7.25-11.33	0.002
SL	5.03±0.19	4.49-5.58	4.87±0.15	3.81-6.14	4.17±0.22	3.34-5.11	0.030
IO	10.11±0.41	8.81-11.40	8.79±0.29	6.60-11.22	10.85±0.68	8.59-13.63	0.005
HLL	46.96±0.96	43.71-49.44	40.87±1.09	33.72-49.53	41.66±3.36	30.03-56.67	0.042
FLL/SVL	0.52±0.02	0.46-0.58	0.51±0.01	0.44-0.59	0.54±0.01	0.52-0.61	0.049
NSA	28±0.94	25-30	37.11±1.01	31-47	27.14±1.77	21.-36	0.000
NPV	53.20±2.10	46-59	54±0.77	49-59	48.85±1.07	46-54	0.014

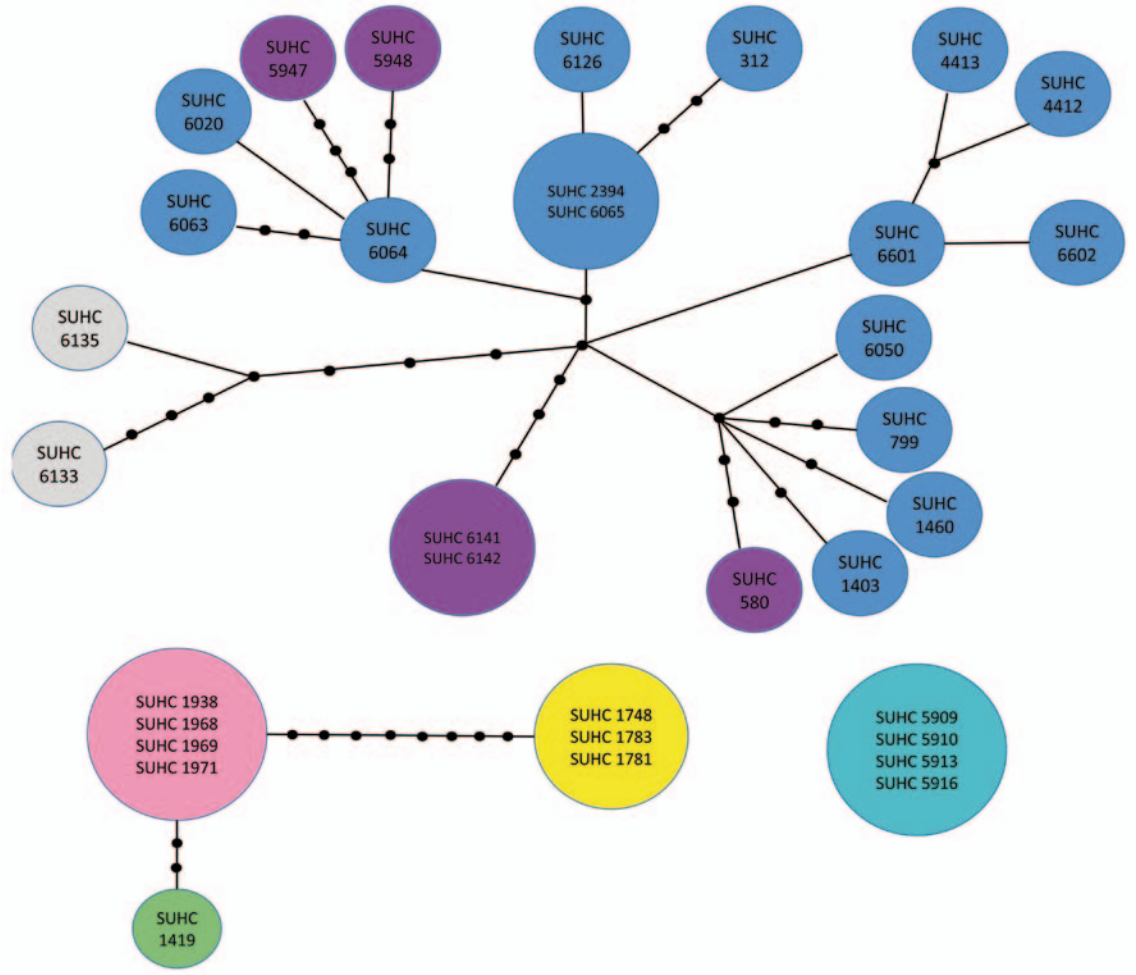

Figure 3. Unrooted haplotype network of MC1R as nuclear marker. Circle size is proportional to the number of samples pre-haplotype, with colours corresponding to species in Fig. 2. Codes correspond to the specimens presented in Table S1.

Table 4. Factor loadings of the first three principal components (PCs). The characters are defined in Table S4.

Characters	PC1	PC2	PC3
HH	0.891	-0.079	0.172
SL	0.936	-0.005	0.034
IO	0.857	0.298	0.110
HLL	0.892	0.312	0.156
NSA	-0.342	0.772	0.430
FLLSVL	-0.636	0.620	-0.056
NPV	0.410	0.487	-0.748
Eigenvalues	3.892	1.410	0.815
Accumulated percent of trace	55.606	75.747	87.389

Table 5. Factor loadings of the first three canonical variates (CVs). Characters were defined in Table S4.

Characters	CV1	CV2
FLLSVL	-0.269	0.314
NSA	0.701	-0.323
NPV	0.110	0.209
HH	-0.320	0.980
SL	0.922	-0.048
IO	-1.149	-1.103
HLL	0.492	0.706
Eigenvalue	4.744	1.028
Accumulated percent of trace	82.2	100.0

haplotypes were distinguished within the concatenated mitochondrial dataset and the nuclear MC1R marker included 32 unique haplotypes.

Phylogenetic analyses and network construction

The Bayesian and Maximum Likelihood trees showed identical topologies with high Bayesian posterior probabilities and bootstrap values (Figs. 2, S1). According to the phylogenetic analyses, *Agamura* is monophyletic (Fig. 2). *Agamura* is divided into three clades (Fig. 2) consisting of *A. cruralis* (the previously known species from eastern Iran), *A. persica* as a western clade (as mentioned by Szczerbak & Golubev, 1996) and *A. kermanensis*. Genetic distances (*p*-distance) of the two mitochondrial markers between clades reveals high diversity among the three lineages (i.e., 12S: 3.6-4.2%; Cyt*b*: 8.3-13.2%). Variation within each clade is very low (12S: 0-0.8%; Cyt*b*: 0.8-1.4%) especially within *A. persica*. The *Cyrtopodion* clade consists of four species that are distinctly separated from *Agamura* and each species is delimited from others with high bootstrap values and posterior probabilities support, but based on the Cyt*b* tree (Supplementary Materials, Fig. S3) the genus was paraphyletic and *Cyrtopodion gastrophole* situated far

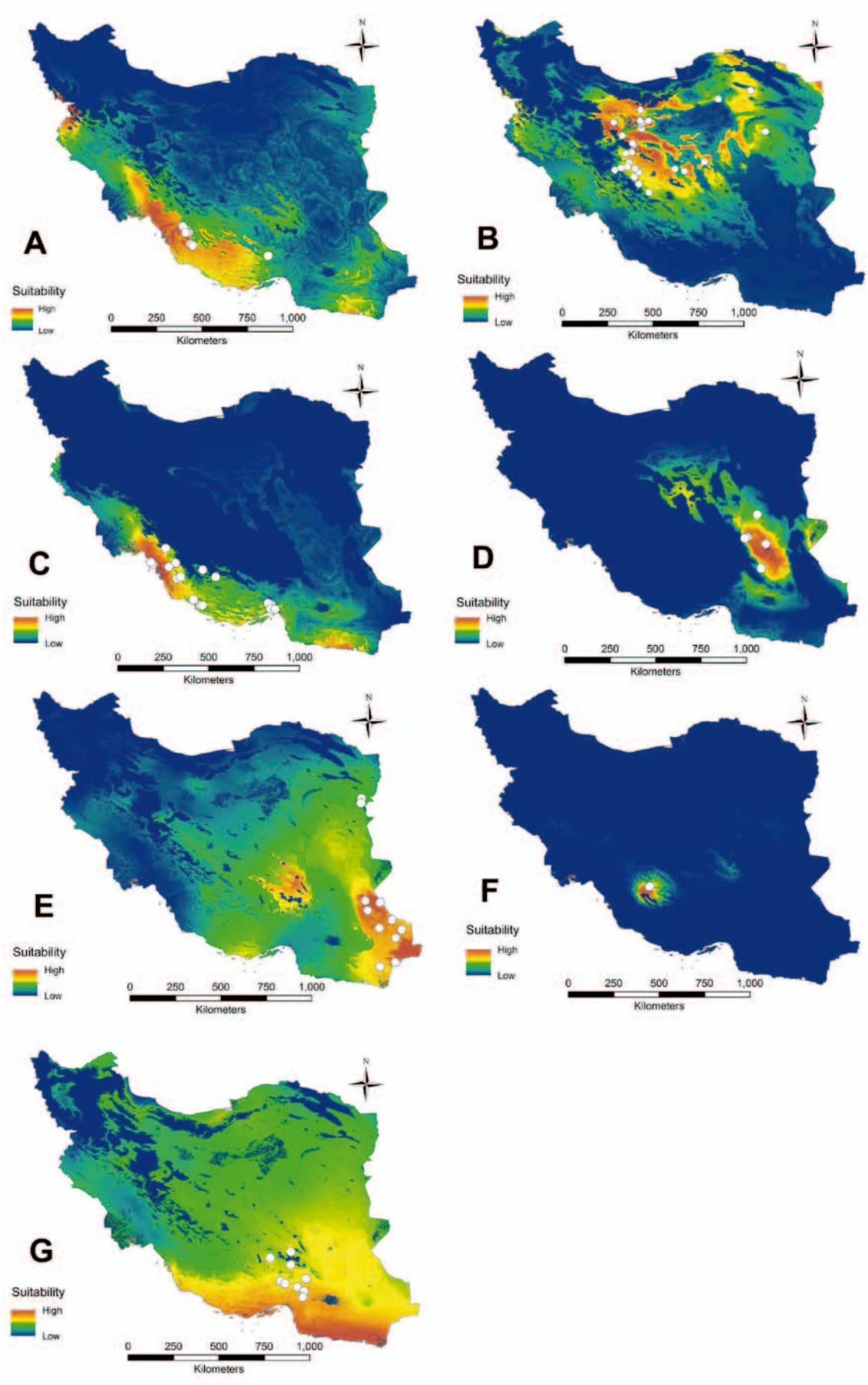

Figure 4. Potential species distribution models of spider geckos on the Iranian Plateau. A) *C. agamuroides*; B) *A. persica*; C) *C. gastrophole*; D) *R. misonnei*; E) *A. cruralis*; F) *C. persepolense*; G) *A. kermanensis*. The colours refer to the level of suitability as presented in the figure legends.

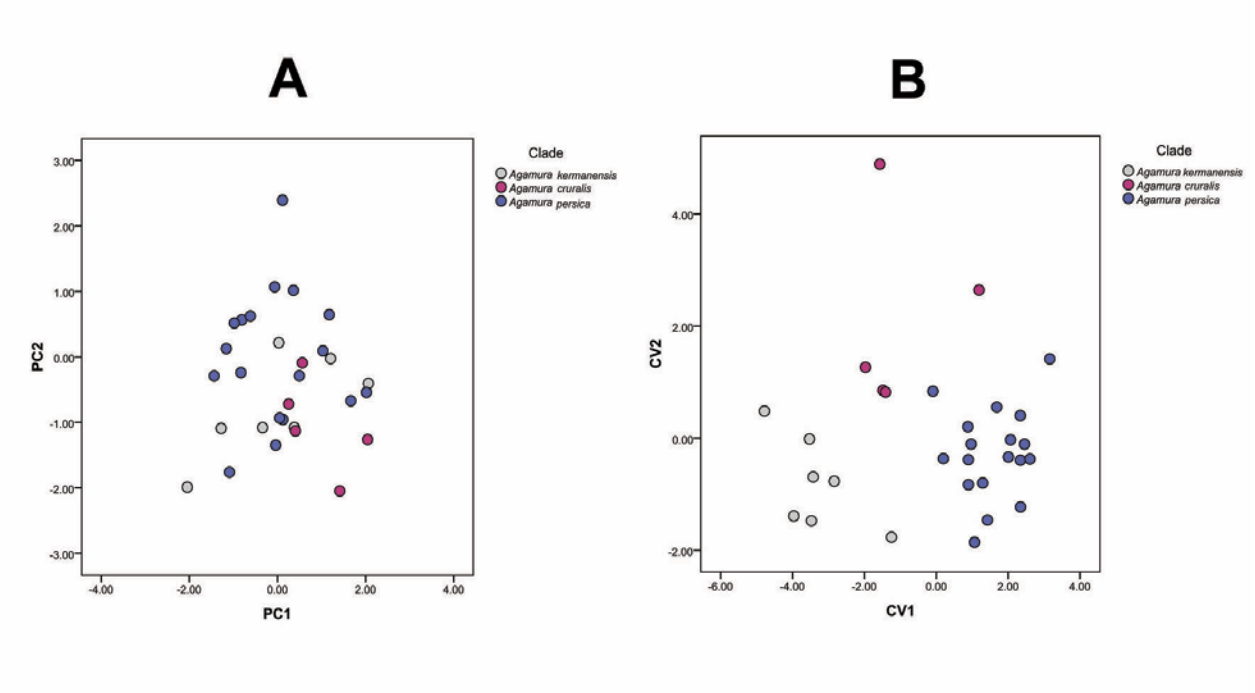

Figure 5. Ordination of principal component 1 (PC1) against PC2 (A) and canonical variate 1 (CV1) against CV2 (B) for all significant characters among *Agamura* clades in the Iranian Plateau

from the genus *Agamura*. The Genus *Rhinogekko* is considered as sister genus to *Bunopus* and confirm the previous assumptions (Szczerbak & Golubev, 1996) (Fig. S3) and separated from the *Agamura* clade with high posterior probability and bootstrap support values.

The haplotype network constructed for nuclear marker MC1R showed the similar pattern of a concatenated phylogenetic tree. According to the network (Fig. 3), separation of *Rhinogekko* and *Cyrtopodion* from the genus *Agamura* is confirmed, but the complexity in the genus *Agamura* remains. *Agamura kermanensis* is clearly differentiated from other clades with more mutations, but other two species have more similarity to each other.

Divergence time estimates

The analyses were run based on two mitochondrial genes (12S and Cyt*b*) and finally, both trees linked together (Figs. 2, S2) and the results indicated that *Rhinogekko* split from angular-toed geckos around 17 million years ago (Mya; 95% HPD: 9.5-31.0 Mya). Divergence between *Cyrtopodion* and *Agamura* started through early-mid-Miocene ca. 15 Mya (95% HPD: 7.8-25.8 Mya). Speciation within the *Cyrtopodion* clade appears to have occurred 11 Mya (95% HPD: 5.2-18.5 Mya). The split between *C. gastrophole* and *C. agamuroides* group took place around 7 Mya (95% HPD: 3.5-12.7 Mya) and the separation between *C. agamuroides* and *C. persepolense* occurred 2.8 Mya (95% HPD: 1.1-5.0 Mya). The cladogenesis of spider geckos started approximately in early Pliocene ca. 4.3 Mya (95% HPD: 2.2-7.8 Mya) and the divergence of *A. persica* and *A. cruralis* have occurred 3.2 Mya (95% HPD: 1.6-5.6 Mya).

Spider geckos in the Iranian Plateau are distributed in all areas within the plateau across the mountains, plains and deserts. The ancestor of spider geckos was retrieved from the central part of the Iranian Plateau as the distribution of most of them covered the central part (Supplementary Materials, Fig. S4). Among spider geckos, *Rhinogekko misonnei*, *Agamura persica*, *A. cruralis* and *A. kermanensis* are distributed in the Central Plateau. *Cyrtopodion* species that were used in this study are from the Zagros area. It seems the spider gecko clade first appeared from the central part of the Plateau, because few of them are distributed in other parts.

Species distribution modelling

The results of Maxent modelling shows good AUCs for all models: *A. cruralis* AUC = 0.896±0.117; *A. persica* AUC = 0.879±0.028; *A. kermanensis*. AUC = 0.865±0.071; *Cyrtopodion persepolense* AUC = 0.997±0.001; *C. agamuroides* AUC = 0.864±0.188; *C. gastrophole* AUC = 0.962±0.013; *Rhinogekko misonnei* AUC = 0.969±0.029. The predicted map indicated that suitable areas confirmed the current distribution of the species (Figs. 1, 4). The most important climate variables are presented in Table 2.

Morphological examination

Twenty-seven morphological characters were examined among three distinct clades of the genus *Agamura*. Based on the analysis of variance (ANOVA) for metric characters, five characters were distinguished as significant characters ($P < 0.05$) (Head Height (HH), Snout Length (SL), Interorbital distance (IO), Hind Limb Length (HLL) and Fore Limb Length/Snout-Vent length (FLL/SVL)). The meristic characters were analysed by Kruskal-Wallis H test and two characters were distinguished as significant (Number of scales across widest part of

abdomen (NSA), Number of scales between postmental scales and vent (NPV) (Table 3). PCA and CVA were calculated using significant characters obtained in the previous stage. In the PCA, the first three components explained 87.38% of total variance and in the CVA, the first two components explained 100% of total variance of characters (Fig. 5; Table 4, 5).

DISCUSSION

Phylogeny, diversity and endemism

The molecular results confirmed the previously known and described species belong to the genus *Cyrtopodion* (Nazarov et al., 2009; Nazarov et al., 2012) and revealed three distinct lineages of the genus *Agamura* and confirmed their species status (Fig. 2). *Agamura* was defined as a monophyletic genus with four distinct species (Szczerbak & Golubev, 1996), but was recently revised morphologically and three of them were excluded from the genus (Anderson, 1999). Two subspecies were considered for *Agamura persica*: *A. p. cruralis* for the eastern population and *A. p. persica* for the western population, with some differences in morphological characters including the number of dorsal scales and shape of the tubercles (Anderson, 1999). *Rhinogekko misonnei* is a representative of the genus *Rhinogekko* and was distinctly separated from *Agamura* (high bootstrap support and posterior probability values). It has previously been considered in the genus *Agamura* by Szczerbak & Golubev (1996) and its distinction presented recently (Anderson, 1999; Krysko et al., 2007; Sindaco & Jeremčenko, 2008). A high level of genetic differentiation between *Rhinogekko* and *Agamura* populations in our study strongly supports the exclusion of *R. misonnei* within the genus *Agamura*. *Agamura gastropholis* was one of the members of *Agamura* according to Szczerbak & Golubev (1996), but in the present study, this species was clustered within the genus *Cyrtopodion*. These geckos are common in the Iranian Plateau and have been recorded several times from arid regions of central and southern parts of Iran (Anderson, 1999; Nazarov et al., 2009; Moradi et al., 2011), but there were, until this study, no documented morphological or molecular evidence, and indeed no taxonomic study, on these geckos in the area.

The Zagros Mountains are presented as an important region of endemism in west and southern Iran (Gholamifard, 2011; Hosseinzadeh et al., 2014). Recently, several species of lizards and snakes have been described, uncovering a deep history of radiations in the region (Nazarov et al., 2009; Ahmadzadeh et al., 2012; Rajabizadeh et al., 2012). All of the described species are endemic to the Iranian Plateau and their cladogenesis began in the Miocene. Interspecific variation of 12S and Cyt*b* genes among the studied species is about 14-15% and 19-20% respectively, which is very high (Table 1). Species distribution modelling revealed that potential areas of distribution of these species did not have any overlap (Fig. 4), but different climate variables affect a species presence in a precise region (Hosseinian Yousefkhani et al., 2016). BIO6 (minimum temperature in coldest month) is the important variable for the presence of three species (*Cyrtopodion agamuroides*, *C. gastrophole* and *R. misonnei*) (Table 2). Divergence between *Cyrtopodion* and *Agamura* took place 15 Mya by uplifting of the Zagros Mountains in the mid-Miocene and then cladogenesis within these clades occurred during the Pliocene (Fig. 2) with different climate conditions.

Morphological variations between Agamura clades include the size of the body and especially in the length of limbs, which is longer in the eastern population than the western one. Ground structures in different parts of the range were observed directly during fieldwork and there are large rocks in the eastern and southern part of Iran, whereas the size of rocks decreases in central Iran. Habitat features like larger rocks are related to the structure of adjacent mountains produced by geological events and pressures over millions of years (Anders et al., 2010).

Biogeography of spider geckos

South-eastern and southern Iran are the probable regions for the spider gecko's diversification. The collision of the Arabian and Eurasian Plates about 35 - 20 Mya (Dercourt et al., 1986; Mouthereau, 2011; McQuarrie & Van Hinsbergen, 2013) and the Zagros Mountain orogeny and consequent climate change are likely to have played an important role in diversification within spider geckos. According to our results, long branches with high bootstrap supports indicated a deep divergence of Rhinogekko from other spider geckos (17 Mya) and the Cyrtopodion from *Agamura* (15 Mya). But divergence time less than 4 Mya among *A. persica* and *A. cruralis* clades indicated to the historical taxon that originated in south-east Iran. *Rhinogekko* is restricted to the Lut Desert boundary and limited dispersal apparently caused by climate factors such as hot and dry conditions in the Lut area (Pourkhorsandi & Mirnejad, 2000). Closure of the new Tethys by collision with Arabia created the Zagros Basin (Khadivi, 2010), but contact with the Lut Block (Lut Block is a rigid plate on the Iranian Plateau) created a hot and dry region. The *Cyrtopodion* clade diversified in the late Miocene as a result of uplifting of the Zagros Mountains and aridification, in addition to trapping and isolating populations of this clade in the south Zagros valleys, which directly affected the variation among them. All species examined in this study are sensitive to temperature (Table 2) in different months of year except *C. persepolense*, which is dependent on annual precipitation. This isolation and diversification is directly reflected by the Zagros uplifting which affects precipitation in south and central Iran (Ramstein et al. 1997).

The main splits among spider geckos took place by vicariance in the mid-Miocene and other divergences within clades mainly represent different dispersal waves, aridification, climate change and restriction among valleys. Climate change and aridification directly affect *R. misonnei*; restriction among valleys is a common method for cladogenesis in the *Cyrtopodion* clade, as the newly described species belonging to this genus

are isolated among valleys and restricted to their small range. Cladogenesis in the genus *Agamura* may occurred by both vicariance events and dispersal waves on the Iranian Plateau from 15 Mya (Fig. 2). The southern clade (taxa. kermanensis) was isolated by the uplifting of the Lalezar Mountain in southern Kerman and the two others (*A. persica* and *A. cruralis*) diverged from each other about 3 Mya by occupying different niches.

Taxonomic implications

The monophyly of *Cyrtopodion* and *Agamura* is confirmed and *Rhinogekko* is placed at the root of the tree as the first diverged spider geckos in south-east Iran. However, the Agamura divided into three clades that are phylogenetically distinct from others, and each clade might represent a distinct species (Fig. 2). This is confirmed by our analyses of the three major clades of *Agamura* represented on the Iranian Plateau; two of them refer to the species previously known as *A. cruralis* (eastern clade, including the type locality of *A. cruralis* from southern Baluchestan, Bahukalat) and *A. persica* (western clade) (Hora, 1926; Szczerbak & Golubev, 1996). The third clade in *Agamura* (*A. kermanensis*) diverged widely from the two others (13.2% and 11.7%; genetic differentiation from *A. cruralis* and *A. persica* in Cyt*b*, respectively). *Agamura kermanensis* can be considered as a new taxon based on morphological and molecular evidence and will be described soon by authors. The morphological variation between the newly explored clade and two other *Agamura* clades is clearly explained in Figure 5. Species niche modelling confirmed this separation by estimating separate suitable areas (Fig. 4). The haplotype network based on the nuclear marker clearly showed separation of *Agamura kermanensis* (Fig. 3).

CONCLUSIONS

The present study provides a biogeographical view of the spider geckos in the Iranian Plateau. The phylogenetic history and divergence times of spider geckos support the geological events during the Miocene period. The monophyly of *Agamura* and *Cyrtopodion* indicate an important role of Zagros orogeny in the isolation of these lineages during the mid-Miocene. Following dispersal, *Agamura* rapidly diversified in the Iranian Plateau. The southern population in Kerman province showed a distinct clade from other *Agamura* populations. The southern Zagros slopes were involved in different collision events with Arabia and then new valleys appeared. Radiation within *Cyrtopodion* clade refers to these valley creations taking place during the late Miocene. *Rhinogekko* is a distinct clade that first diverged from all other spider geckos by closing the new Tethys about 17 Mya in the Lut region. Finally, our phylogenetic analyses suggest that morphological diversity among these geckos arose by historical process such as Zagros orogeny, which can explain the basal divergence within the group. The southern clade will be described as a new taxon in a separate article by authors in future.

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Monitoring amphibian species with complex chromatophore patterns: a non-invasive approach with an evaluation of software effectiveness and reliability

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The estimation of demographic parameters in wild populations is strengthened by individual identification. For amphibians, various techniques are used to either temporarily or permanently mark individuals for identification. Photo-identification of body patterns offers a non-invasive technique. However, the reliability of photo-recognition software is key to the reliable estimation of the true demographic parameters. In the current study, we assessed the effectiveness of fully-automated and semi-automated software: Wild-ID and APHIS. We used the cryptic salamander *Hydromantes strinatii* as our study species. We used the False Rejection Rate (FRR) of Top 1, Top 5 and Top 10 matches of chest and cloaca pictures. Finally, we assessed the bias induced by our FRR for the estimation of population size through simulation. Wild-ID FRRs ranged from 0.042 to 0.093 while APHIS' ranged from 0.227 to 0.547. Wild-ID was equally efficient with pictures from the chest and from the cloaca, while APHIS was significantly more efficient with chest pictures than cloaca pictures. Cropping pictures did not significantly improve Wild-ID effectiveness. Our Wild-ID FRRs are among the lowest ever obtained from pictures of an amphibian with a complex chromatophore pattern. Simulation showed that the Top 10 FRR from selected software Wild-ID induced a low bias 2.7% on the estimation of population size. The effectiveness and plasticity of Wild-ID provides opportunities for reliably monitoring amphibian species with complex colour patterns.

Key words: Photographic identification, Wild-ID, APHIS, False Rejection Rate, *Hydromantes strinatii*, demographic parameters, monitoring

INTRODUCTION

The assessment of demographic parameters using standardised methods is a key part of conservation biology, especially to understand population trends and make reliable predictions about their viability (Griffiths et al., 2015). Capture – mark – recapture models (CMR) that estimate population size, survival and detectability are useful tools for obtaining data for conservation (McCrea & Morgan, 2014). This method requires that individual marks are perennial in order to allow individual recognition over time (Chao, 1989; Nichols, 1992). For amphibians, marking techniques can be either invasive or non-invasive. For some species, evident and consistent colour patterns such as spots or stripes can be used as natural marks for individual recognition (Arntzen et al., 2004; Wengert & Gabrial, 2006; Ferner, 2010). Although visual matching can be an efficient method for monitoring a small population over a limited time, this technique becomes tedious and time-consuming (and thus sensitive to mistakes) when used to monitor large populations over several years (Morrison et al., 2011; Cruickshank & Schmidt, 2017).

The development of algorithms for pattern recognition allows pattern mapping, and therefore pattern matching, to be automated (Sacchi et al., 2016). However, two types of error are likely to induce a bias in the assessment of demographic parameters (Yoshizaki et al., 2009; Morrison et al., 2011). The first, namely False Acceptance (FA), consists of assigning the same identification to two different individuals. The second, False Rejection (FR), consists of the failure of software to match two pictures of the same individual (Jain, 2007). The rates at which these two types of error occur (respectively FAR and FRR) can be used to assess software effectiveness, the latter being more commonly used (Bolger et al., 2012).

There are three types of software that can be distinguished: (1) software, such as I3S series (Van Tienhoven et al., 2007) and AmphIdent (Matthé et al., 2008), that require laborious and time consuming pre-processing treatment, which consists of tracing (i.e. with the computer's mouse) the pattern outlines on each

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picture, (2) software not requiring any pre-processing of pictures (except the recommended cropping of pictures), and consequently time-saving, such as Wild-ID (Bolger et al., 2012) and Hotspotter (Crall et al., 2013), and (3) intermediate software, such as APHIS (Moya et al., 2015), which has been recently developed especially for herpetofauna, i.e., with a reduced pre-processing approach consisting of identifying two landmarks on each picture that the software can use as reference points (e.g. the base of left and right hind legs). Type 1 software is only used for species with gross body patterns (e.g. *Triturus carnifex*, *Paramesotriton hongkongensis*) because patterns need to be characterised easily (Fu et al., 2013; Sannolo et al., 2016). Type 2 requires a good quality of individual pictures, otherwise the accuracy of recognition may not be reliable (Morrison et al., 2016). While there is most probably a continuum rather than a true dichotomy in the specific pattern complexity, the user-friendly software Wild-ID is generally used for the identification of amphibians showing large and/or contrasted spots (e.g. Caorsi et al., 2012; Elgue et al., 2014; Morrison et al., 2016; Romiti et al., 2016), but has been assessed only once with a complex colour (i.e. fine spotted) pattern, using a neotenic salamander (Bendik et al., 2013). Also, the lowest FRRs were obtained using pictures taken with a "digital single-lens reflex" camera (DSLR) and in highly standardised conditions (e.g. cropped pictures, laboratory conditions) (Mettouris et al., 2016). Since APHIS benefits from two reference spots indicated by hand, it theoretically allows for a lower standardisation level compared to fully automated software and could be a useful compromise between type 1 and 2 software in terms of time and handling needed to process the pictures. Yet to date, APHIS has rarely been used for amphibian monitoring (but see Romano et al., 2017, 2018 with *Salamandrina perspicillata*).

In this study, we used Wild-ID and APHIS to identify the French cave salamanders *Hydromantes strinatii*. This species bears a complex chromatophore network resulting in a stippled colour pattern and thus provides a useful study species for testing software effectiveness and reliability. To assess software effectiveness (i.e. the FRR values) and the reliability of the entire photographic identification process (i.e. the FRR variability), we took pictures in field conditions with a "point and shoot" camera; in other words, we rejected the option of using a heavy-handling set-up in the field. We considered two different regions of the salamander body (the chest and the cloaca) to identify differences in FRR between body sites. We first compared the respective FRR and, secondly, we assessed the effect of the lowest FRR on the estimation of population size, as demographic parameters are the ultimate goals of CMR. This allowed us to determine whether the best combination of software and body region lead to acceptable errors in this estimation.

MATERIALS AND METHODS

Study species

Hydromantes strinatii is a salamandrid endemic to south-eastern France (Alpes-Maritimes and Alpes-de-Haute-Provence departments) and north-western Italy (Ligure region). It has a complex chromatophore pattern with a discrete phenotypic variability in its design and thus, it is a good model for the assessment of non-invasive identification methods, such as photo-identification, used to compute the demographic parameters needed for population viability analyses (Fig. 1). Further, it is important to study this species because it has been evaluated as "Near Threatened" and is therefore of conservation interest (Temple & Cox, 2009; Renet & Delauge, 2012). Its snout-vent length does not exceed ca. 75-80 mm, while maximal total length is 123 mm (Lanza et al., 1995). Individuals shy away from daylight in natural and artificial cavities. During the night, they come out and can be found in epigeous rocky habitats.

Population sampling and individual marking

During April 2015, we sampled a population in an epigeic habitat south of Roquebillière city (43°59'50.05''N, 7°18'41.57''E, Alpes-Maritimes department). We searched for individuals on six occasions with an equal sampling effort along a 94 m section of retaining walls, during the maximal activity period of the species, i.e., during nights with relative humidity ranging from 70 % to 100 % and temperature ranging from 5 °C to 15 °C (Lanza et al., 2006). As we repeatedly sampled the same section of wall, some individuals were captured on more than one sampling occasion (i.e. recaptured). Pattern resolution (i.e. scale) is likely to vary with body size and hence with individual growth. In order to avoid this potential bias, we captured adults only and avoided juveniles. Each individual was stored in a 5×10×10 cm box, with holes so that it could breathe, until it could be photographed. At the end of each sampling session, the animals were moved to the nearby photography setup. This consisted of a box lined on the bottom with polypropylene foam and equipped with a swinging glass plate. The animals were placed one by one upside down on the foam and held in place with the glass plate so that their ventral surface was flattened against the glass. For each individual, we took 4-5 pictures of the chest and 4-5 of the cloaca (Fig. 1). They were released immediately after photography at the exact location of capture, which was marked on each storage box. Manipulation of each individual did not exceed 5 minutes and total storage duration did not exceed 2 hours. Permission for this programme was issued by order of the Prefet [2015-227], according to French law.

Pictures were taken with a TG-3 Olympus© digital camera "point and shoot" (sensor: BSI CMOS 16 Mpx; zoom: 4 × 25-100mm f/2-4.9). We used the camera under the automatic "super-macro" mode with an additional LED LG-1 ring, which allowed the pictures to be taken 1 cm from the individuals. Pictures were taken with a 3456 × 4608 px resolution. No further processing was done, not even cropping; however, we also assessed whether cropping improved Wild-ID effectiveness (see below).

For each combination of sampling occasion x individual × body region, we selected the best picture among the 4-5 we had taken, thereby obtaining n_1 = 314 pictures for each

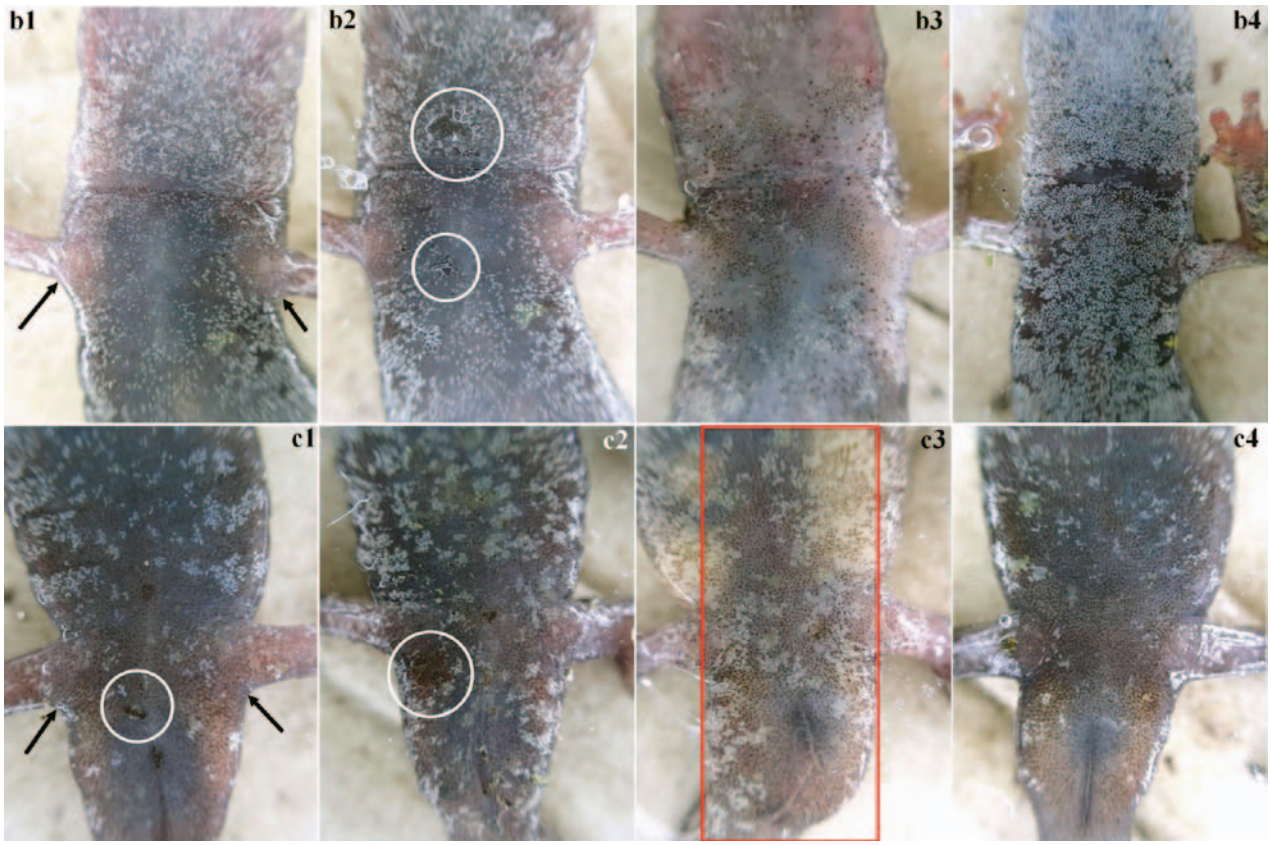

Figure 1. Variability of the complex chromatophore pattern of the French cave salamander *Hydromantes strinatii*, photographed at the two body regions considered in this study: chest (b) and cloaca (c). Picture b1 and b2 concern an individual during capture and recapture, respectively, the latter being identified as the first rank matching picture by Wild-ID during the five replicates despite minimal-standardisation conditions (see impurities and air bubbles indicated by circles). Black arrows indicate the armpit (b1) and the groin (c1) which we used as reference spots required by APHIS ITM approach. Red lines indicate the frame (1700 × 4608 px) used during the cropping test.

body region. We then allocated each pair of chest and cloaca pictures, among which some were capture pictures and others were recapture pictures, to an individual animal by visual matching, which we assume to be 100 % effective in discriminating each individual (i.e. FRR = 0). Indeed, the 314 pairs of chest and cloaca pictures were examined by two naive and independent observers who both recognised the same 253 individuals. There were 314 – 253 = 61 recaptures of 52 individuals; 61 – 52 = 9 and 52 – (61 – 52) = 43 individuals were, respectively, recaptured twice and once, and therefore photographed at three and two occasions; 314 – (43 × 2 + 9 × 3) = 201 individuals were captured and photographed once. We accordingly attributed an identification number (Id) to each of the 52 recaptured individuals.

Software characteristics and effectiveness assessment

APHIS (ver. 1.0; Moya et al., 2015) is available for free from https://imedea.uib-csic.es/bc/gep/docs/aphis/APHISPROGRAM/program/. It offers two individual matching processes: the Spots Pattern Matching (SPM) and the Image Template Matching (ITM). The former is based on I3S algorithm (Van Tienhoven et al., 2007) while the latter is based on algorithms developed by OpenCV (Open source Computer Vision). In this study, we used the ITM process, which requires the definition of two landmarks on the body of each individual; we used the armpit and the vent for the chest and the cloaca regions respectively (Fig. 1: arrows). From there, the software creates a rectangle divided into six areas, which are analysed independently (Moya et al., 2015).

Wild-ID (ver. 1.0.1; Bolger et al., 2012) is available for free from http://software.dartmouth.edu/Macintosh/Academic/Wild-ID_1.0.0.zip. It uses the SIFT algorithm (Scale Invariant Feature Transform; Lowe, 2004) to extract the body pattern, and then compares the geometric arrangement of the SIFT features for each couple of pictures (i.e. matches) without pre-processing.

APHIS and Wild-ID offer, respectively, 100 and 20 "top-ranked" matches following a decreasing calculated score provided by their algorithms (i.e. the picture proposed at the 1st rank is that from the dataset showing the highest score with the submitted picture). One has to then search visually for the correct individual among the software proposals.

We assessed effectiveness of each software for each body region. For this, we determined, for each of the 61 recaptures, the rank at which the software proposed the correct picture (i.e. the 100 % true matched picture, identified from the visual assessment) among the top-ranked matches. Hence, we were able to state whether the software programme failed to rank each of the 61 recapture pictures within the Top 1, 5 or 10 matches; in other words, failed to correctly identify the

recaptured individuals in the first one, five or ten hits. Corresponding error rates were reported in terms of FRR (Jain, 2007). Both APHIS and Wild-ID compare one submitted picture to all pictures recorded in the dataset. During the matching process for APHIS and Wild-ID, each new recapture picture is compared against all pictures that have been processed previously; this means, for instance, that the first recapture pictures we processed was compared to the 253 pictures of 1st capture, and that the 61st recapture picture we processed has been compared to the 253 + 60 = 313 pictures of both 1st captures and recaptures. This means that the size of the dataset each new recapture picture is compared to increases for each subsequent picture. When assessing the FRR according to this method, i.e. by comparing one picture only to other pictures that had been processed so far rather than against all other existing pictures (all capture and recapture picture), the false rejection assignation, and therefore the FRR, may be strongly affected by the order of processing of the pictures. To take this potential bias into account, we decided to assess the software reliability, i.e. the variability of the FRR by repeating the process five times (i.e. five replicates). For each replicate the recapture pictures were processed in a different random order. Each of the three Top rank datasets therefore contained 1220 data points (5 replicates × 2 softwares × 2 body regions × 61 recapture pictures).

Wild-ID manual recommends to crop pictures so as to remove the background as much as possible, however, this can be a time consuming and laborious process. Thus, we assessed whether cropping was require to improve effectiveness. For this, we cropped cloaca pictures using a 1700 × 4608 px frame (Fig.1). We then randomly selected 30 recapture pictures and compared each of these to a database composed by the 253 + 60 = 313 pictures of both 1st captures and recaptures; for each comparison, the database was therefore renewed and slightly different from the previous (i.e. by one picture out of 313). This was done with the cropped pictures and with the original pictures, using the same 30 randomly selected individuals.

Statistical analysis

Failures, i.e. false rejections, were coded as 1 and successes coded as 0 in a new data frame, with software, body region and replicate as co-variables. We used generalised linear mixed models to test differences of FRR (dependent variable) between software and between body regions (explanatory variables, i.e. fixed factors). Mixed models were used to account for (1) the pseudo-replication induced by the fact that some individuals were recaptured and photographed more than once (Hurlbert, 1984) and (2) the replication scheme of the FRR computing. "Id" and "Replicate" were thus included as random factors. To assess whether FRR was influenced by the software and/or body region, we used each Top rank match and analysed the datasets using a binomial error using the following function:

$$logit(FRR) = \alpha + \beta_1.Soft + \beta_2.Reg + \beta_3.(Soft * Reg) + \varepsilon_{Id} + \varepsilon_{Repl}$$

where Soft is the software, Reg is the body region and Soft*Reg is their interaction, Id is this individual and Repl is the replicate. Contrast analyses were performed in order to assess whether the differences between the best combination of software and body region was significantly different from other combinations or not. To assess the variability of the FRR regarding the order in which recapture pictures were processed in, we computed the FRR for each replicate independently using the following function:

$$logit(FRR_i) = \alpha_i + \beta_1^i.Soft + \beta_2^i.Reg + \beta_3^i.(Soft * Reg) + \varepsilon_{Id}^i$$

where i is the replicate number. We then computed the standard deviation (SD) of the 5 FRR we obtained. Results are given as FRR estimated by the models together with their 95 % CI. Significance of fixed factors is given by a range of corresponding estimates ± standard error which does not overlap zero (i.e. meaning a null effect of the fixed factor). All analyses described above were performed in R 3.4.4 (R Core Team, 2018) using packages 'lme4' (Bates et al., 2014), 'lmerTest' (Kuznetsova et al., 2017) and 'effects' (Fox et al., 2016).

To assess the bias on the estimation of population size induced by the best software and body region FRRs obtained for each of the Top 1 and 10 matches, 1000 CMR datasets were simulated to which we applied the FRRs obtained from the generalised linear mixed model. The datasets were created assuming a population of 600 individuals, six occasions of sampling with a mean capture probability of 0.1 varying randomly for each sampling occasion. Those assumptions were based on the results of Mt standard closed capture model (Otis et al., 1978) applied to the real dataset with a time effect on detection probability and run in a Bayesian framework (population size = 543 ± 44 SD; mean detection probability = 0.098 ± 0.050 SD). The same Mt closed capture model was then applied to the 1000 simulated datasets with respectively no FRR, Top10 and Top1 FRRs. MCMC was applied using JAGS (Plummer, 2003) via the package R2jags (Su & Yajima, 2012) in R (R Core Team, 2018). We used a burn-in of 500 iterations, three chains, a thinning rate of two and 2000 iterations for each posterior distribution. Convergence was reached and mixing of the chains was good.

RESULTS

Fixed effects driving False Rejection Rate

For all three types of Top rank matches ($n_1 = n_2 = n_3 = 1220$), the FRR was significantly influenced by software (Top 1: |estimate| = 1.640 ± 0.234; Top 5: |estimate| = 1.909 ± 0.273; Top 10: |estimate| = 1.891 ± 0.286) and body region (Top 1: |estimate| = 0.826 ± 0.192; Top 5: |estimate| = 0.868 ± 0.196; Top 10: |estimate| = 0.914 ± 0.200) and their interaction (Top 1: |estimate| = 0.961 ± 0.324; Top 5: |estimate| = 0.596 ± 0.360; Top 10: |estimate| = 0.914 ± 0.390). Hence, we hereafter provide the FRR of each combination of software and body region separately.

Table 1. Software effectiveness: False Rejection Rate (FRR) when matching individual pictures of the French cave salamander *Hydromantes strinatii*, comparing software programmes and body regions. Contrast analyses were made between (1) the smallest FRR and others, and (2) between APHIS FRRs: ns = not significant ($p > 0.05$), * = significant ($p < 0.05$), *** = highly significant ($p < 0.001$).

High-scoring matches	Software	Body region	FRR estimated	Contrast analysis (1)	Contrast analysis (2)
Top 1	Wild-ID	Cloaca	0.082 (0.055-0.122)		
		Chest	0.093 (0.063-0.135)	|z| = 0.519 p = 0.604 ns	
	APHIS	Cloaca	0.547 (0.472-0.620)	|z| = 10.329 p < 0.001 ***	
		Chest	0.346 (0.279-0.419)	|z| = 7.363 p < 0.001 ***	|z| = 4.298 p < 0.001 ***
Top 5	Wild-ID	Chest	0.050 (0.030-0.081)		
		Cloaca	0.064 (0.041-0.100)	|z| = 1.008 p = 0.313 ns	
	APHIS	Chest	0.261 (0.203-0.328)	|z| = 7.702 p < 0.001 ***	
		Cloaca	0.457 (0.385-0.531)	|z| = 10.922 p < 0.001 ***	|z| = 5.058 p < 0.001 ***
Top 10	Wild-ID	Cloaca	0.042 (0.025-0.071)		
		Chest	0.042 (0.025-0.071)	|z| = 0 p = 1 ns	
	APHIS	Cloaca	0.422 (0.351-0.396)	|z| = 9.529 p < 0.001 ***	
		Chest	0.227 (0.173-0.291)	|z| = 6.613 p < 0.001 ***	|z| = 5.244 p < 0.001 ***

False Rejection Rate and its variability: the effects of software and body region

Wild-ID produced the smaller FRR for Top 1, Top 5 and Top 10 matches i.e. 0.082, 0.050 and 0.042, respectively (Table 1). These FRR values were significantly smaller than those of APHIS, for both chest and cloaca. Wild-ID FRR values were not significantly different for the two body regions (Table 1). In contrast, APHIS FRRs were significantly smaller for the chest than the cloaca region for Top 1, Top 5 and Top 10 matches. The standard deviation of the FRR across the five replicates ranged from 0.004 to 0.057 (Fig. 2). The FRR variability regarding the order of processing of the recapture pictures (i.e. across replicates) was greater with APHIS (SD range: 0.022–0.057) than with Wild-ID (SD range: 0.004–0.016).

Influence of cropping of pictures on Wild-ID effectiveness

Cropping did not significantly affect Wild-ID effectiveness: removing the background reduced the rank at which Wild-ID proposed the correct picture in only two recapture pictures out of 30: 7th instead of 8th rank and 18th instead of >20th rank. Hence, when coding these data as binary (false rejection or success), the two new data frames for cropped and original pictures were absolutely similar for the Top 1, Top 5 and Top 10 matches.

Influence of error in Wild-ID on the estimation of population size

With the selected software programme (Wild-ID) used on chest, FRR had a weak influence on the estimation of population size of *Hydromantes strinatii* (Table 3). Mean estimated population size for Control, Top 10 and Top 1 matches (the latter being the highest FRR and therefore inducing the strongest bias during the estimation of demographic parameters) all included the known population size of 600 individuals within their 95% confidence interval. Mean population size estimated from simulated data without FRR was underestimated by 2.0% [-16.6;14.5] while mean estimated population sizes were overestimated by 2.7% [-12.9;18.7] using Top 10 FRR, and by 9.0% [-7.7;25.8] using the Top 1 FRR.

DISCUSSION

Software effectiveness in recapture identification

Our results, obtained in field conditions, showed that Wild-ID was more effective and reliable than APHIS in matching pictures of a complex chromatophore pattern salamander (Table 1). Despite the field conditions and the use of a point and shoot camera, our Top 10 Wild-ID FRR values are among the lowest ever obtained and our more stringent Top 1 Wild-ID FRR values are close to the first quartile (i.e. among the lowest) of those found throughout the literature (Table 2). These low values are undoubtedly influenced by the small size of our database (N = 253 captures + 61 recaptures = 314) but there is probably also some influence of technological progress in the quality of sensor and optic lenses since the other studies were conducted, which helps to cope

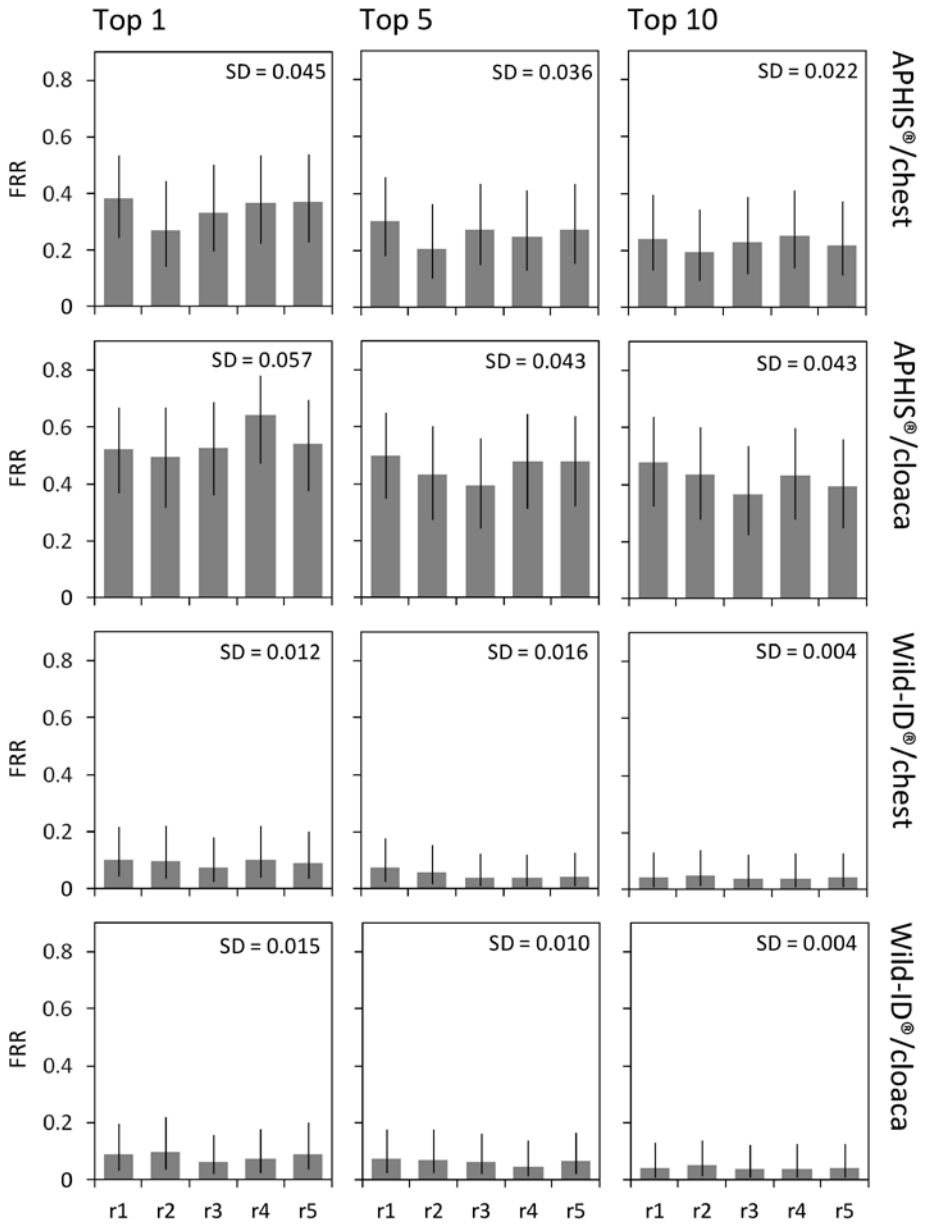

Figure 2. Software reliability: variability for each combination of software, body region and Top rank matches of the False Rejection Rate (FRR) across five replicates (r1–r5) of the matching of individual recapture pictures of the French cave salamander *Hydromantes strinatii*. Grey columns show the estimated mean FRR and black bars their 95% CI.

with poorly-standardised conditions. Even in our study, software effectiveness could have been improved further with even higher quality pictures produced by (i) holding individuals manually or using a shutter with a hole without glass (i.e. allowing a direct view on a selected body region) in order to avoid bubbles and impurities, (ii) improving lighting, and therefore the depth of field, for instance using a powerful flash, and (iii) holding the camera still with a tripod. Also, cropping pictures is supposed to improve software effectiveness. By comparing here FRRs obtained with uncropped pictures (Wild-ID) vs pictures where we restricted the searching algorithm to the focus area by generating a set of landmarks (APHIS), we potentially skewed our results in favour of APHIS. However, the little improvement cropping pictures provided in Wild-ID picture ranking had no effect on the computing of FRR values. If it had, it only had strengthened the dominance of Wild-ID over APHIS.

Using APHIS with the ITM procedure on chest pictures of a salamander, Moya et al. (2015) detected 100 % of recaptures (i.e. FRR = 0, n = 305). In contrast, we obtained with APHIS poor FRR values (i.e. lower effectiveness), which were in addition highly variable (i.e. lower reliability) (Fig. 2). This is probably due to our low level of standardisation (e.g. body distortion, variable lighting), for which the software cannot fully compensate despite pre-processing of images, combined with human error since pre-processing is executed by hand during each replicate by positioning two new reference points on the same pictures.

Wild-ID was as effective with pictures of the chest as with those of cloaca. On the other hand, APHIS appeared to be less effective with cloaca pictures than with chest

Table 2. Review of FRRs obtained with Wild-ID when monitoring amphibia in different studies. PAS = "point and shoot" camera; DSLR = "digital single lens reflex" camera; CC = Camcorder

High-score matching range	Species	Pattern	Type of camera	FRR (sample size)	References
Top 100	*Eurycea tonkawae*	Dorsal head	DSLR	0.008 (1367)	Bendik et al., 2013
			PAS	0.159 (965)	
Top 20	*Anaxyrus baxteri*	Dorsal side	DSLR	0.200 (822)	Morisson et al., 2016
			PAS	0.470 (822)	
	Melanophryniscus cambaraensis	Ventral side	PAS	0.091 (492)	Coarsi et al., 2012
	Melanophryniscus montevidensis	Ventral side	PAS	0.100 (410)	Elgue et al., 2014
	Salamandrina perspicillata	Ventral side	?	0.650 (760)	Romiti et al., 2016
Top 10	*Hydromantes strinatii*	Cloaca region	PAS	0.042 (253)	This study
		Chest region	PAS	0.042 (253)	
	Ichthyosaura alpestris	Body (left) side	DSLR	♀: 0.001 (721) ♂: 0.000 (517)	Mettouris et al., 2016
	Lissotriton vulgaris	Ventral side	DSLR	♀: 0.031 (125) ♂: 0.025 (77)	
	Salamandra salamandra + S. infraimmaculata	Ventral side	PAS - CC	0.732 (500) 0.770 (2000) 0.774 (2197)	Matthé et al., 2017
	Triturus carnifex + T. cristatus	Ventral side	PAS - CC	0.146 (500) 0.161 (2000) 0.169 (4000) 0.186 (7000) 0.188 (7458)	Matthé et al., 2017
	Ambystoma opacum	Ventral side	PAS - CC	0.177 (500) 0.217 (2000) 0.264 (4000) 0.304 (7000) 0.341 (12488)	Matthé et al., 2017
	Bombina variegata	Ventral side	PAS - CC	0.027 (500) 0.033 (2000) 0.036 (4000) 0.036 (4063)	Matthé et al., 2017
Top 1	*Hydromantes strinatii*	Cloaca region	PAS	0.082 (253)	This study
		Chest region	PAS	0.093 (253)	
	Ichthyosaura alpestris	Body (left) side	DSLR	♀: 0.017 (710) ♂: 0.006 (514)	Mettouris et al., 2016
	Lissotriton vulgaris	Ventral side	DSLR	♀: 0.186 (105) ♂: 0.076 (73)	Mettouris et al., 2016
	Salamandra salamandra + S. infraimmaculata	Ventral side	PAS - CC	0.863 (500) 0.882 (2000) 0.884 (2197)	Matthé et al., 2017
	Triturus carnifex + T. cristatus	Ventral side	PAS - CC	0.274 (500) 0.300 (2000) 0.324 (4000) 0.352 (7000) 0.355 (7458)	Matthé et al., 2017
	Ambystoma opacum	Ventral side	PAS - CC	0.350 (500) 0.449 (2000) 0.505 (4000) 0.553 (7000) 0.604 (12488)	Matthé et al., 2017
	Bombina variegata	Ventral side	PAS - CC	0.047 (500) 0.059 (2000) 0.068 (4000) 0.068 (4063)	Matthé et al., 2017

Table 3. Population size estimated from a simulated French cave salamander population of 600 individuals. 1000 datasets with randomly varying detection probabilities over six closed capture events (mean detection probability of 0.1) were simulated. False Rejection Rates (FRRs) obtained from Table 1 (chest Wild-ID Top 10 and Top 1 matches) were applied to simulated datasets. Population sizes were estimated applying standard closed capture model with a time effect on detection probability.

Dataset	FRR	Mean estimated population size [95 % CI]
Control	0	588 [500-687]
Top 10	0.042	618 [522-712]
Top 1	0.093	655 [554-755]

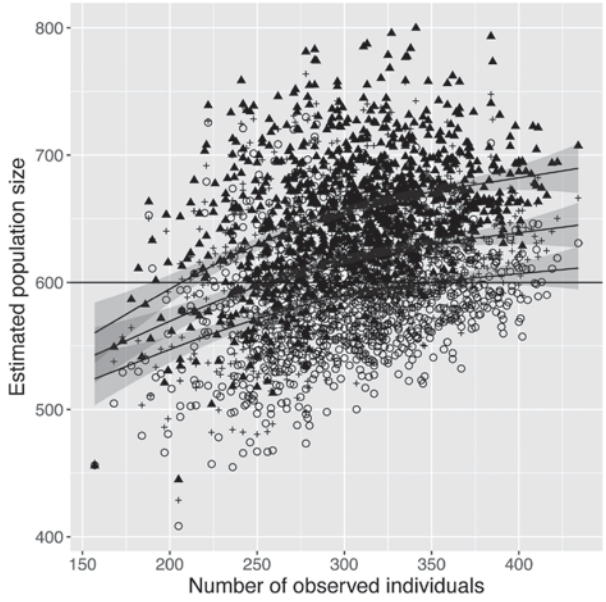

Figure 3. Impact of false rejections on estimation of abundance of a simulated population of 600 French cave salamanders. Capture-recapture data were collected over six closed capture events with mean detection probability of 0.1. Estimated population size from simulated data with no error are presented with open circles, from simulated data with False Rejection Rate (FRR) corresponding to the use of Top 10 Wild-ID on the chest are presented with crosses and with FRR corresponding to the use of Top1 Wild-ID on the chest are presented with black triangles. A regression spline smoother with 95% confidence intervals was added for each group of data to help with visual interpretation. The smoother was fitted using the mgcv package (Wood, 2006) and explains respectively 10%, 12% and 17% of the variation in the estimated population sizes for the Control, Top10 and Top1 datasets.

pictures. When flattened against the glass, the soft cloaca can vary in shape, therefore modifying patterns. The higher sensitivity of APHIS to distortion is most probably once again due to the pre-processing conducted by hand. Pictures of the less mobile and more stable body regions such as the chest produced results at least as good as the cloaca pictures in our study, so we suggest using pictures from such stable body regions with all software. Though I3S straighten software copes with body contortion, it requires a manual pre-processing approach (Den Hartog & Reijns, 2015), which is time consuming.

Consequences for the estimation of population size

One assumption necessary for valid inference from capture-mark-recapture studies is that marks are correctly read (Lindberg, 2012). In case of non-invasive sampling, errors in the identification of individuals may lead to bias in estimation of demographic parameters (Creel et al., 2003). Here we showed that considering first Wild-ID proposals (Top 1 high-score matches) leads to an overestimation of population size of 9%. Underestimation of population size without FRR was due to the low detection probability found in this species (<0.1, see Methods section) and applied to the simulated dataset. Indeed, datasets created from simulations that implied lower number of recaptures induced higher uncertainty and underestimation of the population size (Fig. 3). As a consequence, we found wide 95 % confidence intervals in the estimated population size (Table 3).

A 9% overestimation of population size is however twenty times less than the overestimation of population size found by Morrison et al. (2016) using Wild-ID. This is consistent with the much higher FRR (0.47 for Top 20) found by Morrison et al. (2016) on Wyoming toad (*Anaxyrus baxteri*) than those found in this study. In order to limit bias in estimation of population size when individuals are misidentified, closed capture models incorporating misidentification as implemented in the programme MARK may be used but, as a consequence in such case, confidence intervals would become much wider (Lukacs & Burnham, 2005). When a long-term monitoring programme is launched, a further important point to consider is the possibility that chromatophore patterns vary over time in amphibia (Church et al., 2007; Drechsler et al., 2015; Balogova & Kyselova, 2016).

Management implications

The assessment of bias in individual recognition is an essential step when software is used to estimate the demographic parameters, especially in a threatened species. Indeed, these values can greatly influence the decisions regarding conservation strategies. To limit this bias without spending too much time reviewing all available pictures, especially with huge sample sizes (i.e. $n > 1000$), we recommend considering the Top 10 high-score matches proposed by Wild-ID, at least for *H. strinatii* and most certainly for several other *urodela* species that have a similar pigment pattern. Our study suggests that in the conditions described here (low detection probability), overestimation of the population size is 3 %, and true population size falls within the 95% confidence interval.

Although Wild-ID was initially developed for large terrestrial mammals, we show that the software is able to cope effectively with a small sized amphibian with a complex chromatophore pattern. We have also shown that it is effective in field conditions and without pre-processing treatment of pictures. Therefore, it can work

well even with limited equipment and time. This also means stress to the animals and the cost of the study may be reduced using Wild-ID than other software – two aspects which are nowadays of primary concern for reasons of ethics and efficiency. The low FRR values obtained through the present study in *H. strinatii* provide encouraging prospects for using Wild-ID to identify and monitor species with complex pigment patterns. Even if the FRR may increase with the size of the dataset, this rate can be reduced by increasing the quality of the pictures (Gamble et al., 2008; Matthé et al., 2017).

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A new species of *Contomastix* (Squamata, Teiidae) supported by total evidence, with remarks on diagnostic characters defining the genus

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Formerly *Cnemidophorus* was thought to be the most speciose genus of Teiidae. This genus comprised four morphological groups that were later defined as four different genera, *Ameivula*, *Aurivela*, *Cnemidophorus* and *Contomastix*. The last appears as paraphyletic in a recent phylogenetic reconstruction based on morphology, but monophyletic in a reconstruction using molecular characters. Six species are allocated to *Contomastix*. One of them, *C. lacertoides*, having an extensive and disjunct geographic distribution in Argentina, Uruguay and Brazil. Preliminary analyses revealed morphological differences among its populations, suggesting that it is actually a complex of species. Here, we describe a new species corresponding to the Argentinian populations hitherto regarded as *C. lacertoides*, by integrating morphological and molecular evidence. Furthermore, we demonstrate that the presence of notched proximal margin of the tongue is a character that defines the genus *Contomastix*.

Key words: C. lacertoides species group, lizards, Reptilia, South America, systematics

INTRODUCTION

The family Teiidae is distributed throughout the New World, from the northern United States to Argentina, in a wide variety of habitats ranging from extremely arid deserts to tropical rainforests (Pough et al., 2004; Vitt & Caldwell, 2014). One of the most speciose genera of this family was formerly known as *Cnemidophorus* Wagler. Cei (1993) proposed three species groups for the Argentinian taxa of *Cnemidophorus* on the basis of pholidosis and anatomical characters; (a) the *C. longicaudus* (sic) species group for lizards exhibiting a unique preauricular flap, a markedly bilobate posterior margin of the tongue, and granular supraorbital semicircles, (b) the *C. lacertoides* species group for those with a notched posterior margin of the tongue, lacking granular supraorbital semicircles and a preauricular flap, and (c) the *C. lemniscatus* species group for taxa exhibiting a markedly bilobate posterior margin of the tongue and granular supraorbital semicircles, without a preauricular flap. This last group was later split by Cabrera (2004). Thus, the *C. lemniscatus* species group exclusively included all of the species in which males bear preanal spurs, whereas the *C. ocellifer* species group included all the species in which males lack preanal spurs.

Reeder et al. (2002) inferred a phylogeny of the family Teiidae analysing diverse lines of evidence (data derived from mitochondrial ribosomal RNA genes, allozymes, and morphological characters). These authors demonstrated that *Cnemidophorus* was polyphyletic and assigned all the species of this genus from North America to the resurrected genus *Aspidoscelis* Fitzinger. However, this new systematic arrangement did not completely solve the polyphyly of *Cnemidophorus*, as the clades of three of the four morphological species groups mentioned above (*longicauda*, *lacertoides* and *lemniscatus*) were more closely related to clades of other genera of the family than among themselves. The phylogenetic position of the *C. ocellifer* species group could not be accurately determined in their study due to inadequate data.

While the polyphyly of *Cnemidophorus* remained unresolved, their diversity was noticeably increased with the description of many new species [e.g., Colli et al., 2003 (*Cnemidophorus mumbuca*); Cabrera, 2004 (*Cnemidophorus tergolaevigatus*); Colli et al., 2009 (*Cnemidophorus jalapensis*); Cabrera & Carreira, 2009 (*Cnemidophorus charrua*); Ugueto et al., 2009 (*Cnemidophorus senectus* and *Cnemidophorus flavissimus*); Ugueto & Harvey, 2010 (*Cnemidophorus leucopsammus* and *Cnemidophorus rostralis*); Arias et al., 2011a (*Cnemidophorus confusionibus* and

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Cnemidophorus venetacaudus); Arias et al., 2011b (*Cnemidophorus nigrigula* and *Cnemidophorus cyanurus*); Cabrera, 2012 (*Cnemidophorus abalosi*); Silva & Ávila-Pires, 2013 (*Cnemidophorus pyrrhogularis*)]. In general, these descriptions of new species were based only on morphology, coloration and/or meristic variables. Furthermore, some new species were described from just one or few localities, and/or their characteristics were compared to a limited number of species of congeners, some of which belonged to a different morphological group from the new lineage.

Harvey et al. (2012) conducted a phylogenetic analysis of the family Teiidae based on morphological characters and resolved the polyphyly of *Cnemidophorus*, assigning each previously known species group described by Cei (1993) and Cabrera (2004) to a different genus: (a) the name *Cnemidophorus* remains for the species of the *C. lemniscatus* species group; (b) the species of the *C. ocellifer* species group now correspond to the new genus *Ameivula*; (c) the new genus *Aurivela* includes the two apomorphic species of the *C. longicauda* species group formerly known as *Cnemidophorus longicauda* and *Cnemidophorus tergolaevigatus*, and (d) the new genus *Contomastix* comprises the species of the *C. lacertoides* species group.

The arrangement of Harvey et al. (2012) has been supported by two recent phylogenetic studies using several molecular markers, 48 mitochondrial and nuclear loci in Goicoechea et al. (2016), and 316 nuclear loci in Tucker et al. (2016). However, Goicoechea et al. (2016) split the genera *Ameivula* and *Ameiva*, resurrecting the genera *Glaucomastix* to include the species of the *Ameivula littoralis* species group, and *Pholidoscelis* for all the West Indies *Ameiva*.

The genus *Contomastix* includes the species *Contomastix lacertoides* (Duméril & Bibron), *Contomastix leachei* (Peracca), *Contomastix serrana* (Cei & Martori), *Contomastix vacariensis* (Feltrim & Lema) and *Contomastix charrua* (Cabrera & Carreira). Harvey et al. (2012) added *Cnemidophorus vittatus* Boulenger to the group, indicating that this species, together with *C. lacertoides* and *C. leachei*, lack a lingual sheath and have a straight posterior margin of the tongue rather than a heart-shaped one. However, they did not include tongue morphology in their phylogenetic reconstruction, arguing that this character is unreliable and difficult to assess. In their study, *Contomastix* was not recovered as monophyletic, with *C. lacertoides* placed outside the clade containing *C. serrana* and *C. vittata*. Based on their results, Harvey et al. (2012) considered that there is "no single unique character that distinguishes *Contomastix* from all other teiids" and suggested that additional research is needed to determine if the distinctive characters of *C. lacertoides* exclude it from the genus *Contomastix*. However, in a more recent phylogenetic analysis, Tucker et al. (2016) recovered a clade that includes *C. vacariensis*, *C. serrana*, and *C. lacertoides*.

Contomastix lacertoides is a small long-tailed striped terrestrial lizard. This species is distributed in a large region encompassing southern Brazil (the coast of the state of Santa Catarina and the southern half of the state of Rio Grande do Sul), almost all of Uruguay, and two small disjunct areas in Argentina: the south end of the Sierras de Córdoba system, in Córdoba province, and the Sierras de Ventania system, in Buenos Aires province (Cei, 1993; Lema, 1994; Vrcibradic et al., 2004; Carreira et al., 2005). The type locality for the species is Montevideo, Uruguay (Duméril & Bibron, 1839). Our preliminary revisions indicated that there are morphological differences among the populations of *C. lacertoides* from southern and eastern of Uruguay in relation to the other populations of the species, suggesting that *C. lacertoides* is actually a complex of species (Cabrera & Carreira, 2009; Cabrera, 2015).

One of the basic tasks in systematic research is to reach a more stable taxonomy and accurate species delimitation. Therefore, the use of multiple lines of evidence in order to provide stronger support for proposals of nomenclatural action is advisable. In this paper we describe a new species corresponding to the Argentinian populations hitherto regarded as *C. lacertoides*, by integrating morphological and molecular evidence. We also aim to determine if there are any morphological characters that define the genus *Contomastix*.

METHODS

Morphological analyses

We examined 40 specimens (23 males, 13 females, and 4 juveniles) of *C. lacertoides* sensu stricto from 23 localities in Uruguay, 15 specimens (7 males, 5 females, and 3 juveniles), and 23 specimens (12 males, 6 females, and 5 juveniles) of *C. lacertoides* sensu lato from one locality in the Sierras de Córdoba, Argentina and 4 localities in the Sierras de Ventania, Argentina, respectively. These samples comprised all the available Uruguayan specimens of this lizard known to us, and most of the Argentinian ones lodged in the national museums of both countries. This lizard is harder to find in the wild nowadays than before. We also examined other species of the genus: *C. charrua* (3 males, 6 females, one locality), *C. leachei* (2 males, 2 localities), *C. serrana* (5 males, 5 females, and 4 juveniles, 10 localities) and *C. vacariensis* (4 males, 3 females, 4 localities) (Fig. 1). Since we had no access to specimens of *C. vittata*, morphological data were obtained from the original description (Boulenger, 1902, one specimen) and from the studies of Vance (1978, the same specimen, re-evaluated) and Harvey et al. (2012, n = 9, all from Bolivia). Additional data for *C. leachei* and *C. vacariensis* were taken from Cei & Scrocchi (1991) and Feltrim & Lema (2000), respectively. The tongue morphology in *Contomastix* spp. was compared to that of *Ameiva ameiva*, *Ameivula abalosi*, and *Aurivela longicauda*. The specimens examined and the institutions in which they are housed are detailed in Appendix 1.

A standard morphological protocol for lizard taxonomy study was made following the definitions of Peters (1964), Smith (1995), Markezich et al. (1997), and Cabrera (2012). Measurements were taken to the nearest 0.1 mm with digital callipers under a dissecting stereomicroscope. The minimum size at which these

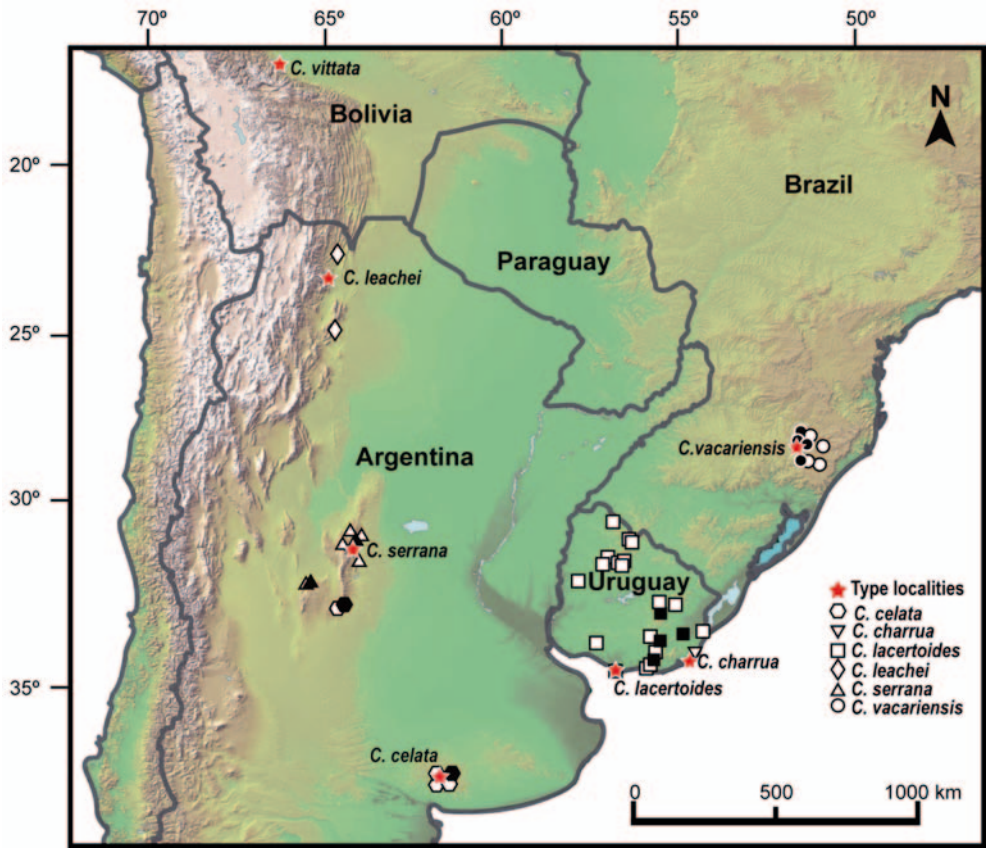

Figure 1. Geographic distribution of the specimens examined. Open symbols identify lizards whose morphology was analysed; black symbols, those used in molecular analyses. Each reference may represent more than one lizard in cases of close localities. The samples widely cover the range of all the species except for *C. vittata*. Base map by courtesy NASA/JPL Caltech.

species reach sexual maturity is unknown; however, specimens with snout-vent length (SVL) less than 45 mm were not considered for morphometric and meristic analyses in order to exclude allometric bias. Sex was determined by gonad inspection through a short incision on the left side of the belly in intact specimens without everted hemipenis. Coloration data were obtained from live specimens, field notes, literature, and photographs.

Morphometric characters included are: snout-vent length (SVL), along the midventral line from the tip of snout to the posterior edge of the preanal flap; head length (HL), from the tip of snout to the posterior margin of the ear, along the medial axis of the head; head depth (HD), measured vertically at the level of contact between frontal and frontoparietal scales; snout length (SL), from the tip of snout to the anterior limit of the frontal scale; axilla to groin distance (AG), from the posterior margin of the forelimb insertion to the anterior margin of the hindlimb insertion, on the left side of the body.

Meristic characters recorded were: dorsal scales around the body but excluding enlarged ventrals (DS), counted at mid axilla to groin distance; scales at midbody between the medialmost light stripes (SPV); dorsal scales along the body (DAS), counted on midline from behind the occipitals to the first transverse row of tail scales; transverse rows of ventral scales along midventral line (TVS), from behind the granular scales posterior to the gular fold to the anterior margin of the hindlimbs; longitudinal rows of ventral scales (LVS), at mid axilla to groin distance; supralabial scales (SLB), counted on left side from behind rostral to the last scale bordering the upper edge of the mouth; infralabial scales (ILB), counted on left side from behind mental to the angle of mouth (rictus ori); chin shields in contact on midline (CH); supraocular scales (SOC), counted on left side; parietal plates (PAP), counted as: interparietal + frontoparietals + parietals, but excluding the postparietals (= occipitals); gular folds (GF), including gular and pregular folds, if present; total number of femoral pores (FP); lamellar scales under the fourth finger of the left hand including the one below the claw (FFS); lamellar scales under the fourth toe of the left foot including the one below the claw (FTS).

Categorical characters included are: presence or absence of spots on infralabial scales (SIL); presence or absence of vertebral light stripe (VLS); condition (continuous, broken, or spotted) of the dorsolateral light stripe (the stripe running from the superciliaries along the body onto the tail) (DLS); condition (continuous, broken, or spotted) of the lateral light stripe (the stripe running from the suborbital region to the hindlimb) (LLS); presence or absence of dark pigment in any portion of ventral scales (VM); morphology (notched or bilobate) of the proximal margin of the tongue (PMT). This set of characters has proved to be reliable and robust in works dealing with cnemidophorine lizards at species group-level. Harvey et al. (2012) proposed a large set of character definitions for the phylogenetic analysis of the family Teiidae. We found most of them inapplicable to the present work, either because they are invariant

within the genus *Contomastix* (83 characters) or within *C. lacertoides* sensu lato (13 characters) or inapplicable to the genus (5 characters).

Comparisons were made among the geographical groups of *C. lacertoides* (Sierras de Córdoba, Sierras de Ventania and Uruguay) and among the different lineages within the genus *Contomastix*. All statistical analyses were performed using the InfoStat software v. 2015p (Di Rienzo et al., 2015) at a $p \leq 0.05$ significance level.

DNA extraction, PCR amplification and sequencing

Sample tissues (liver and/or muscle) were collected from four specimens of *C. lacertoides* sensu stricto from four localities in Uruguay and from three specimens of each of the two disjunct areas of distribution in Argentina (Fig. 1). We also obtained samples from two specimens of *C. serrana*, one from *Aurivela longicauda*, one from *Teius teyou* and one from *Salvator merianae*. Total genomic DNA was extracted from alcohol-preserved tissues, using a saline extraction method (Bruford et al., 1992). Fragments of the 16S, ND4, c-mos, and NTF3 genes were amplified using the primers and conditions specified in Appendix 2. The PCR products were sequenced in an automated DNA sequencer (ABI PRISM 3730x1 DNA) by Macrogen Korea Inc.

Phylogenetic analyses

Phylogenetic analyses were performed at the nucleotide level based on a matrix that included new sequences obtained in this study, and available sequences from GenBank of species with at least two of the genes analysed from almost all genera of the subfamily Teiinae. Taking into account that the phylogenetic hypothesis of Harvey et al. (2012), based on morphological characters, differs substantially from the hypotheses based on DNA (Reeder et al., 2002, Giugliano et al., 2013, Tucker et al., 2016), we used the genus *Salvator* as outgroup. Species, specimens, and GenBank accession numbers are listed in Appendix 3.

We performed multiple-sequence alignments for each gene using MAFFT software version 7 (Katoh & Standley, 2013). Phylogenetic relationships for the combined matrix of mitochondrial and nuclear DNA were analysed using Maximum Parsimony (MP), Maximun Likelihood (ML), and Bayesian inference (BI). MP analysis was made with PAUP 4.0.B10 (Swofford, 2003), with equal weighting for all characters and gaps treated as missing data. The Wagner algorithm was used for the heuristic search of the phylogenetic reconstructions with the TBR branch swapping algorithm. Then, the minimum length trees were summarised in a majority-rule consensus tree. The node support was evaluated by 1000 bootstrap replicates.

The best-fitting model of sequence evolution was selected using JModeltest2 (Darriba et al., 2012), under the Akaike information criterion (AIC) for ML and the Bayesian information criterion (BIC) for BI (Appendix 2). ML trees were constructed using the online version of PhyML 3.0 (http://www.atgcmontpellier.fr/phyml/; Guindon et al., 2010). The selected model was GTR+G, and it was used for the analyses; 1000 bootstrap replicates were performed. Bayesian analyses were performed using MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003), conducting two independent Markov chain Monte Carlo simulations (with four chains each) for 2 million generations, sampling every 1000 generations, and discarding the first 25% of the samples as burn-in. The convergence to stable values and Effective Sample Size (ESS) were checked with Tracer v1.5 (Rambaut & Drummond, 2007). The two runs converged on very similar posterior estimates with an average standard deviation of split frequencies of 0.01.

The Kimura 2 parameter genetic distances (K2P) were calculated for the 16S gene using Mega 7.0 software (Kumar et al., 2016). The nuclear genes were not used to calculate genetic distances due to their low level of variability.

RESULTS

Morphological analyses

There were no significant differences (Wilcoxon test) in any morphometric character between the Argentinian populations of *C. lacertoides*, so they were pooled as one. Between the Uruguayan and Argentinian populations, five morphological characters differed significantly (Kruskal-Wallis tests): DS, ($H=15.52$, $p<0.001$), SPV, ($H=28.14$, $p<0.001$), DAS ($H=39.96$, $p<0.001$), FFS ($H=8.78$, $p<0.01$), and FTS ($H=12.84$, $p<0.001$) (Fig. 2). These data indicate that Uruguayan lizards have smaller and more numerous dorsal scales than the Argentinian ones and more lamellae under the fourth finger and fourth toe (Table 1).

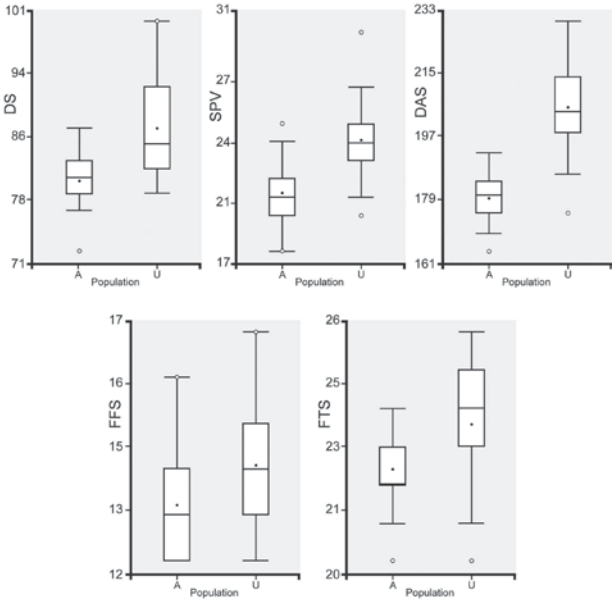

Figure 2. Box-plots illustrating meristic differences between the Argentinian (A) and Uruguayan (U) samples of *Contomastix lacertoides* sensu lato. DAS, dorsal scales along the body; DS, dorsal scales across midbody; FFS, lamellae under the fourth finger; FTS, lamellae under the fourth toe; SPV, scales at midbody between the medialmost light stripes.

Phylogenetic analyses

The database used includes a total of 2486 base-pairs.

Table 1. Character variation in all the species of *Contomastix*. Measurements expressed in mm. Values indicate Mean ± SD, except for *C. vittata* (Mean ± SE), and range in parentheses. Data for *C. vittata* extracted from Boulenger (1902), Vance (1978), and Harvey et al. (2012); tongue morphology of *C. leachei* according Cei & Scrocchi (1991).

	***Contomastix celata* new sp.**	***C. charrua***	***C. lacertoides***	***C. leachei***	***C. serrana***	***C. vacariensis***	***C. vittata***
n (sex)	20 (M), 9 (F)	3 (M), 6 (F)	23 (M), 13 (F)	2 (M)	5 (M), 5 (F)	4 (M), 3 (F)	9
SVL (Max.)	70.3	75.2	74.0	66.8	61.4	72.1	76
HL	14.2 ± 1.3 (11.4-16.3)	15.6 ± 0.4 (13.7-16.9)	14.8 ± 1.2 (12.4-17.3)	15.4 ± 2.9 (12.5-18.2)	13.2 ± 0.2 (12.2-14.4)	15.9 ± 0.4 (14.5-17.6)	12
HD	6.5 ± 0.6 (5.4-7.5)	7.5 ± 0.3 (6.6-8.6)	6.7 ± 0.6 (5.6-8.0)	6.3 ± 0.9 (5.4-7.2)	5.8 ± 0.2 (5.1-6.4)	7.0 ± 0.3 (6.2-8.0)	?
SL	5.0 ± 0.5 (4.2-6.1)	5.2 ± 0.1 (4.5-5.7)	5.0 ± 0.4 (4.0-5.9)	5.3 ± 1.0 (4.3-6.3)	4.5 ± 0.1 (4.3-4.9)	5.5 ± 0.2 (4.9-6.3)	?
AG	29.6 ± 3.8 (22.5-35.5)	33.4 ± 1.5 (27.6-39.2)	30.3 ± 3.2 (21.6-37.5)	27.9 ± 3.2 (24.7-31.1)	28.5 ± 1.1 (23.6-34.1)	32.5 ± 0.8 (29.9-36.3)	?
DS	80.8 ± 3.8 (72-87)	90.2 ± 2.2 (81-98)	86.8 ± 6.0 (79-100)	87.0 ± 2.0 (85-89)	69.7 ± 1.3 (63-75)	95.7 ± 1.2 (89-98)	64
SPV	21.1 ± 1.6 (18-25)	41.3 ± 11.5 (12-94)	24.1 ± 1.9 (20-30)	26.0 ± 0.0 (26)	20.0 ± 0.6 (16-22)	25.0 ± 0.9 (22-29)	?
DAS	179.0 ± 6.9 (164-193)	203.7 ± 1.5 (201-206) M, 216.8 ± 3.1 (208-229) F	204.7 ± 13.6 (175-230)	182.0 ± 4.0 (178-186)	166.0 ± 3.7 (146-177)	218.9 ± 2.8 (212-233)	?
TVS	31.9 ± 1.2 (30-34)	33.4 ± 0.3 (32-35)	32.1 ± 1.0 (31-35)	33.0 ± 0.0 (33)	31.5 ± 0.3 (30-33)	32.4 ± 0.7 (31-36)	32 ± 1 (30-33)
LVS	9.9 ± 0.4 (8-10)	10.0 ± 0.0 (10)	10.0 ± 0.2 (9-10)	10.0 ± 0.0 (10)	8.3 ± 0.2 (8-9)	9.1 ± 0.4 (8-10)	10
SLB	6.8 ± 0.8 (6-8)	7.1 ± 0.2 (6-8)	6.9 ± 0.5 (6-8)	7.5 ± 0.5 (7-8)	6.0 ± 0.0 (6)	6.7 ± 0.2 (6-7)	6-7
ILB	5.6 ± 0.6 (5-7)	5.9 ± 0.3 (5-7)	5.7 ± 0.6 (5-7)	6.0 ± 0.0 (6)	5.2 ± 0.1 (5-6)	5.6 ± 0.2 (5-6)	5-7
CH	2.0 ± 0.5 (0-4)	1.8 ± 0.2 (0-2)	1.8 ± 0.6 (0-2)	2.0 ± 0.0 (2)	2.0 ± 0.0 (2)	1.4 ± 0.4 (0-2)	0-2
SOC	3.7 ± 0.5 (3-4)	3.1 ± 0.1 (3-4)	3.4 ± 0.5 (3-4)	3.5 ± 0.5 (3-4)	3.2 ± 0.1 (3-4)	3.1 ± 0.1 (3-4)	2-4
PAP	5.0 ± 0.0 (5)	5.0 ± 0.0 (5)	5.0 ± 0.0 (5)	5.0 ± 0.0 (5)	5.0 ± 0.0 (5)	5.0 ± 0.0 (5)	5
GF	2.0 ± 0.0 (2)	1.8 ± 0.2 (1-2)	2.0 ± 0.3 (1-3)	2.0 ± 0.0 (2)	2.0 ± 0.0 (2)	2.0 ± 0.0 (2)	?
FP	19.9 ± 1.6 (16-23)	20.2 ± 0.3 (19-22)	19.6 ± 1.3 (16-22)	23.5 ± 1.5 (22-25)	19.7 ± 0.3 (18-21)	19.9 ± 0.5 (18-22)	19-25
FFS	13.1 ± 1.2 (12-16)	14.0 ± 0.2 (13-15)	14.1 ± 1.2 (12-17)	15.5 ± 1.5 (14-17)	14.5 ± 0.2 (14-16)	15.0 ± 0.4 (14-16)	14 ± 1 (13-15)
FTS	22.2 ± 1.2 (20-24)	22.6 ± 0.6 (20-25)	23.6 ± 1.5 (20-26)	27.5 ± 0.5 (27-28)	25.3 ± 0.5 (23-28)	23.7 ± 0.7 (21-27)	25 ± 1 (24-28)
SIL	Present	Present	Present	Present	Present	Present	Present
VLS	Absent	Absent	Absent	Absent	Absent	Absent	Absent
DLS	Broken/ continuous	Continuous (if present)	Mostly continuous	Continuous	Continuous	Spots/dashes	Continuous
LLS	Mostly broken	Continuous (if present)	Broken/ continuous	Continuous	Continuous	Spots/dashes	Continuous
VM	Present	Present	Present	Present	Mostly present	Present	Present
PMT	Notched	Notched	Notched	Notched	Notched	Unknown	Notched

The phylogenetic trees obtained with MP, ML and BI yielded highly similar estimates of phylogenetic relationships among the taxa; in general, the nodes received less support in the MP analyses (Fig. 3). The specimens of *C. lacertoides* from the two regions in Argentina group together with high support values (1.00/97/100 with BI, ML, and MP, respectively). The Argentinian group is the sister clade of that formed by the specimens of *C. lacertoides* from Uruguay and both group together in a cluster with the clade of *C. vacariensis*. *Contomastix serrana* appears to be basal for the genus. The genus *Contomastix* is recovered as monophyletic with a high support values (1.00/100/100) and with none of its specimens grouping with any other teiid genus. *Cnemidophorus* and *Ameivula* are also recovered as monophyletic (0.99/100/84 and 0.98/59/--, respectively).

Regarding the phylogenetic relationships among the genera, three clades can be observed: i) *Contomastix* grouping together with *Aurivela* (0.99/62/--); ii)

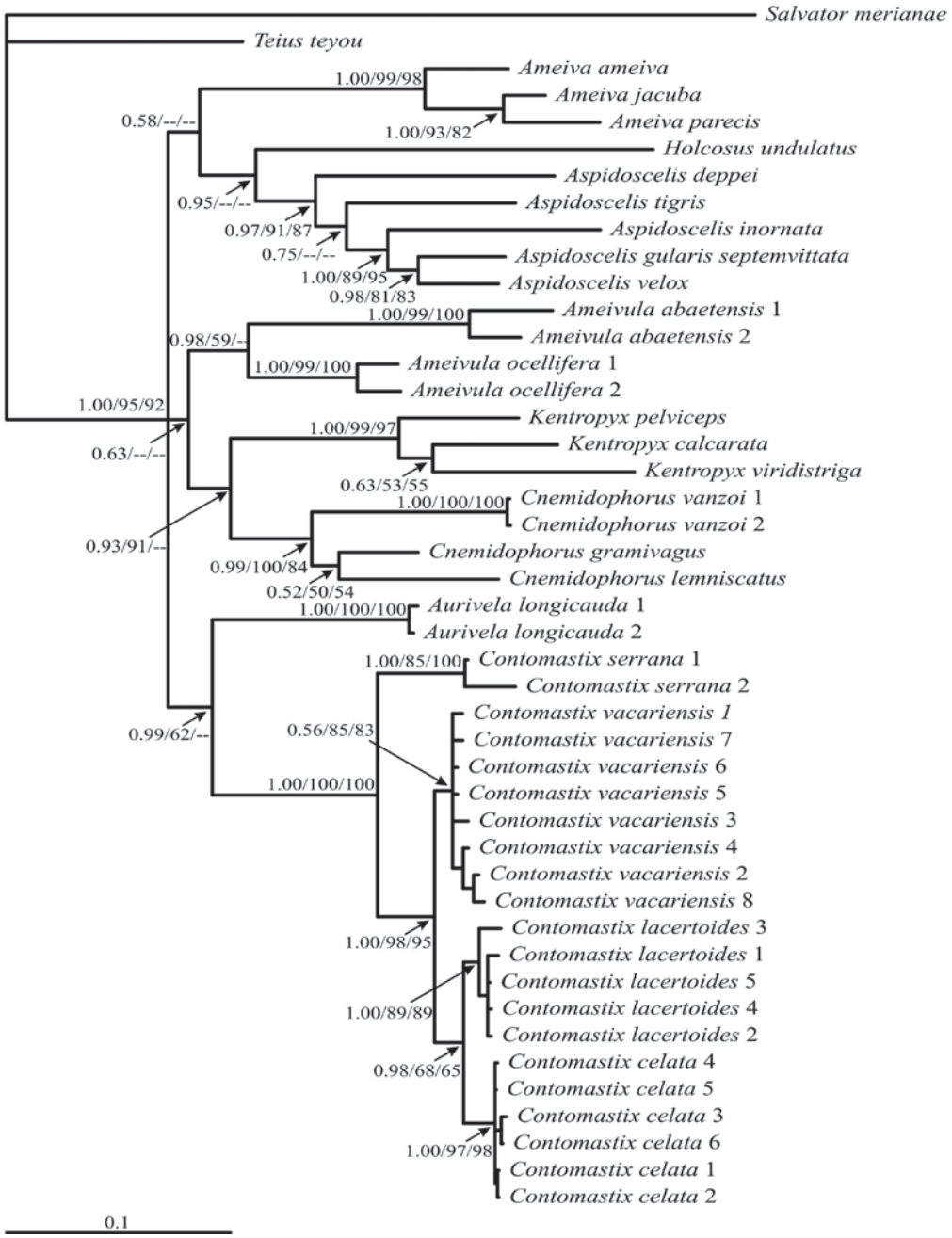

Figure 3. Bayesian phylogram of the 50% majority-rule consensus tree of the subfamily Teiinae based on the 16S, ND4, c-mos and NTF3 data sets. The node supports are: Bayesian posterior probabilities/bootstrap support after 1000 replicates in ML analysis/bootstrap support after 1000 replicates in MP analysis.

Cnemidophorus as the sister taxon of *Kentropyx* (0.93/91/--) and both grouping together with *Ameivula*, although without support (0.63/--/--); iii) *Aspidoscelis* as the sister taxon of *Holcosus* (0.95/--/--) and both grouping together with *Ameiva* with low support (0.58/--/--). *Teius* is recovered basal to these three clades.

The mean K2P genetic distances within clades of *Contomastix* were 0.4%/0.5% for genes 16S and ND4, respectively. Among clades of *Contomastix* the genetic distances were 3.3%/7.7% for genes 16S and ND4, respectively. Such distances range from 1.6%/4% (Uruguayan *C. lacertoides* - Argentinian *C. lacertoides*) to 6.7% /11.8% (*C. serrana*- Argentinian *C. lacertoides*). In general, the genetic distance within each clade was one order of magnitude lower than the distance between them in *Contomastix*. The genetic distances among species within the genera of the subfamily Teiinae were on average 7.9%/16.1% and range, for the gene 16S, from 6.9% for species of *Ameiva* to 9.5% for species of *Kentropyx*. The genetic distances for the gene ND4 range from 15.1% for species of *Kentropyx* to 17.5% for species of *Ameivula*.

Based on the morphological differences among Argentinian and Uruguayan populations, and on the results of the phylogenetic analyses and genetic distances, the Argentinian populations are recognised as a new species and described here.

Contomastix celata new species (Figs 4–7, Table 1)

Holotype. MZUC-C 672, adult male, from Villa La Arcadia (38˚06'50.42" S, 61˚46'22.4" W, 423 m a.s.l.), Partido de Coronel Suárez, Buenos Aires Province, Argentina. 15 September 2012. Collected by D. Di Pietro. The acronym MZUC corresponds to Museo de Zoología, Universidad Nacional de Córdoba, Argentina.

Paratypes. MACN 32867 (adult male) and MLP.S 1049/1050 (adult female and male, respectively) from Sierra de Ventania, Ernesto Tornquist Provincial Park, Partido de Tornquist, Buenos Aires Province, Argentina. 16 March 1985. Collected by J. Cranwell, G. Gnida and J. Soroka. MLP.S 1166 (adult female) from Achiras, Río Cuarto Department, Córdoba Province, Argentina. 19 November 1991. Collected by R. Martori and L. Aun. MZUC-C 563 (adult male) from Achiras, Río Cuarto Department, Córdoba Province, Argentina. 4 November 1990. Collected by L. Avila and A. Pettinichi. MZUC-C 567 (adult female) from Achiras, Río Cuarto Department, Córdoba Province, Argentina. 29 December 1990. Collected by L. Avila. MZUC-C 676 (juvenile) from Piedra del Aguila (33˚09'33.7"S, 64˚59'10.6"W, 828 m a.s.l.), Achiras, Río Cuarto Department, Córdoba Province, Argentina. 11 March 2013. Collected by M. R. Cabrera and R. Torres.

Diagnosis. A small-to-medium sized lizard (70.3 mm maximum SVL), recognisable by the following combination of characters in both sexes: 72–87 granular dorsal scales across midbody; 164–193 dorsal scales along midline; 18–25 scales at midbody between the medialmost light stripes; 10, rarely 8, longitudinal rows of quadrangular ventral scales; 16–23 femoral pores in total; 12–16 subdigital lamellae under fourth finger; 20–24 lamellae under fourth toe; 3–4 supraoculars on each side. *Contomastix celata* can be distinguished phenotypically from *C. leachei* (character states in parenthesis) by having fewer lamellar scales under fourth toe (20–24 vs. 27–30), lateral light stripe broken (continuous), and dorsal ground colour brown (greenish). It is distinguishable from *C. serrana* (character states in parenthesis) in having fewer lamellar scales under fourth toe (20–24 vs. 23–28), more dorsal scales across (72–87 vs. 63–75) and along the body (164–193 vs. 146–177), 10, rarely fewer, longitudinal rows of ventral scales versus generally 8 in *C. serrana*, and dorsolateral and/or lateral light stripes usually broken (both invariably continuous). *Contomastix celata* is distinguishable from the probably extinct *C. charrua* in having a smaller body (SVL up to 70.3 mm vs. 75.2 mm), fewer dorsal scales across midbody (72–87 vs. 81–98) and along the body (164–193 vs. 201–229), and in always having a striped pattern, whereas *C. charrua* is completely unstriped or has two thin light stripes on each side of the body, with feeble to no expression of black bars between them. It is distinguishable from *C. vacariensis* in having fewer dorsal scales across midbody (72–87 vs. 89–98) and along the body (164–193 vs. 212–233), and a different pattern of the light stripes, which in *C. vacariensis* is formed by dots or spots. *Contomastix celata* is distinguishable from *C. vittata* in having a smaller body (SVL up to 70.3 mm vs. 76 mm), more dorsal scales across midbody (72–87 vs. 64), and fewer lamellar scales under the fourth toe (20–24 vs. 24–28), and frequently broken light lateral stripes (continuous in *C. vittata*). *Contomastix celata* most closely resembles *C. lacertoides* in size, body habitus, colour and pattern. It is distinguishable from the latter in having fewer dorsal scales across midbody (72–87 vs. 79–100) and along the body (164–193 vs.175–230), fewer scales at midbody between the medialmost light stripes (18–25 vs. 20–30), and (statistically) fewer subdigital lamellae on both fourth finger and fourth toe.

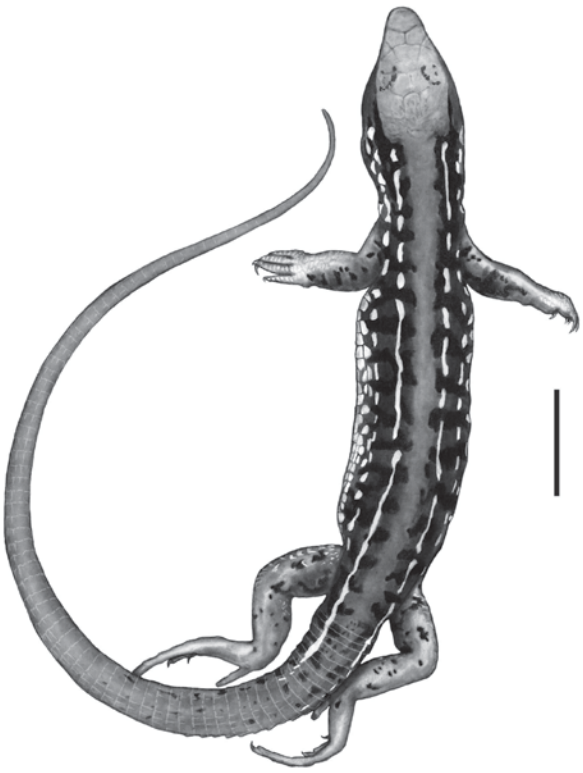

Figure 4. *Contomastix celata* new sp. Body aspect and pattern of the holotype (MZUC-C 672) in dorsal view. Scale bar = 1 cm.

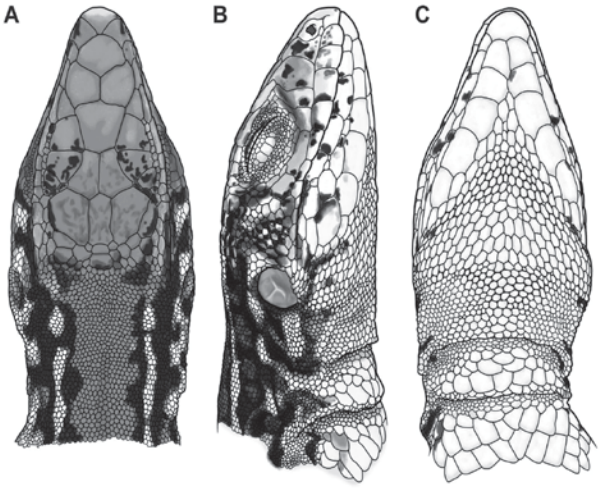

Figure 5. *Contomastix celata* new sp. Head of the holotype in dorsal (A), lateral (B), and ventral (C) view.

Figure 6. Coloration in life of *Contomastix celata* (MZUC-C 677) from Achiras, Córdoba, Argentina.

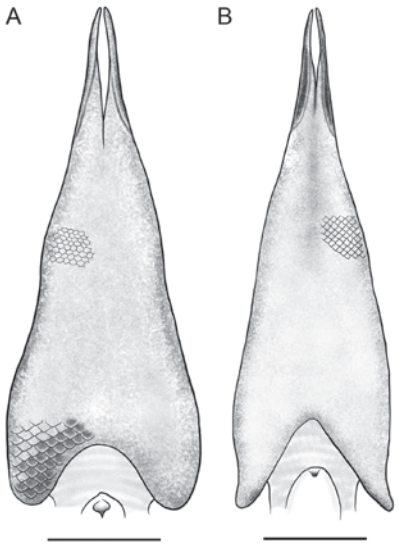

Figure 7. Dorsal view of the tongue and glottis of *Contomastix celata* (MZUC-C 671), detailing juxtaposed papillae anteriorly and imbricate papillae to behind, and its notched proximal end (A); Tongue and glottis of *Ameivula abalosi* (LECOH 00579), with subimbricate papillae covering the whole surface (here partly highlighted) and bilobate proximal end (B), a condition shared with the species of the genus *Aurivela*. Scale bars = 2.5 mm.

Description of holotype. MZUC-C 672, adult male, snout-vent length 62.9 mm, head length 15.4 mm, tail complete. Head triangle-shaped in dorsal aspect, with sides slightly concave. Canthus rostralis blunt but evident, snout length 5.7 mm, 1.5 times longer than eye length. Rostral convex, visible from above and below, partially incised on both sides in front of the anterior nasal scale. Nasals large, paired, with the nostril situated almost entirely in each anterior nasal scale; middorsal contact between anterior nasals prevented by the posterior angle of the rostral. Each anterior nasal contacts rostral, frontonasal, posterior nasals, and supralabial 1 scales. One posterior nasal on each side, slightly convex and smaller than the anterior nasal, contacts anterior nasal, frontonasal, prefrontal, loreal, and supralabials 1–2. Loreal large, concave, single, contacting postnasal, prefrontal, supraocular 1, first superciliary, preocular, and supralabial 3. Frontonasal rhomboidal, wider than long. Two large prefrontals, in broad contact with each other in the midline, each also contacting frontonasal, posterior nasal, loreal, frontal, and supraocular 1. Frontal single, flat, large, subhexagonal with straight borders, narrow behind; its two anteriormost sides contacting prefrontals, its lateral sides contacting supraoculars 1 and 2, and its two posterior sides contacting the anterior side of frontoparietals. Two frontoparietal plates, subpentagonal with external border concave, in broad contact with each other along midline, its lateral sides contacting supraoculars 2, 3 and a small postorbital scale, and its posteriormost sides contacting parietal and interparietal plates. Two broad parietals, rugose, subpentagonal separated by a rugose interparietal on the midline, subpentagonal, two times longer than wide, with lateral sides long and parallel; its anterior and posterior sides, straight and short. The right parietal limited externally by a large, ovate, convex scale. Occipitals polygonal to oval scales, wider than long, behind parietal and interparietal plates, followed by much smaller granular scales on the neck. Supraoculars convex, four on each side, the first contacting loreal, prefrontal, frontal, supraocular 2, and first superciliary scales. Supraoculars 2 to 4 separated from superciliaries by a single row of small granules. Superciliaries 5/5 in a row, the first two longer than the others. Eyelids finely granular, lower eyelid with a group of 3/4 quadrangular scales in its centre, surrounded by granular scales. Suboculars 4/4, large, all contacting supralabials, the first higher than long, in broad contact with loreal and touching the first superciliary. First three suboculars markedly keeled near their upper borders. Supralabials 6/7, with rounded free border, notched at the margin where each scale contacts its neighbours. Temple and cheek with swollen granular to polygonal scales. Ear opening oval nearly round, slightly higher than wide, surrounded by tiny granular scales.

Mental subtriangular, wider than long, followed in the midline by the first pair of chin shields. Postmental

absent. Infralabials 6/6. All, except the first one, longer than high; first infralabial contacting mental and first chin shield, the second contacting first and second chin shields, the third contacting second and third chin shields. The other infralabials separated from the chin shields by a row of sublabials in a single or double row. Five pairs of large, subquadrangular chin shields, the first two pairs in broad contact in the midline. A field of oval scales between chin shields, replaced in the gular region by larger and flatter polygonal scales in the plane between the ears, followed by nine or ten rows of rounded, smaller scales and by a field of large imbricate mesoptychial plates prior to the gular fold. Well defined gular fold, lined by granules. Scales on nape and sides of the neck granular.

Dorsal and flank scales granular, convex, 83 across midbody and 193 in the middorsal line from the nape to the base of tail. At ventral, a field of large polygonal imbricate scales, roughly arrayed in three rows, on upper chest between the insertion of humeri. Posterior to this field a series of ventral plates, smooth, mostly rectangular, wider than long, the external ones in each row having curved lateral sides. Seven scales in the row between axillae, 10 midventrally and 8 in the last transverse row, near groin. One or two scales smaller than ventrals at the extreme of some rows; 31 transverse rows of ventrals on the midventral line. Four large preanal plates, polygonal, preceded and flanked by smaller flat scales. A field of granules posterior to vent. Anal spurs absent.

On forelimbs, suprabrachial and postbrachial scales large, imbricate, in longitudinal rows to elbow, those of the anteriormost row larger and wider than long. Prebrachials rounded, sub-imbricate. Axillary and infrabrachial scales granular, very small. Infra-antebrachial and post-antebrachial scales granular, small, juxtaposed. Two rows of large preantebrachials, imbricate, wider than long, gradually increasing in width towards the hand. Hand pentadactyl, with long, sharp claws. Subdigital lamellae smooth, 14 under left fourth finger, those under first finger followed by a row of three prominent scales proximal to wrist. Palm granular. Two prominent scales forming an outer metacarpal tubercle. Dorsum of manus with rows of imbricate plates wider than long, arrayed along the axis of each digit as supradigital lamellae. A row of granular scales between supradigital and subdigital lamellae, continued on digits 3–5, interrupted on digit 2, and absent on first finger. Suprafemoral and postfemoral scales granular, juxtaposed. Prefemoral and infrafemoral scales large, imbricate, organized in rows, reaching the knee. Eighteen femoral pores in total. Supratibial, pretibial, and post-tibial scales granular and juxtaposed. Infratibial scales large and imbricate, arrayed in three rows. Pes pentadactyl, thin digits with sharp claws shorter than those in hands. Subdigital lamellae smooth, 21 under left fourth toe. Sole granular. Foot dorsum with imbricate supradigital lamellae in rows over each digit.

Scales on tail dorsum quadrangular, longer than wide, keeled, becoming progressively mucronate from tail base to the tip; keels on scales forming continuous carinae. Ventral and lateral tail scales longer than wide, imbricate, becoming progressively keeled distally, but less markedly than in dorsals. Twenty-seven scales around tail on its fifth complete postcloacal whorl.

Coloration and pattern. Dorsal head scales shiny, light brown. Dorsal neck surface and central field along the body dorsum light brown, clearing gradually to greyish light brown on tail. No vertebral stripe. A thin white dorsolateral stripe, unevenly interrupted, along each side of the body, starting on the neck behind superciliaries and continuing to the tail, where it fades out. A lateral white string of dashes and spots along each side of body, starting below and behind the eye, touching upper border of the ear and running along flank, parallel to the dorsolateral stripe. The lateral white markings extend on to the thigh anterodorsally and posterodorsally and continue on the lateral side of the body up to the tail. It becomes indiscernible on the tail because it is located at the limit of the greyish brown dorsum and the white venter of tail. A white ventrolateral string of dots starts below the tympanum and runs along flank almost parallel to the lateral white string of markings. Both lateral and ventrolateral traces are generally broader than the dorsolateral stripe. Colour of flanks between dorsolateral stripe and lateral strings, brown, with a series of bold black bars, some of them bifurcated as inverted "Vs" or "Ys". A series of bold black indentations above the white dorsolateral stripe, fading on tail. Dorsum of arms and legs brown, with irregular black marks and occasional lighter blotches. Supradigital lamellae of hand and foot ivory. Dorsum and upper sides of tail light greyish brown, with feeble dark marks. Sides of head brown, lighter than dorsum. Supra- and infralabials white, with black marks on the margins of most scales. Ventral surfaces of head, neck, body, limbs and tail pearly white, with black marks on the two or three external rows of ventral scales.

Variation. The white dorsolateral stripe is continuous in some individuals, while in others it is fragmented as long dashes all along its extension (Fig. 6). In the former the lateral white trace (i.e., the second in dorsum-to-venter order) is neither continuous nor a string of dots but a stripe with few sparse breaks. The ventrolateral white trace is never continuous. There is no difference between male and female adult colour patterns. Juveniles of both sexes have the lateral and ventrolateral light stripes yellowish instead of white, and the field between them is darker than in adults. No appreciable changes in pattern or colour in preservative (70% ethanol).

Geographic distribution. *Contomastix celata* is endemic to Argentina and is associated to rocky grassland habitats in two mountain systems: the south of Sierras de Córdoba in the centre of the country, and Sierras de Ventania in Buenos Aires province (Fig. 1). Both mountain systems are separated by 560 km of vast plains (Pampas landscape). These lowlands seem unsuitable for the species, according to its saxicolous habits and the lack of reliable records of its presence there. The nearest population of the sister species *C. lacertoides* (in San José Department, Uruguay) is approximately 600 km distant from the *C. celata* populations in the Sierra de Ventania

system. The basal species, *C. serrana*, is geographically the closest to *C. celata*, but they are not sympatric.

Etymology. The specific epithet is an adjective derived from the Latin word celatus (celata in feminine form), meaning hidden or concealed, in reference to the phenotypical similarity to *Contomastix lacertoides*, under whose name these populations have been hitherto masquerading.

Cytogenetics. The karyotype of the population of *C. celata* in Sierras de Córdoba is 2n = 52 with 26 macrochromosomes (12 pairs telocentric, 1 pair submetacentric) and 26 microchromosomes (Delia Aiassa, pers. comm.). It is different from the karyotype of *C. lacertoides* from Uruguay described by Cole et al. (1979), which is 2n = 50 with 26 macrochromosomes (12 pairs telocentric or essentially so, and one pair of submetacentric) and 24 microchromosomes. The karyotype of the populations of *C. celata* from Sierras de Ventania remains unknown.

Phylogenetic relationships of the new species. In the morphological analysis, meristic characters easily distinguish *C. celata* from *C. lacertoides* sensu stricto and from the other species of the *Contomastix* genus. However, *C. celata* and *C. lacertoides* show greater similarity between them, suggesting that these two lineages are sister species, which is evidenced in the phylogenetic reconstruction (Fig. 3). The tree shows two well supported clades, one including the sequences of specimens from Argentina (now *C. celata*) and another comprising the sequences of individuals from Uruguay (*C. lacertoides* sensu stricto).

Conservation status. In the more recent evaluation of conservation status for the Argentinian species of lizards and amphisbaenids (Abdala et al., 2012) the populations of *C. lacertoides* in Argentina (now *C. celata*) were listed as Vulnerable, based mainly on anthropogenic effects on the areas where this species distributes in the country. Now, having recognised those populations as a new species with a restrictive range, the status of *C. celata* should be carefully analysed in the next re-evaluation.

DISCUSSION

Harvey et al. (2012) transferred the content of the *C. lacertoides* species group as well as *Cnemidophorus vittatus* to a new genus, *Contomastix*. However, they did not identify any unique characters to distinguish *Contomastix* from all other teiid lizards. They did not consider tongue morphology in their phylogenetic reconstruction, although this character defines the *C. lacertoides* species group, according to Cei (1993:371). We examined the lingual morphology in *C. celata* (Fig. 7) and compared it to the tongues of *C. charrua*, *C. lacertoides* sensu stricto and *C. serrana* (Cabrera & Carreira, unpubl. data), *C. leachei* (fide Cei & Scrocchi, 1991), and *C. vittata* (fide Harvey et al., 2012). We confirm that at least six of the seven species of the genus *Contomastix* present a notched proximal margin of the tongue and lack of lingual sheath around its base. As for *C. vacariensis*, there is no published information about its tongue morphology, and specimens for dissection were unavailable to us. Regarding *C. vittata* there is some disagreement among different authors. This species was originally described by Boulenger (1902) as *Cnemidophorus vittatus*, although he did not mention the tongue morphology in his description. Later, Vance (1978) redescribed the holotype and indicated that it shows a lingual sheath between the tongue and the larynx, and therefore he transferred it to the genus *Ameiva*. Harvey et al. (2012) confirmed that *Contomastix vittata* has the same tongue morphology as *C. lacertoides* and *C. leachei*, as originally stated by Cei & Scrocchi (1991), i.e., lingual sheath absent and notched proximal margin of the tongue instead of bilobate as it is seen in *Ameivula*, *Aurivela* and *Cnemidophorus* (Fig. 7). We verified that the tongues of both *Ameivula* and *Aurivela* have the proximal end bilobate, and scale-like papillae subimbricate. The papillae on the tongue of *C. celata* are juxtaposed anteriorly but progressively imbricate towards posterior. *Ameiva ameiva* (MZUC-C 470, not pictured) markedly differs from these two types by bearing clearly imbricate papillae all along the dorsal surface of its elongate tongue, and by the presence of lingual sheath. Based on this information, we consider the presence of a notched proximal margin of the tongue a synapomorphy of the genus *Contomastix*, which distinguishes it from all the other teiid genera.

In our phylogenetic analysis *Contomastix* appears as monophyletic, in agreement with the results of Tucker et al. (2016). However, we recovered the clade of *C. lacertoides* - *C. celata* as the sister taxa of *C. vacariensis*, with *C. serrana* in a basal position in the genus. Tucker et al. (2016) recovered *C. serrana* as the sister taxon of *C. lacertoides* (although considering the provenance of its specimen this would correspond to *C. celata*) and *C. vacariensis* as the basal species for the genus. New phylogenetic analyses including all the lineages of the genus will help to understand their relationships. Feltrim & Lema (2000) indicated that *C. vacariensis* is morphologically similar to *C. lacertoides*, which is in accordance with our phylogenetic reconstruction. This does not agree with Harvey et al. (2012) findings, who recovered *Contomastix* as paraphyletic, placing *C. lacertoides* outside the clade containing *C. serrana* and *C. vittata*. They mention having analysed two more species of *Contomastix* (*C. leachei* and *C. charrua*), but they did not include them in their phylogenetic analysis. Harvey et al. (2012) indicated that *C. lacertoides* differs considerably from its congeners in scutellation, hemipenis morphology and coloration. This discrepancy could have originated because they grouped as *C. lacertoides* specimens that correspond, according to their provenance, to *C. lacertoides* sensu stricto (Uruguay: Maldonado Department, Sierra de Animas), to *C. lacertoides* sensu lato (Brazil: Rio Grande do Sul State, Osório), and to the new species described here as *C. celata* (Argentina: Córdoba Province, Achiras).

The relationship between *Contomastix* and *Aurivela* was not recovered in previous molecular reconstructions

(Reeder et al., 2002; Giugliano et al., 2013; Goicoechea et al., 2016) in which *Aurivela* appears as the sister taxon of *Aspidoscelis*. This could be due to the fact that only one specimen of *C. lacertoides* was included in those analyses. Tucker et al. (2016) in their study included *C. lacertoides*, *C. serrana* and *C. vacariensis*; *Aurivela* appears basal to a clade including *Contomastix*, *Ameivula* and *Glaucomastix*. Phylogenetic reconstructions grouping cis-Andean South American distribution clades imply a more coherent biogeographic hypothesis than those grouping these clades with a Central and North American-distribution clade, *Aspidoscelis*.

The conservation status of the species of *Contomastix* is important because their distributions are relatively restricted, most being localized endemics and rare. According to the criteria used by the International Union for Conservation of Nature (IUCN, 2018), four of its species are categorised: *C. charrua* (EX = Extinct), *C. vittata* (CR = Critically Endangered), *C. vacariensis* (DD = Data Deficient) and *C. serrana* (LC = Least Concern). Categorisations for *C. lacertoides* and *C. leachei* remain unpublished.

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APPENDIX 1

Specimens examined are referred to by their catalogue number except otherwise indicated. Acronyms: CH-UNSL, Colección Herpetológica de la Universidad Nacional de San Luis, Argentina; LECOH, Laboratorio de Ecología y Conservación de la Herpetofauna, Instituto de Diversidad y Ecología Animal (IDEA-UNC), Córdoba, Argentina; MACN and MACN (exCENAI), Museo Argentino de Ciencias Naturales, Buenos Aires, Argentina; MLP.S, Museo de La Plata, La Plata, Argentina; MNHN, Museo Nacional de Historia Natural, Montevideo, Uruguay; MZUC-C, Museo de Zoología, Universidad Nacional de Córdoba, Argentina; PQDN, Proyecto Quebradas del Norte, Facultad de Ciencias, Universidad de la República, Uruguay; SC, Field collection of Santiago Carreira; UFRGS, Laboratório de Herpetologia, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Brazil; ZVC-R, Colección Zoología de Vertebrados, Facultad de Ciencias, Universidad de la República, Uruguay.

Ameiva ameiva
ARGENTINA. El Chaco Province: Comandante Fernández Department: Presidencia Roque Sáenz Peña, MZUC-C 470.
Ameivula abalosi
ARGENTINA. Córdoba Province: Tulumba Department: About 10 km W from Lucio V. Mansilla, LECOH 00579.
Aurivela longicauda
ARGENTINA. San Juan Province: Caucete Department: Médanos Grandes, MZUC-C 446.
Contomastix celata new sp.
ARGENTINA. Buenos Aires Province: Partido de Coronel Suárez: Villa La Arcadia, MZUC-C 671, 672 (holotype), 673; Partido de Tornquist: Abra de la Ventana, MACN 20862. Sierra de Ventania, MACN 32199, 32883, 32997, MACN (exCENAI) 336, 339, MLP.S 967. Sierra de Ventania, Ernesto Tornquist Provincial Park, MACN 32864, 32867 (paratype), 32868, 32874/76, 32878, MLP.S 1049 (paratype), 1050 (paratype), 1051, 1564/65. Sierra de Ventania, Villa Ventana, MLP.S 1052/54. Córdoba Province: Río Cuarto Department: Achiras, MZUC-C 559/62, 563 (paratype), 564/66, 567 (paratype), 568, 676 (paratype), 677/78, MLP.S 1165, 1166 (paratype).
Contomastix charrua
URUGUAY. Rocha Department: Cabo Polonio, MNHN 03423 (holotype), 03422 and 03424 (paratypes); ZVC-R 1856 and 1865 (paratypes), 2505/06, 2519/20.
Contomastix lacertoides
URUGUAY. Artigas Department: Nacientes del Arroyo Pintado, ZVC-R 4835/36. Lavalleja Department: Asperezas de Polanco, ZVC-R 5042/43; Environs of Lascano, SC-406; Predio Papazián, near Mariscala, MNHN 9744, SC-413; Route 8, Km 131 Establecimiento "El Penitente", ZVC-R 5350. Maldonado Department: Cerro de Animas, MLP.S 965; Route 60, ZVC-R 5304; Sierra de Animas, ZVC-R 3891, 4358/59. Montevideo Department: Cerro de Montevideo, ZVC-R 1265/66. Paysandú Department: Route 90, Establecimiento "El Refugio", ZVC-R 4889; Route 26, Km 147, between Arroyo Laureles and Arroyo Perdido, ZVC-R 5361. Rivera Department: Gajo Arroyo Lunarejo, ZVC-R 5119; Puntas del Arroyo Lunarejo, ZVC-R 4518/19. Rocha Department: Castillos, MACN 1126/28; San Miguel National Park, ZVC-R 1810. San José Department: Sierra de Mahoma, ZVC-R 5566. Tacuarembó Department: Road to Valle Edén, ZVC-R 5306; Pozo Hondo, ZVC-R 5139, 5413; Pozo Hondo, Route 26, Km 200, ZVC-R 5233; Valle Edén, ZVC-R 4504. Treinta y Tres Department: Cuenca del Arroyo Avería, 20 km E Valentines, PQDN 370; Quebrada de los Cuervos, ZVC-R 1348, 1351, 1353, 1355, 1382, 4569/70, 4578, 4751; Santa Clara de Olimar, ZVC-R 1263.
Contomastix leachei
ARGENTINA. Salta Province: Orán Department: Río Pescado and Serranía Las Pavas, southwestern end of Baritú National Park, MACN 32299. Rosario de la Frontera Department: Rosario de la Frontera, MLP.S 1064.
Contomastix serrana
ARGENTINA. Córdoba Province: Colón Department: Cabana, MACN 12509; MLP.S 1055. Punilla Department: Road to Pampa de Olaen, MZUC-C 243; Carlos Paz, MZUC-C 572/73; Carlos Paz, Estancia Vieja, MLP.S 1164; Cosquín, MACN 36176; Los Chorrillos, MZUC-C 571, 574, MLP.S 1163; Tanti, MZUC-C 569/70; Valle Hermoso, CH-UNSL 0558. Santa María Department: Alta Gracia, MLP.S 1066, 1305. San Luis Province: Ayacucho Department: Río Nogolí, CH-UNSL 0457.
Contomastix vacariensis
BRAZIL. Rio Grande do Sul State: Bom Jesus, UFRGS 4564, 4780, 4783; Jaquirana, UFRGS 5273; Vacaria, UFRGS 4723/24. Santa Catarina State: Capão Alto, UFRGS 4843.

APPENDIX 2

Locus, primer sequence, source, aligned fragment length (FL), and models of sequence evolution selected for the four loci used in this study.

Locus	*Primer sequence*	Source	FL	Selected model
16S	F: CGCCTGTTTATCAAAAACAT R: CCGGTCTGAACTCAGATCACGTA	Gifford et al. (2004)	548	GTR+I+G
ND4	F: TGACTACCAAAAGCTCATGTAGAAGC R: TACTTTTACTTGGATTTGCACCA	Forstner et al. (1995)	889	HKY+G
NTF3	F: ATGTCCATCTTGTTTTATGTGATATTT R: ACRAGTTTRTTGTTYTCTGAAGTC	Townsend et al. (2008)	653	HKY+I+G
c-mos	F: GCGGTAAAGCAGGTGAAGAAA R: TGAGCATCCAAAGTCTCCAATC R*: AGRGTGATRWCAAANGARTARATGTC	Saint et al. (1998)	396	HKY+G

* Used only for *Teius teyou*

APPENDIX 3

List of species, identification codes of specimens analysed and GenBank accession numbers for the four loci used in this study.

Species	Specimen voucher	16S	ND4	c-mos	NTF3
Ameiva ameiva		AY359493	AF151206	KC109625	---
Ameiva jacuba	CHUNB 47996	JQ762444	---	KC109626	---
Ameiva parecis	CHUNB 11655	JQ762442	---	KC109632	---
Ameivula abaetensis 1	MZUSP 104240	KF957470	KF957534	---	KF957566
Ameivula abaetensis 2	MZUSP 104250	KF957485	KF957549	---	KF957581
Ameivula ocellifera 1		AF420759	AF420914	AF420862	---
Ameivula ocellifera 2		AY217992	AF151205	AY217890	---
Aspidoscelis deppei		AY046473	KF555555	---	---
Aspidoscelis gularis septem-vittata		AY046485	AF026179	---	---
Aspidoscelis inornata		AY046478	AF026174	---	---
Aspidoscelis tigris		AY046494	AF026172	AF039481	EU390903
Aspidoscelis velox		KC621326	KC621494	EU116675	EU108017
Aurivela longicauda 1	CH-UNSL 0561	KY020123	MF039743	MF039730	KY020108
Aurivela longicauda 2		AY046481	---	KC109630	---
Cnemidophorus gramivagus		AY046474	---	KC109627	---
Cnemidophorus lemniscatus		AY046480	AF026171	KC109629	---
Cnemidophorus vanzoi 1	Co140	DQ168986	DQ168990	---	---
Cnemidophorus vanzoi 2	Co141	DQ168987	DQ168991	---	---
Contomastix celata 1	MZUC-C 671	KY020117	MF039738	MF039724	KY020102
Contomastix celata 2	MZUC-C 672	KY020118	MF039739	MF039725	KY020103
Contomastix celata 3	MZUC-C 673	KY020119	---	MF039726	KY020104
Contomastix celata 4	MZUC-C 676	KY020120	MF039740	MF039727	KY020105
Contomastix celata 5	MZUC-C 677	KY020121	MF039741	MF039728	KY020106
Contomastix celata 6	MZUC-C 678	KY020122	MF039742	MF039729	KY020107
Contomastix lacertoides 1	AMNH R-115938	AY046479	---	---	---
Contomastix lacertoides 2	PQDN 370	KY020113	MF039734	MF039720	KY020098
Contomastix lacertoides 3	SC 406	KY020114	MF039735	MF039721	KY020099
Contomastix lacertoides 4	SC 413	KY020115	MF039736	MF039722	KY020100
Contomastix lacertoides 5	MNHN 9744	KY020116	MF039737	MF039723	KY020101
Contomastix serrana 1	CH-UNSL 0457	KY020111	MF039732	MF039718	KY020096
Contomastix serrana 2	CH-UNSL 0558	KY020112	MF039733	MF039719	KY020097
Contomastix vacariensis 1	UFRGST 124	KY933592	---	---	---
Contomastix vacariensis 2	UFRGST 130	KY933593	---	---	---
Contomastix vacariensis 3	UFRGST 132	KY933594	---	---	---
Contomastix vacariensis 4	UFRGST 134	KY933595	---	---	---
Contomastix vacariensis 5	UFRGST 144	KY933596	---	---	---
Contomastix vacariensis 6	UFRGST 156	KY933597	---	---	---
Contomastix vacariensis 7	UFRGST 158	KY933598	---	---	---
Contomastix vacariensis 8	UFRGST 191	KY933599	---	---	---
Holcosus undulatus		HM012699	---	JN090144	---
Kentropyx calcarata		AF420760	AF420913	AF420864	---
Kentropyx pelviceps		AY046501	---	KC109633	---
Kentropyx viridistriga		EU345182	AF151207	---	---
Teius teyou	CH-UNSL 0478	KY020110	MF039731	MF039717	KY020095
Salvator merianae	M303	KY020109	KF034085	MF039716	KY020094

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Population genetic structure of the endangered yellow spotted mountain newt (*Neurergus derjugini*: Amphibia, Caudata) inferred from mitochondrial DNA sequences

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The yellow spotted mountain newt (*Neurergus derjugini*) is a critically endangered species restricted to fragmented habitats in highland streams of the middle Zagros Mountain in Iran and Iraq. We examined the species phylogeography by investigating sequences of a mitochondrial fragment of the ND2 gene for 77 individuals from 15 locations throughout the species known distribution. We found relatively high haplotype diversity (0.82 ± 0.025) but low nucleotide diversity (0.0038 ± 0.00022) across all populations. Phylogenetic trees supported monophyly, and the segregation of haplotypes was concordant with the haplotype network. We found a significant correlation between geographical and genetic distances among populations ($r = 0.54$, $P < 0.01$), suggesting restricted gene flow. Molecular dating suggested that haplogroups diverged during the early or middle Pleistocene. Bayesian skyline plot provided evidence for an expansion of populations during the Pleistocene-Holocene transition period. Taken together, isolation by distance due to low dispersal capability, habitat fragmentation, and historical factors have shaped the current population structure of *N. derjugini*.

Key words: phylogeography, endangered species, demography, evolutionary history, climate oscillation, conservation.

INTRODUCTION

Environmental and geographic heterogeneity are significant factors contributing to spatial genetic diversity (Manel et al., 2003; Palo et al., 2003). In addition, genetic differentiation among populations can be interpreted as the result of historical evolutionary processes such as genetic drift, founder effects, and acclimatisation to past ecological conditions including climatic oscillations during the Pleistocene glacial cycles (Hewitt, 2000; Weese et al., 2012). Physical and geographical barriers that separate populations may reduce population connectivity and gene flow, and, as a result, populations diverge due to natural selection and random genetic drift (Zhang et al., 2016). Isolation-by-distance (IBD), the correlation of genetic divergence and geographic distances, is further inversely liked to effective population size (Sexton et al., 2014). Population divergence can also occur in different environments with evolving reproductive isolation due to ecologically-based divergent selection (Dyer et al., 2010; Freeland et al., 2010; Wang et al., 2013), a process termed isolation by ecology or isolation by environment (IBE; Zellmer et al., 2012; Shafer & Wolf, 2013).

Population genetic divergence originating from geographical or environmental factors can be demonstrated through correlations of genetic distance measures with geographical or environmental distances (Wang et al., 2013). Assessing casual relationships between environmental and geographic factors and the genetic structure of populations is difficult (Balkenhol et al., 2009) because the interactions among various factors cannot always be detected by isolation-by-distance alone (Kittlein & Gaggiotti, 2008). An integration of genetic and environmental data has been employed for many different goals, including the exploration of population genetic structure (Mota-Vargas & Rojas-Soto, 2012), selecting re-introduction sites (Martinez-Meyer et al., 2006), mapping the habitat of threatened species (Chunco et al., 2013), and designing appropriate management plans and conservation strategies (Gebremedhin et al., 2009). However, the ecological and geographical data which are necessary for devising the species conservation action plan are as yet lacking for many species (Farasat et al., 2016).

Neurergus derjugini (Nesterov, 1916) is a urodele species confined to living in highland streams of the mid-Zagros Mountains (630 and 2,057 masl), and a distribution range covering western Iran and parts of eastern Iraq. Inhabited streams are surrounded by open oak forest and other plants such as amygdales scrublands, deciduous dwarf-scrublands, and cushion shrub land. In the northern part of its distribution, *N. derjugini* can live in streams without natural vegetation cover, including flooding meadows, agricultural lands, rangelands and orchards (Afroosheh et al., 2016). However, drought, the collection of *N. derjugini* for the national and international pet trade, and habitat degradation are threats for this

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species. Fragmented habitats, the diminished number of subpopulations, a small area (< 10 km^2) for occupancy and a continuing decline in the range and quality of habitats are factors for listing *N. derjugini* as a Critically Endangered species by the International Union for Conservation of Nature (IUCN; Red List criteria: A3cde+4cde; B2ab [iii, iv, v] ver. 3.1) (Sharifi et al., 2009).

Here, we test the current genetic and geographical structure among populations of *N. derjugini* throughout the known distribution range in Iran and Iraq, based on partial sequences of the mtDNA ND2 genes, 1) to identify associations between genetic diversity and ecological-geographical differences, 2) to identify links between population genetics and climatic oscillations in the Quaternary and 3) to estimate levels of genetic variation within and among different populations of *N. derjugini*, in order to provide management plans for future conservation.

METHODS

Population sampling and sequencing

Population sampling was conducted for 15 populations throughout the range of species distribution in Iran and Iraq, via 22 sampling occasions during 2012 - 2014 (Table 1, Fig. 1a). Tissue samples were obtained from 77 individuals by removing a small section of the tail tip or toe using sterilised scissors. All tissue samples were stored in 95 % ethanol immediately after removal and then frozen at -20˚ C until processing. Genomic DNA was extracted using the GenNetBioTM tissue kit (Seoul, South Korea) following the manufacturer's instructions. A mitochondrial fragment of ND2 gene (1036pb) was amplified and sequenced using primer L3780, 5' TCG AAC CTA CCC TGA GGA GAT and H5018, 5' TCT GGG TTG CAT TCA GAA GA (Babik et al., 2005). PCR conditions used for this region consisted of an initial denaturation step at 94˚ C for 3 minutes, followed by 35 cycles of 30s denaturation at 94˚ C, 30 s annealing at 58˚ C and 60 s extension at 72˚ C, and a final extension step of 4 min at 72˚ C. Sequencing was performed by Macrogen Korea Laboratories. All sequences of partial mitochondrial ND2 have been deposited in the GenBank databases (accession numbers MK035716- MK035726 for Haplotypes 1-11).

Nucleotide polymorphisms

DNA sequences were aligned using Clustal W in the BioEdit v.7.0.5.3 (Hall, 1999) and by Muscle in MEGA 6 (Tamura et al., 2013). Five closely related taxa, *N. crocatus*, *N. kaiseri*, *N. strauchii*, *Triturus karelinii*, and *Ommatotriton vittatus* were used as outgroups, using existing GenBank under accession numbers (DQ517788, DQ517789, DQ517790, DQ517837 and, DQ517844). As an additional outgroup, ND2 of *N. kaiseri* was sequenced in the present study as described above. The number of haplotypes, polymorphic sites, parsimony informative sites, haplotype diversity (Hd) and nucleotide diversity (pi) were determined using DnaSP v 5.10.01 (Rozas et al., 2010) and Arlequin v 3.1 (Excoffier et al., 2005). Pairwise sequence divergence between haplotypes was calculated using the Kimura 2-parameter (K2P) model (Kimura, 1980) using MEGA 6 (Tamura et al., 2013), with standard errors calculated for 1000 bootstrap replicates.

Phylogenetic analyses

Phylogenetic relationships among haplotypes were determined by Bayesian analysis in MrBayes v3.2.2 (Ronquist et al., 2012) with 10,000,000 generations, sampling each 1000th generation, and Maximum likelihood (ML) analyses conducted in PhyML, v 3.0 (Guindon et al., 2010) with 1500 bootstrap replicates. jModelTest v 0.1.1 (Posada, 2008) was used to determine the best-fit substitution model for BI and ML analysis using the with Akaike information Criterion (AIK), and GTR+I

Table 1. List of sampling locations used in this study and haplotypes with genetic diversities and frequencies. SS: sample sizes, H: haplotypes and *Pi*: nucleotide diversity and *Hd*: haplotype diversity.

	Locality	Latitude	Longitude	Elev. (m)	Haplotypes and their frequencies	SS	H	*Pi*	*Hd*
1	Kavat	34º 52' N	46º 30' E	1601	Hap1(5), Hap2(1)	6	2	0.00032	0.333
2	Ghori ghale	34º 52' N	46º 29' E	1600	Hap1(5)	5	1	0.00000	0.000
3	Gholani	34º 54' N	46º 27' E	1575	Hap1(5)	5	1	0.00000	0.000
4	Dourisan	35º 01' N	46º 23' E	1600	Hap1(5)	5	1	0.00000	0.000
5	Darrenajjar	35º 05' N	46º 18' E	1472	Hap1(5)	5	1	0.00000	0.000
6	Lashkargah	35º 00' N	46º 08' E	1415	Hap3(1), Hap4(4)	5	2	0.00039	0.400
7	Nowdeshe	35º 11' N	46º 14' E	1760	Hap5(3), Hap6(2)	5	2	0.00174	0.600
8	Hani garmale	35º 14' N	46º 08' E	1383	Hap6(4)	4	1	0.00000	0.000
9	Tawale	35º 11' N	46º 11' E	1400	Hap6(4)	4	1	0.00000	0.000
10	Balkha	35º 12' N	46º 09' E	1482	Hap6(4)	4	1	0.00000	0.000
11	Penjwin	35º 36' N	45º 58' E	1421	Hap7(4), Hap8(1)	5	2	0.00039	0.40
12	Siya gwez	35º 47' N	45º 47' E	1689	Hap7(6)	6	1	0.00000	0.000
13	Shalmash	36º 05' N	45º 29' E	1622	Hap9(5), Hap10(1)	6	2	0.00032	0.333
14	Saqez	36º 03' N	46º 02' E	2168	Hap9(6)	6	1	0.00000	0.000
15	Benjun	36º 32' N	45º 31' E	2152	Hap11(6)	6	1	0.00000	0.000
	Total					77	1	0.00389	0.82399

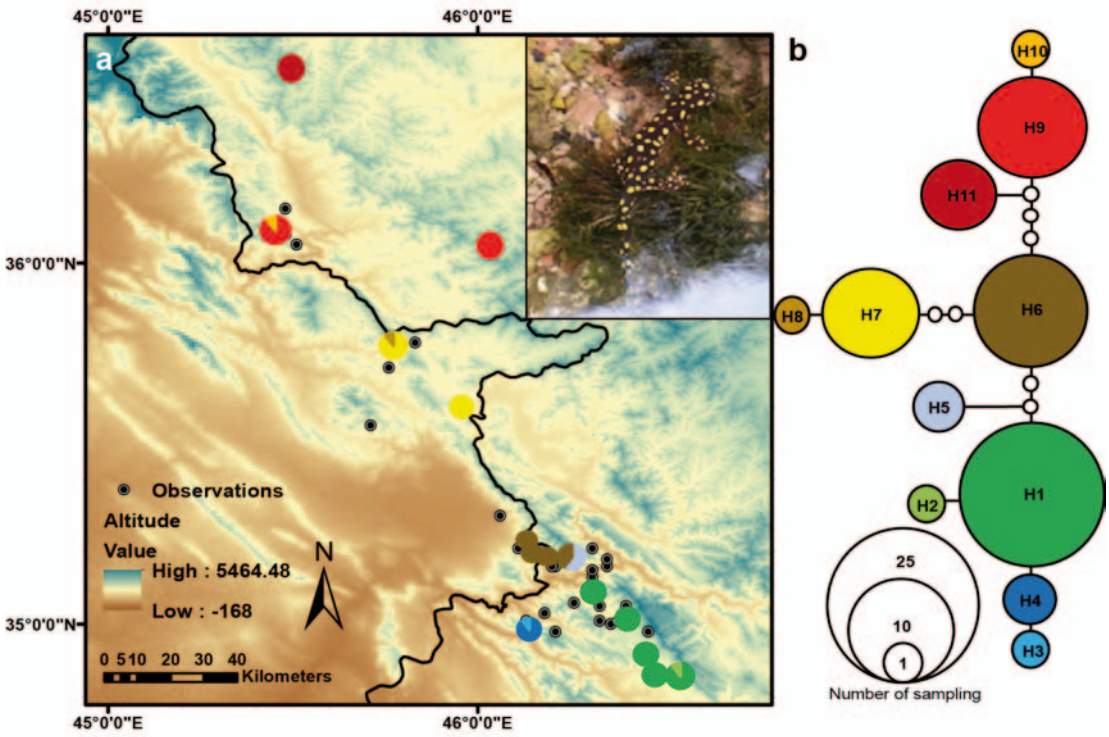

Figure 1. (a) Map illustrating the geographic distribution of *N. derjugini* and the 15 sampling localities in the study area (numbers show localities as indicated in Table 1) in Iran and Iraq; pies represent the haplotype frequency in each population that their colours are in accordance with haplotypes (H1-H11) in the haplotype network. **(b)** Haplotype network showing the phylogenetic relationships among the 11 haplotypes. Different haplotypes in the haplotype network have different colours. Sizes of circles are representative of the haplotype frequencies. Open dots represent missing intermediate haplotypes.

substitution models supported by our data. A consensus tree with posterior probabilities was generated using FigTree v1.4.0 (Rambaut, 2012). Furthermore, we applied a nested clade phylogeographic analysis (NCPA) using TCS v 1.21 for phylogeographic interpretation of relationships among haplotypes, (with a 95% parsimony connection limit, Clement et al., 2000).

Population analysis

Analysis of molecular variance (AMOVA) was conducted on (1) populations from three geographical regions as southern (populations 1-7), central (populations 9-12) and northern (populations 13-15), where selection of population groups was based on the haplotype groups designated in the phylogeny trees, and (2) all populations as one group to determine the level of genetic differentiation within and among *N. derjugini* populations, using Arlequin v 3.1 (Excoffier et al., 2005) with 10,000 permutations. Arlequin 3.1 (Excoffier et al., 2005) was also used to measure pairwise FST between populations.

Isolation by geographical and environmental distance

Mantel tests were used to evaluate the connection between geographical and environmental distances with genetic distances using Arlequin 3.1 (Excoffier et al., 2005). This analysis was performed based on a matrix of pairwise FST and a matrix of geographical distances as well as environmental distances with 10000 random permutations. We measured geographic distances between populations using DIVA-GIS v 7.5.0 (Hijmans et al., 2012). We used eight climate and land cover variables for our analysis that had previously been evaluated by Sharifi et al. (2017). These variables included precipitation of warmest quarter, precipitation of coldest quarter, temperature seasonality (standard deviation×100), isothermally (BIO2/BIO7×100), temperature annual range (BIO5–BIO6), mean temperature of driest quarter, mean temperature of wettest quarter and elevation. Environmental variables were processed in ArcMap 10.3 software and data matrices were analysed using SPSS version 16.0. In addition, a three-way Mantel test was performed between matrices of pairwise genetic distances and environmental distances, adding the matrix of geographical distances among populations.

Demographic analysis

Past population dynamics of *N. derjugini* was estimated with a Bayesian skyline plot (BSP) using BEAST, v 2.4.5 (Bouckaert et al., 2014). This analysis was carried out with the uncorrelated lognormal relaxed clock and the Bayesian skyline as a coalescent model with the mutation rate of 0.64% Myr (Weisrock et al., 2001). We ran the MCMC procedure with 100,000,000 generations, and the genealogy and parameters of the model were stored every 10,000 iterations. We used Tracer v 1.6 (Rambaut et al., 2014) to assess the effective population size through time.

Divergence time estimate

Four estimates of divergence between lineages of *N. derjugini* were obtained using BEAST v 2.4.5 (Bouckaert

et al., 2014). In all calibrations, we used a Bayesian Markov Chain Monte Carlo (MCMC) approach with the uncorrelated lognormal relaxed clock and the constant size as a coalescent model. The substitution model for each partition was obtained by Partition Finder v 2.1.1 (Lanfear et al., 2016). Runs were carried out based on 100 million generations, sampled every 1000 generations with the first 10% discarded as burn-in. We checked convergence and parameter estimates with ESS values >200 by Tracer v 1.6 (Rambaut et al., 2014). TreeAnnotator v1.8.4 (Drummond & Rambaut, 2007) was used to find the maximum credibility tree. In the absence of a fossil record of Neurergus and internal calibration points to calibrate the rate of divergence, we used external calibration points based on the estimated divergence time between *N. kaiseri* and *N. struchii* by Zhang et al. (2008). The root ages were 19.1 (12.1-26.4) Myr and 9.5 (5.4-13.8) Myr in calibration I and calibration II, respectively. Calibration III was carried out based on the evolutionary rate of the ND2 gene in salamanders identified by Weisrock et al. (2001) as 0.64% per Myr per lineage. Finally, calibration IV was carried out based on one fossil by approximating the crown of the genus *Triturus* dated at 24 Myr (Weisrock et al., 2001). In this analysis, in addition to the previous outgroups (*N. crocatus*, *N. kaiseri*, *N. strauchii*, *T. karelinii*, and *Ommatotriton vittatus*), we used two sequences of *T. carnifex* and one sequence each from *T. dobrogicus*, *T. cristatus*, *T. pygmaeus* and, *T. marmoratus* (available in GenBank under accession numbers: GQ258952, GQ258962, JN831597, NC_015790, GU982456 and, GQ258948).

RESULTS

We identified 11 unique haplotypes among 77 *N. derjugini* individuals based on the mitochondrial ND2 sequence (1036 base pairs), with 63 base pairs of the tRNA-Met gene at the beginning of the sequence. The ND2 mtDNA fragment contained a low polymorphism with only 16 variable sites, of which 14 were parsimony informative and 2 were singleton-variables including 14 transitions and 2 transversions (Table 2). Mean nucleotide compositions were A: 36.15 %, T: 25.36 %, C: 26.55 % and G: 11.94 %. The haplotype and nucleotide diversities across all populations of *N. derjugini* were 0.82399 and 0.00389, respectively. The haplotypes were allocated to different localities across the species' range. Seven of the eleven haplotypes were unique to their population, and two were shared only in two populations. Haplotype 1 was most widespread and abundant, shared among five of the fifteen populations. Ten populations had a single haplotype and the highest haplotype diversity (*Hd* = 0.60) occurred in Nowdeshe from the southern portion of the distribution range (Table 1). Average sequence divergence among *N. derjugini* haplotypes was low (0.54 ± 0.06%), whereas divergence of *N. derjugini* with *N. kaiseri* and *N. crocatus* was 6.8 ± 0.7% to 5.9 ± 0.7% respectively.

Bayesian and ML phylogenetic analyses based on 11 haplotype sequences of *N. derjugini* and five outgroup taxa had the identical topology (Fig. 2). All samples from 15 populations throughout the distribution range formed

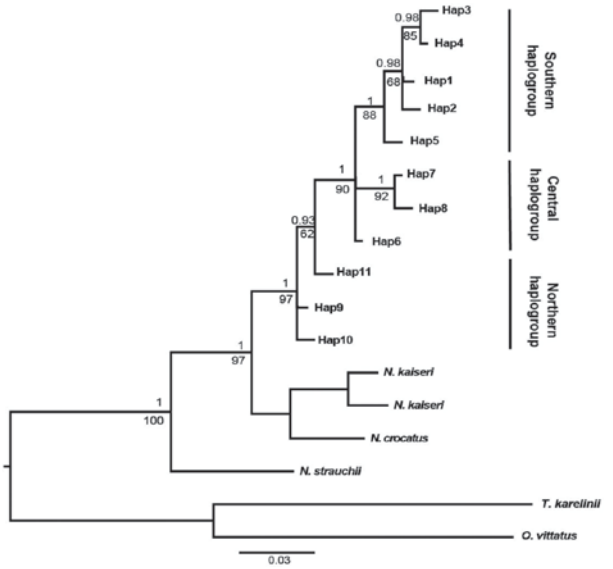

Figure 2. Phylogenetic trees of haplotypes implemented in PhyML and MrBayes based on partial ND2 gene sequence of 77 individuals for *N. derjugini* (Bayesian posterior probability values are above the branches, maximum likelihood bootstrap values are below the branches).

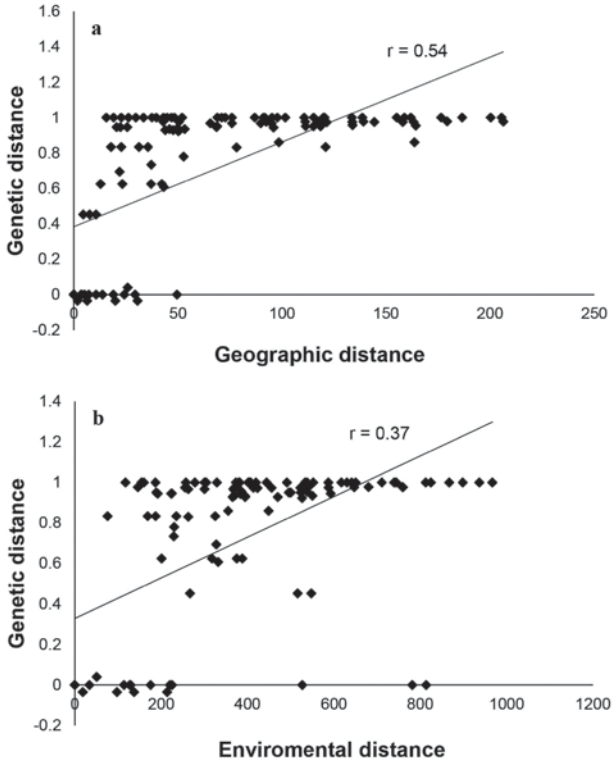

Figure 3. The plot of simple Mantel test indicating the correlation between **(a)** the geographic and genetic distances **(b)** environmental distance with genetic distance among 15 populations of *N. derjugini*.

a monophyletic group with high support (posterior probability = 1.00, likelihood bootstrap proportion = 97). The distribution of haplotypes was consistent with a northern, central, and southern part of the range. The TCS analysis (Fig. 1b) supported this phylogenetic tree. The geographic distribution of haplotypes suggests that gene

Table 2. Variable nucleotide sites and genetic variation within the partial sequences of the ND2 gene for 11 haplotypes of 77 Neurergus derjugini individuals in different localities

Hap	Polymorphic site																	Locality													Total
1	183	249	319	342	370	453	567	661	723	756	774	813	828	930	939	984	Ka	Ghor	Ghol	Do	Da	La	No	Ha	Ba	Ta	Pe	Si	Sh	Sa	Be	
2	A	G	A	A	G	A	A	T	T	C	G	C	A	C	C	C	5	5	5	5	5	0	0	0	0	0	0	0	0	0	0	25
3	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
4	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	T	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
5	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	4
6	.	.	G	G	.	.	.	.	.	.	.	.	.	.	.	.	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	3
7	C		.	G	.	.	.	C	.	.	.	.	.	.	.	.	0	0	0	0	0	0	2	4	4	4	0	0	0	0	0	14
8	C	.	.	G	.	.	.	C	.	.	A	T	G	.	.	.	0	0	0	0	0	0	0	0	0	0	4	6	0	0	0	10
9	C		.	G	.	.	.	C	.	T	A	T	G	.	.	.	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
10	C	.	.	G	.	C	G	C	C	.	.	.	.	.	T	.	0	0	0	0	0	0	0	0	0	0	0	0	5	6	0	11
11	C	.	.	G	.	C	G	C	C	T	.	.	.	.	T	.	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
	C	.	.	G	.	C	.	C	C	.	.	.	.	T	T	.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	6
Sample size																	6	5	5	5	0	5	4	4	4	4	5	6	6	6	6	77
Number of polymorphic sites																	1	0	0	0	0	1	3	0	0	0	1	0	1	1	0	16
Number of transitions																	1	0	0	0	0	1	2	0	0	0	1	0	1	0	0	14
Number of transversions																	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	2
Number of parsimony informative sites																	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	14
Number of nucleotide difference																	0.3	0	0	0	0	0.40	1.80	0	0	0	0.40	0	0.33	0	0	4.03
																	3					0	0				0		3			

Ka =Kavat, Ghor= Ghori ghale, Ghol= Gholani, Do= Dourisan, Da= Darrenajjar, La= Lashkargah, No= Nowdeshe, Ha= Hani garmale, Ta= Tawale, Ba= Balkha, Pe= Penjwin, Si= Siya gwez, Sh= Shalmash, Sa= Saqez, Be= Benjun.

Table 3. Results of analysis of molecular variance (AMOVA) using partial ND2 gene

Structure	Source of variation	d.f.	Variation (%)	F_{SC}	F_{ST}	F_{CT}
Three region	Among regions	2	74.91	0.84**	0.96**	0.74**
	Among populations within regions	12	21.13			
	Within populations	62	3.97			
The studies samples	Among populations		94.79			
	Within populations		5.20		0.94**	

Significant values are shown for $p < 0.05$ (*) and $p < 0.01$ (**)

flow between northern and southern regions is low.

The AMOVA revealed that most genetic variation was explained by differences among regions (74.91 % genetic variation: F_{CT} = 0.74, P < 0.01) followed by populations within regions (21.13 % genetic variation: F_{SC} = 0.84, P < 0.001; Table 3). Similar significant genetic differences exist among populations without the grouping (Table 3). Table 4 shows pairwise *F*st between populations.

There was a significant correlation between pairwise genetic distances and Euclidean distances (two-way Mantel tests, r = 0.54, P < 0.0001; Fig. 3a), a well as environmental distances (r = 0.37, P = 0.0012; Fig. 3b). Results of the three-way Mantel test revealed that the correlation between genetic and geographical distances remained significant even after accounting for the effect of environmental distance (r = 0.42, P = 0.0001). On the other hand, the elimination of the influence of geographical distance in the partial Mantel test resulted in a non-significant association between genetic and environmental distances (r = -0.097, P = 0.183).

The Bayesian skyline plot (Fig. 4) suggests that the population size was relatively stable from about 80,000 years ago (the middle Pleistocene) until approximately 25,000 years ago near the Last Glacial Maximum (LGM), followed by a contraction and an increase in effective population size starting at about 12,000 years ago near the Pleistocene-Holocene transition period.

Estimates of divergence between haplogroups in four calibrations are shown in Fig. 5. Calibrations I and II were the youngest and oldest, and estimation times by calibrations III and IV were between calibration I and II, while divergence times of calibration III was closer to

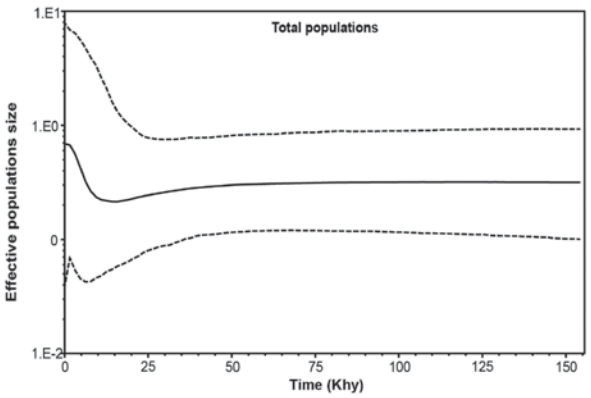

Figure 4. Bayesian skyline plot (BSP) based on partial ND2 sequences of *N. derjugini*. The x-axis shows time in the past in thousands of years, and the y-axis shows Ne (effective population size). Dashed lines show the median estimates, and white areas between the blue lines show the 95 % highest posterior density (HPD) limits.

calibration I. Taken together, divergence between the south-central and northern haplogroups took place in the early or middle Pleistocene (95% HPD, approximately 0.66 – 1.03 Myr), and divergence between southern and central haplogroups took place in the middle Pleistocene (95% HPD, approximately 0.41 – 0.83 Myr).

DISCUSSION

The present study revealed a low nucleotide diversity and a relatively high haplotype diversity for the total populations of *N. derjugini*. Haplotype diversities within ten populations of *N. derjugini* in our study were zero. Additionally, mean sequence divergence between all haplotypes (0.54 ± 0.06%) was low. Also, population genetic analyses exhibited significant phylogeographic structure in this species. There are reports of strong phylogeographic structure and low level of genetic divergence in several species of amphibians (Matsui et al., 2008, Richter et al., 2009, Farasat et al., 2016), which has been attributed to high frequency of inbreeding due to small population sizes, habitat loss, low dispersal ability (Allentoft & O'Brien, 2010), relatively short evolutionary history (Wang et al., 2017), a recent range expansion from glacial refugia (Makowsky et al., 2009; Pabijan et al., 2015; Vásquez et al., 2013), and a slow evolutionary rate at the genomic level (Chen et al., 2012). We expected genetic diversity of these populations to be low, due to a small geographical range and fragmentation of terrestrial habitat (Afroosheh et al., 2016), local extinctions (Sharifi & Assadian, 2004), and a small home range (Sharifi and Afroosheh, 2014).

Our phylogenetic analyses showed that all sampled populations form a monophyletic group. Average sequence divergence among *N. derjugini* and *N. kaiseri*, and between *N. derjugini* and *N. crocatus*, are 6.8% and 5.9% respectively. However, the average sequence divergence among populations of *N. derjugini* is only 0.4%. Although populations in different regions have specific haplotypes, there are very few mutational steps between the haplotypes. A similar study on *N. derjugini* conducted by Hendrix et al. (2014) based on mitochondrial genes (12S ribosomal RNA and control region) and one nuclear gene (KIAA gene) indicated that there are low genetic differences between populations separated as *N. microspilotus* and *N. derjugini*.

Mantel tests demonstrated that differentiation between populations of *N. derjugini* is more associated with geographic distances rather than environmental distances, a pattern that is typical for species with low dispersal capacity and low habitat availability (Dixo et al., 2009). Due to limited gene flow, geographically separated populations will become isolated even in the absence of barriers. Genetic drift and inbreeding will reduce genetic diversity in such populations (Irwin, 2002; Louy et al.,

Table 4. F_{ST} values between populations. Numbers are representative of localities based on Table 1.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	0.000														
2	-0.034	0.000													
3	-0.034	0.000	0.000												
4	-0.034	0.000	0.000	0.000											
5	-0.034	0.000	0.000	0.000	0.000										
6	0.733	0.833	0.833	0.833	0.833	0.000									
7	0.607	0.625	0.625	0.625	0.625	0.694	0.000								
8	0.934	1.000	1.000	1.000	1.000	0.945	0.452	0.000							
9	0.934	1.000	1.000	1.000	1.000	0.945	0.452	0.000	0.000						
10	0.934	1.000	1.000	1.000	1.000	0.945	0.452	0.000	0.000	0.000					
11	0.942	0.967	0.967	0.967	0.967	0.945	0.780	**0.928**	**0.928**	**0.928**	**0.000**				
12	0.972	1.000	1.000	1.000	1.000	0.975	0.830	**1.000**	**1.000**	**1.000**	**0.040**	**0.000**			
13	0.954	0.974	0.974	0.974	0.974	0.956	0.833	**0.950**	**0.950**	**0.950**	**0.950**	**0.976**	**0.000**		
14	0.976	1.000	1.000	1.000	1.000	0.978	0.860	**1.000**	**1.000**	**1.000**	**0.975**	**1.000**	**-.0.000**	**0.000**	
15	0.976	1.000	1.000	1.000	1.000	0.978	0.860	**1.000**	**1.000**	**1.000**	**0.975**	**1.000**	**0.923**	**1.000**	**0.000**

Node no.	Calibration I	Calibration II	Calibration III	Calibration IV
1	4.03 (2.69-5.23)	8.14 (5.94-10.51)	5.29 (4.08-6.65)	6.29 (4.93-8.33)
2	0.66 (0.35-1.04)	1.33 (0.72-2.11)	0.86 (0.29-0.83)	1.03 (0.57-1.63)
3	0.41 (0.21-0.66)	0.83 (0.44-1.35)	0.53 (0.29-0.83)	0.65 (0.34-1.03)
4	0.30 (0.11-0.53)	0.61 (0.23-1.07)	0.39 (0.16-1.29)	0.47 (0.17-0.82)
5	0.26 (0.06-0.54)	0.54 (0.13-1.09)	0.35 (0.09-0.68)	0.42 (0.11-0.85)
6	0.22 (0.09-0.39)	0.45 (0.18-0.80)	0.29 (0.12-0.49)	0.35 (0.14-0.62)
7	0.13 (0.004-0.27)	0.28 (0.08-0.54)	0.18 (0.06-0.33)	0.21 (0.06-0.41)
8	0.07 (0.003-0.17)	0.15 (0.009-0.36)	0.09 (0.006-0.22)	0.11 (0.006-0.28)
9	0.04 (0.001-0.13)	0.09 (0.002-0.26)	0.06 (0.002-0.17)	0.07 (0.002-0.20)
10	0.07 (0.002-0.20)	0.14 (0.003-0.40)	0.09 (0.004-0.35)	0.11 (0.004-0.31)
11	0.09 (0.002-0.27)	0.20 (0.005-0.55)	0.12 (0.004-0.35)	0.15 (0.004-0.42)

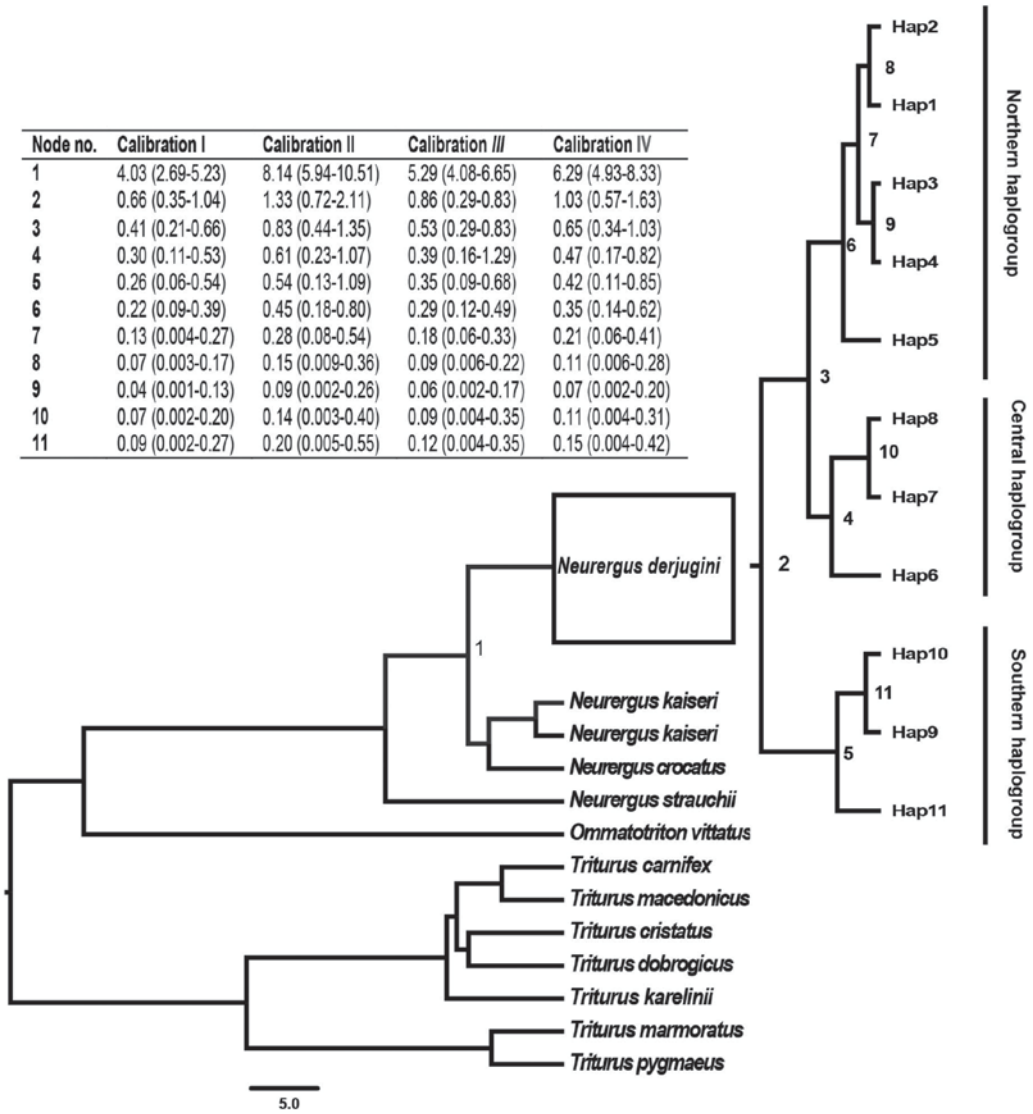

Figure 5. Chronogram of diversification implemented in BEAST based on partial ND2 gene for *N. derjugini*. The table shows three calibrations and the ranges of the divergence times for nodes in millions of years with 95 % highest posterior density (95 % HPD).

2007). The isolation by geographic distance mechanism has been reported in populations of *N. kaiseri*, the sister species of *N. derjugini* (Farasat et al., 2016). In the present study, the Mantel test revealed a positive but statistically non-significant correlation between genetic and environmental distances. Contrary to our expectations, distinct ecological parameters had a less strong influence on the genetic divergence among populations.

BSP analysis indicated an overall stationary historical population size with a contraction around the LGM followed by an expansion at the Pleistocene-Holocene transition. The classic scenario based on glacial contraction and postglacial expansion as is known in some species that are located in regions with higher latitude may not happen in mid-latitude areas such as Iran (Kehl et al., 2009). Different species in the Middle East may also respond in a different way. For example, Najafi et al. (2018) showed that divergence between two major geographical clades of *Rhinolophus euryale* (Chiroptera) in the Pleistocene was congruent with the classic scenario. However, Shahabi et al. (2017) reported contraction of populations in another Rhinolophid bat, *R. euryale*, in glacial periods within glacial refugia in southern Zagros Mts. It was also suggested by Ahmadzadeh et al. (2013) that there was a refuge in a narrow Zagros corridor between the Sabalan and Bozghosh mountain ranges during glacial periods for Iranolacerta brandtii (Reptilia). However, Javanbakht et al. (2017) reported that Transcaucasian tortoises had a long-term range stability and did not show shift in their range during glaciation and interglaciation.

The genetic variance observed within *N. derjugini* populations and its geographical distribution suggests that historical isolation has probably played a role in shaping the genetic structure of *N. derjugini*. Divergence dates based on four calibrations estimated that the most ancient diversification have probably occurred in haplogroups distributed in the south, centre and north during the early or middle Pleistocene, probably relating to the oscillating glacial cycles. Haplogroups of southern region diversified approximately around the LGM. Since the number of first order breeding streams and newt

abundance (as reported by the number of visual counts) are substantially higher in the southern region of the distribution (Afroosheh et al., 2016), it seems that *N. derjugini* expanded to surrounding areas and created extant distribution patterns with the combination of low nucleotide diversity and high haplotype diversity.

The Zagros open woodland of mostly oak in western Iran and eastern Iraq has experienced forest expansion and contraction as the result of fluctuating climate during the Pleistocene (Khalyani et al., 2013). Moreover, this area has been affected by livestock grazing and agricultural development since the beginning of the 5th Millennium BP (Wright et al., 1967). Long term traditional land use for grazing livestock by nomads and other disturbances associated with recent population growth are two main driving factors that have resulted in massive deforestation or changes in the vertical structure, composition, and configuration of forests in the Zagros Mountain Range (Metzger et al., 2005). The remnants of formerly widespread open woodlands are currently present only in the southern part of the geographic range of *N. derjugini*. The few remaining populations of *N. derjugini* in the northern part of its distribution are located in areas that presumably lost their natural vegetation cover decades ago (Afroosheh et al., 2016).

Low levels of genetic variation were observed among most populations of *N. derjugini*. Whether this low diversity is a threat to any of these populations has not been documented, and many of these populations may persist despite this. Nevertheless, a general correlation between population fitness and genetic diversity has been demonstrated in many groups of vertebrates including amphibians (Reed & Frankham, 2003; Jordan et al., 2009). The maximum linear distance between the most segregated breeding streams in the southern and northern parts of the species range is only 205 km. However, localities inhabited by *N. derjugini* are separated with nearest neighbour distances averaging 7.95 km. Surveys on the abundance of *N. derjugini* in 32 of the 42 localities within the Iranian range of the species resulted in the total visual count of 1,379 adults, juveniles, and larvae (mean/stream = 43; range, 1–601). Most of these observations (51%) were found in just two of the localities, 44% were found in 14 streams, and the remaining 5% were scattered among 16 streams (Afroosheh et al., 2016).

Very low levels of genetic variation within each small population and the lack of connectivity among most populations of *N. derjugini* occurring in fragmented habitats suggest that the species is at high risk of becoming extinct. Considering the isolation of many *N. derjugini* populations, it would seem reasonable to focus on management efforts to minimise future genetic drift and inbreeding by increasing population sizes and habitat connectivity. This is probably best accomplished by improving or expanding the available wetland habitats at each site to facilitate a natural population increase. We also recommend the supplementation of extant populations with captive bred individuals, a strategy which is enabled by the existence of a captive breeding facility for this species (Sharifi & Vaissi, 2014; Vaissi & Sharifi, 2018).

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Longitudinal monitoring of turtle trade through Facebook in Vietnam

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Trade of turtles, for both food and pet, represents a substantial business in Vietnam, especially because this country is a cross-bridge for wildlife trade from Indochina to China. Vietnam is also one of the main countries worldwide in terms of the number of Facebook members, and a considerable portion of the business has gone online through Facebook trading, including turtle trade. Here, the advertisements of turtles for sale in Vietnamese Facebook groups were monitored for the period 2013-2018, obtaining a total of 481 advertisement cases concerning 5,758 individuals belonging to 53 species and 12 families. There has been a rapidly rising trade of turtles online, especially in the last two years. Many traded species were allochthonous, but native species accounted for 22 species and over 36 % of the traded individuals. Most allochthonous species were traded as hatchlings and juveniles, whereas most of the native species were traded as subadults and adults, thus suggesting a high frequency of illegal trade in wild caught animals. Five traded native species are considered among the 50 most threatened turtle species in the world. Turtle trade occurred mostly in the two biggest cites of Vietnam (Ho Chi Minh city and Hanoi), which accounted for 68 % of the total trade. Turtle price varied substantially across species and by different lifestages (i.e. hatchlings, juveniles, sub-adults and adults), and increased considerably in comparison to 1993 estimates.

Key words: turtle trade, Facebook, Asia, Vietnam

INTRODUCTION

Trade of turtles, for both food and pet, represents a substantial business, both internationally and within several 'mega-biodiverse' tropical and subtropical countries, and has consequently risen considerably through the last decades (Böhm et al., 2013; Auliya et al., 2016; Luiselli et al., 2016). The turtle trade has been especially massive in Asia (Van Dijk et al., 2000; Turtle Conservation Fund, 2002; Cheung & Dudgeon, 2006), thus causing substantial concern among conservation biologists who defined it as the 'Asian turtle crisis' (e.g., see Van Dijk et al., 2000; Ly et al., 2011; Horne et al., 2012). During the 'Asian turtle crisis', there have been an estimated 300 million turtles traded in the Chinese market in the period 1990s-2000s, including wild caught and farmed individuals (Yiming & Dianmo, 1998; Haitao et al., 2008).

The socialist republic of Vietnam, being a "cross-bridge" for wildlife trade from Indochina to China (Yiming & Dianmo, 1998; Van Song, 2008; Ngoc & Wyatt, 2013), has been the main turtle supplier to the Chinese market (Hendrie, 1998, 2000). Vast amounts of turtles have provided the Chinese market with food, ingredients for traditional medicine, farming and pets (Hendrie, 2000). Conversely, Vietnamese people do not traditionally consume turtles for subsistence and rarely use their parts for traditional medicine (Le Dien Duc & Broad, 1995). The turtle business as a whole is worth USD 750 million annually in China, with more than 300 million turtles sold yearly (Haitao et al., 2008). As a consequence of decades of massive trade, the wild populations of many Vietnamese turtles have been substantially impoverished (Hendrie, 2000; Le, 2007; Ly et al., 2011) with some species on the brink of extinction (Stanford et al., 2018). After decades of overharvesting, the quantity of traded turtles has declined remarkably in Vietnam, presumably due to a massive decline of the wild turtle populations (McCormack & Hendrie, 2007; Van Song, 2008; Linh et al., 2016).

In the last five years, the situation has been changing, whereby increasing numbers of turtles are being imported to Vietnam from China, Thailand and Malaysia to serve the pet market (Thong Pham Van,

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unpublished observations). Before 2010, keeping turtles as pets was rare within Vietnamese households due to the local culture considering any turtle to be a God, thus discouraging any attempt at keeping turtles at home as a source of bad luck to the owners (Espenshade & Le, 2002; Le Thien Duc, 2003). However, there are a few shops, mostly from the tourist areas such as Tam Dao town, Vinh Phuc Province, where turtles were sold as pets, with just few species being kept (i.e. *Geoemyda spengleri*, *Cuora galbinifrons*, *Cuora mouhotii*) (Le Dien Duc & Broad, 1995; Hendrie, 1998). After 2010, young Vietnamese people started to collect turtles as pets, and this trend became a true "fashion" in 2012-2013. Nowadays, there are dozens of turtle species, including native and *allochthonous* species of all ages (hatchlings, juveniles, sub-adults and adults) being traded as pets (Linh et al., 2016). For instance, juvenile turtles (especially *Trachemys scripta*) became frequently traded in animal shops in Hanoi (our unpublished observations). Recently, the online market of wildlife has grown dramatically (e.g., INTERPOL, 2013; Lavorgna, 2014, 2015; Chng & Bouhuys, 2015; Morgan & Chng, 2017), even posing serious issues to biosecurity (Derraik & Phillips, 2010). In Vietnam, turtle trade has also gone online by using social networks such as Facebook (Linh et al., 2016), but this form of trade has not been intensively monitored so far by conservation biologists. To the best of our knowledge, the only study on turtle trade via Facebook was conducted by Linh et al. (2016), who monitored this form of social media for just two months (March to May 2016), recording 346 turtle individuals belonging to 15 different species traded in online markets. Nowadays, Vietnam has about 64 million Facebook users, which account for about two third of the country's population (Ha Phuong, 2017), with an exponentially increasing amount of people conducting business on Facebook (Nguyen, 2017). Thus, following the increasing demand from Vietnamese people, turtle trade on Facebook started to become popular in the country a few years ago. It is presumed that trade through Facebook may severely influence the turtle market in Vietnam in the years to come (see also Linh et al., 2016). Therefore, in this study, we present a longitudinal monitoring of the turtle trade in Vietnam through Facebook in the years 2013-2018, with the aim contributing to the understanding of this emerging source of threats for wild turtle populations.

METHODS

Protocol

We examined Facebook pages of turtle dealers from 1st December 2017 to 7th August 2018. During this period, while examining all the current posts of these Facebook pages, we also carefully scrutinised their retrospective posts for the period 2013-2018. As Facebook (hereby FB) became popular in Vietnam since 2012, here we present data for the whole period of popularity of this form of social media in the country. Nonetheless, and despite all possible efforts for obtaining a comprehensive dataset, it cannot be excluded that several advertisements for the period 2013-2016 may have been missed, thus making our estimates on the numbers of traded individuals conservative for that period of time. We also cannot exclude that illegal advertisement might be deleted after the turtles were sold, thus lowering our counts.

Data searches on FB was updated daily, with the main targets being turtle trade groups, personal FB accounts of turtle traders, fanpages of turtle traders, and confiscation news from fanpages of Non-Government Organizations (NGO). The most popular groups that commonly advertised turtles for sale were: Hội Yêu Rùa Việt Nam, Hội nuôi rùa bảo tồn, bán rùa cảnh, Hội rùa Việt Nam, Rùa cạn ba miền, and Hoi san ban thu 3 mien (i.e. groups of wildlife hunters in three regions of Vietnam). It should be noticed that, on each turtle trade group, there are hundreds of turtle buyers and sellers. For example, the most popular groups are Hội Yêu Rùa Việt Nam (with 11,397 members at time of writing), Hội yêu rùa cạn (10,784 members), and Hội yêu rùa kiểng Việt Nam (9,462 members), etc. Thus, it is assumed that most of the online turtle trade business in Vietnam was monitored during the present study.

In several cases, the same advertisement including the same individual turtles for sale was advertised on two or more FB groups. Usually, the traders used the same pictures for advertising a given turtle sample in the various FB groups, thus making very easy to record only one post for our analyses and to ignore the rest. There were some additional cases in which a trader posted the picture to sell, for example, 10 turtles at one time, and then 10 days later they posted different photos of five turtles (same species as in the previous advertisement) that might be a subgroup of the first traded sample. In these cases, we asked him/her whether the turtles belonged to the same sample that was advertised in the first instance, and then recorded the data on the basis of trader's comments.

For all traded individuals, we identified the species on the basis of the FB pictures (Fig. 1). We did not consider advertisements without appropriate pictures in order to avoid species' misidentifications or fake announcements. The number of turtles on trade were counted on the basis of the visible number of turtles on the pictures posted in the above-mentioned FB sources. These counts were then confirmed by asking the owner, through private message or comment, on how many turtles of each given species he/she had to sell. This means our counts were on the conservative side. The traded turtles were classified into hatchlings, juveniles, sub-adults and adults on the basis of the appearance of the traded individuals in the FB pictures. We recorded the location of trade to determine where are the 'hotspots' of turtle trade within Vietnam. The date of the posts were also recorded, along with the price for each turtle, obtained from the post or by asking privately or in a public comment to the seller.

We also aimed to determine whether a given turtle was legally or illegally traded. In the case of *allochthonous* species, one of us (Thong Pham Van; hereby TPV) privately asked the traders for the legal permits for all the individuals offered for sale. This information was requested using the regular FB account of TPV without hiding his identity. In the case of Vietnam's native

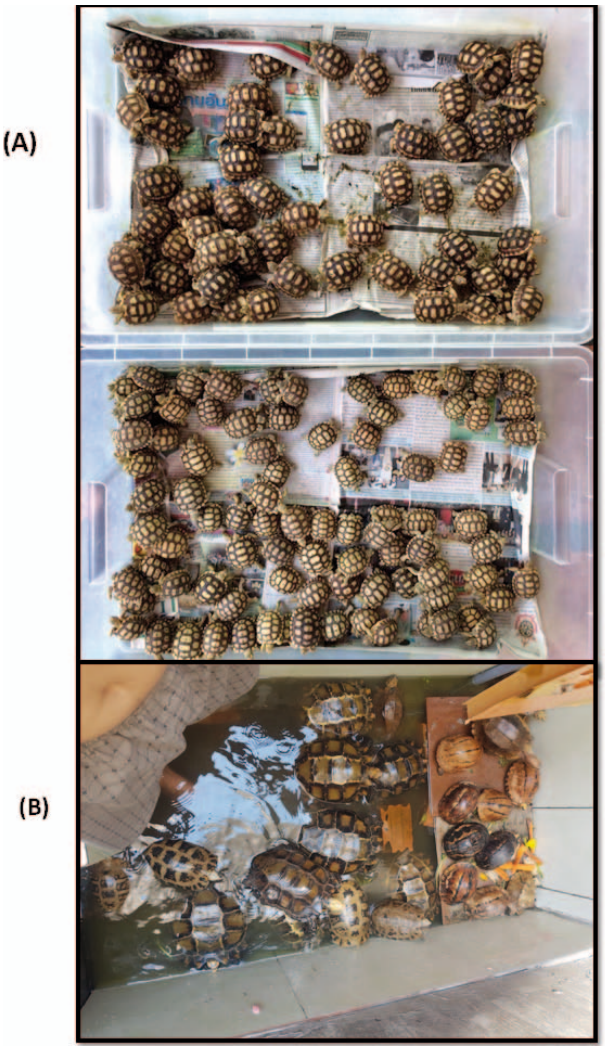

Figure 1. Examples of pictures of Vietnamese turtles on trade through Facebook. **(A)** individuals on legal trade; **(B)** individuals on supposedly illegal trade.

species, when the traders advertised the selling of large numbers of hatchling turtles, mostly aquatic species (i.e. *Mauremys sinensis*, *Heosemys grandis*, *Mauremys mutica*, *Heosemys annandalii*), we assumed that this trade was legal because there were many farms that have legal permits to sell turtles issued by Provincials Forest Protection Department (FPD). These farms normally legally sell hatchling turtles in large numbers (>10 individuals on each occasion; Fig. 1A). When turtles were traded as adults and sub-adults, and usually in small numbers, we classified this trade as illegal as these turtles were most likely wild caught, with no evidence of any legal permit released from competent authorities (Fig. 1B). We also directly asked traders whether they have legal permits for the traded animals. As a general rule, traders explicitly state that an animal is legally traded if they have permit, and they usually sell it for a higher price. In these few cases, the individual was obviously considered 'legal', but this situation appeared extremely rare when adult and subadult native turtles were offered for trade.

Statistical analyses

We used parametric tests only after having verified data normality and homoscedasticity in all variables using a Shapiro-Wilk W test. We assessed the correlation between year and number of traded turtles using Pearson's correlation coefficient, and the correlation between year and yearly number of traders by Spearman's rank correlation coefficient. Contingency table χ^2 test was used to evaluate the frequency differences in terms of traded individuals (hatchlings + juveniles versus subadults + adults) between native and allochthonous species. In the text, the means are presented ± 1 Standard Deviation. All statistical analyses were performed by Past 3.0 software, with alpha set at 5 %.

RESULTS

General data

We recorded 481 advertisement cases of turtles and tortoises for sale on FB from 2013-2018. Overall, these advertisements concerned 5,758 individuals belonging to 53 species and 12 families, including both allochthonous and native species (Table 1). Overall, 71.9 % of the traded individuals were hatchlings and juveniles, and 28.1 % were subadults and adults. In the period 1st January 2018-30th July 2018, there were an average of 16 individual turtles on trade daily.

Turtle price varied substantially across species and by different lifestages (i.e. hatchlings, juveniles, sub-adults and adults) (Table 1). In addition, the price is also varied hugely in relation to the different coloration morphs of each given species: for example, the normal form of *Malayemys subtrijuga* was traded at $6.82 while the albino form (white or golden in colour) was sold for up to $1704.55. The same was true also for *Heosemys annandalii*, with its albino form juvenile being traded at $4545.45. The price for normally coloured individuals was unknown as the traders did not publish the price in their FB pages.

Although Chelonians originated mostly from Asia and North America, species from Africa (for instance, *Centrochelys sulcata*, *Pelomedusa subrufa*) and Europe (*Testudo hermanni*) were also recorded (Table 1). Among them, 45.3 % of the traded species were Vietnam's native species and 54.7 % were allochthonous species. 60.4 % of 53 traded species were listed as Threatened in IUCN (2018) Red List. Specifically, 15.1 % were Critically Endangered, 18.9 % were Endangered, and 26.4 % were Vulnerable. In addition, 7.5 % of them were listed on CITES Appendix I and 54.7% were listed on CITES Appendix II (CITES 2017).

Based on answers provided by the sellers, it appeared that the turtle sources outside Vietnam were China (about 80 % of individuals on trade, mostly belonging to freshwater species), whereas the sources of terrestrial species were Malaysia and Cambodia (5 % of individuals on trade), Thailand (5 % of individuals on trade), Madagascar and countries from Africa (especially Mozambique, Tanzania and Sudan, 5 % of individuals on trade), and other countries (roughly 5 % of individuals).

Table 1. Summary of the chelonian species (listed in alphabetical order) offered for sale in Vietnam on Facebook in the period 2013-2018. The traded amounts and the range in prices per individual is also presented.

Species	Quantity	Lowest ($)	Highest ($)
Aldabrachelys gigantea	24	2272.73	16000.00
Amyda cartilaginea	10	22.73	
Astrochelys radiata	223	909.09	1818.18
Carettochelys insculpta	35	59.09	204.55
Centrochelys sulcata	334	59.09	2000.00
Chelodina mccordi	5	54.55	136.36
Chelonida novaeguineae	1	90.91	-
Chelonida siebenrocki	1	77.27	-
Chelonoidis carbonaria	11	-	1420.45
Chelus fimbriata	4	72.73	118.18
Chelydra serpentina	1179	5.23	363.64
Chrysemys picta	5	31.82	-
Cuora amboinensis	66	13.64	20.45
Cuora bourreti	134	36.36	109.09
Cuora galbinfrons & Cuora bourreti	100	-	-
Cuora galbinifrons	159	56.82	190.91
Cuora mouhotii	91	6.82	22.73
Cuora picturata	71	70.45	90.91
Cuora 'serata'	1	-	-
Cyclemys oldhamii	2	17.27	-
Cyclemys pulchristriata	19	13.64	15.91
Emydura subglobosa	2	54.55	-
Geochelone elegans	69	90.91	909.09
Geochelone platynota	13	718.18	-
Geoclemys hamiltonii	1	272.73	-
Geoemyda spengleri	197	5.00	15.91
Graptemys geographica	1	-	-
Heosemys annandalii	154	-	4545.45
Heosemys grandis	93	22.73	25.00
Indotestudo elongata	354	15.91	113.64
Macrochelys temminckii	2	250.00	318.18
Malaclemys terrapin	10	77.27	-
Malayemys subtrijuga	31	6.82	1704.55
Manouria impressa	26	15.91	45.45
Mauremys annamensis	4	-	-
Mauremys mutica	263	13.18	40.91
Mauremys reevesii	42	7.27	15.91
Mauremys sinensis	253	3.64	22.73
Pelochelys cantori	2	-	-
Pelomedusa subrufa	1	-	-
Phrynops hilarii	2	113.64	-
Platysternon megacephalum	63	50.00	81.82
Podocnemis unifilis	55	25.91	34.09
Pseudemys peninsularis	1	8.64	-
Sacalia quadriocellata	11	45.45	-
Siebenrockiella crassicollis	1	13.64	-
Staurotypus triporcatus	10	127.27	-
Sternotherus carinatus	4	-	-
Sternotherus odoratus	1	-	-
Stigmochelys pardalis	161	120.45	772.73
Testudo hermanni	2	-	-
Trachemys scripta elegans	1177	0.91	20.45
Trachemys scripta scripta	277	1.82	15.91

For allochthonous species, some big shops in Hanoi and Ho Chi Minh city issued CITES permits. However, most of the turtles traded in Vietnam were illegal: we estimated that the illegal trade outweighed the legal one by 88.4 % versus 11.6 % of traded individuals respectively.

The most frequently traded species belonged to the families *Geoemydidae* and *Testudinidae* (43 % and 39 % respectively) (Fig. 2). Turtle trade occurred mostly in the two biggest cites of Vietnam: Ho Chi Minh City (42 % out of a total of 5,758 traded individuals) and Hanoi (26 %). Thus, these two cities accounted for 68 % of the total trade. Other towns still contributed with considerable numbers of individuals: 6 % of individuals were traded in Da Nang, 3 % in Lang Son and the remaining 23 % in many other smaller towns. Overall, the sources of online turtle trade were widespread within the political territory of Vietnam (Fig. 3).

In terms of the number of individual turtles traded by the main FB's groups, Hội yêu rùa Việt Nam traded about 45 % of the total followed by Hội yêu rùa cạn (20 %) and Rùa cạn ba miền (6 %). Other sources (i.e. CLB nuôi rùa bảo tồn, Rùa kiểng Việt Nam, chuyên bán rùa cảnh, Kato pet shop, Viet pet garden and so on) accounted for about 29 % of the total traded turtles.

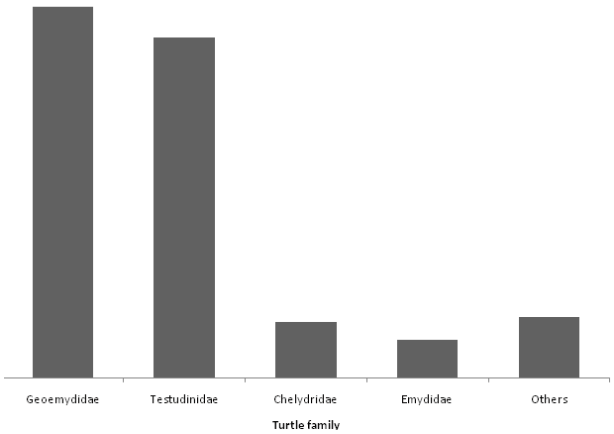

Figure 2. Percentage of turtle individuals on trade through Facebook in Vietnam by family

Native species

22 species and 2,105 traded individuals (36.6 % of the total) belonged to species that are native to Vietnam (Table 2). It is possible that for an undocumented number of individuals, the species is native to Vietnam but the individuals might come from neighbouring countries. Six of these species (i.e. *Indotestudo elongata*, *Mauremys mutica*, *Mauremys sinensis*, *Cuora galbinifrons*, *Geoemyda spengleri*, and *Cuora bourreti*) accounted for the great majority of the traded turtle individuals (Table 2). Among the Vietnamese species, one (*Mauremys annamensis*) is considered among the 25 most endangered turtles in the world, and four species (*Cuora bourreti*, *Cuora galbinifrons*, *Cuora picturata* and *Pelochelys cantori*) are considered among the 50 most endangered turtles in the world (Stanford et al., 2018).

Considering only the species native to Vietnam (n = 22), their yearly traded numbers varied from 3 to 1,192 (mean = 350.8 ± 455.3), and increased significantly year-by-year (r = 0.852, r^2= 0.726, n = 6, P < 0.05). In particular, there was an exponential increase in the number of traded individuals in 2017 and 2018 (Fig. 4). In some species, only hatchlings were traded (e.g. *Amyda cartilaginea*) and in others only adults (e.g., *Geoemyda spengleri*). Among the native species, hatchlings accounted for 19.6 ± 36.3 %, juveniles for 9.2 ± 21.6 %, subadults for 13.7 ± 27 %, and adults for 57.5 ± 37.6 % of the traded individuals. Thus, the great majority of the traded individuals from Vietnamese species were adults. If we consider the five most endangered species, all the traded individuals were adults for *Mauremys annamensis* and *Pelochelys cantori*, whereas adults accounted for 88.5 % of traded *Cuora galbinifrons*, 84.7 % of *Cuora bourreti* and 69 % of *Cuora picturata* (Table 2). Therefore, the great majority of the traded individuals of the most threatened species were adults.

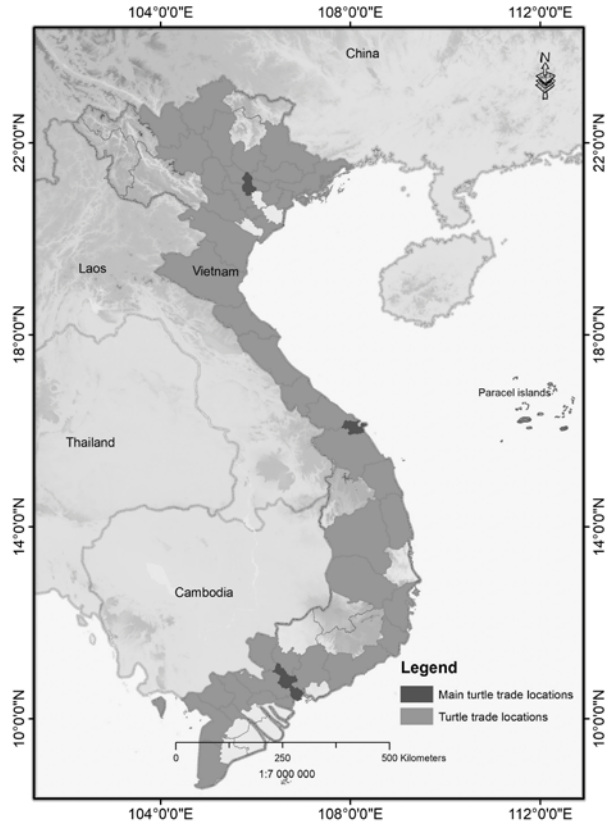

Figure 3. Map of Vietnam showing the areas from which turtle trade originated.

The frequency of hatchlings + juveniles versus subadults + adults on trade was significantly different between allochthonous and native species (χ^2= 37.35, df = 1, P < 0.0001), with the two younger categories dominating the allochthonous species sample and the two older categories dominating the native species sample. The yearly number of FB traders (mean = 53.2 ± 46.8, range = 13-142) increased significantly from 2013 to 2018 (Spearman's rs = 0.942, n = 6, P < 0.005).

DISCUSSION

The social context of turtle trade in Vietnam

The general economy of Vietnam has grown quickly during the recent decades, and the country is now recognised as a middle income country, with a middle class increasing very rapidly in terms of the number of people (World Bank, 2016). Concurrently, there has also been a much higher interest from people for the pet market. Indeed, other than dogs and cats, which have always been routinely kept as pets in Vietnam, it is now normal that middle class people keep reptiles in captivity, especially freshwater turtles and tortoises. Our study showed that, indeed, keeping turtles in captivity is a growing "fashion" in Vietnam, as indicated by the exponential increase of chelonians offered for sale in FB and the high increase in the yearly number of online turtle traders. In this regard, it is possible that Vietnamese tend to prefer allochthonous species as pets rather than native species due to the difficulty of keeping native species, as most of them die easily in captivity (ATP, 2012, 2014).

We also showed that both allochthonous and native species do enter the online trade. However, allochthonous species were primarily young turtles, whereas native species were primarily adult turtles. Why do allochthonous and native species differ in terms of frequency of age classes of traded individuals? For allochthonous species, young turtles (especially the North American *Trachemys scripta* and *Chelydra serpentina*) come from farms situated in China, and are sought after especially by FB teenagers (TPV unpublished data), who can still afford to buy these animals because of their moderate prices. On the other hand, the highly expensive allochthonous tortoises (*Aldabrachelys gigantea*, *Chelonoidis carbonaria*, etc) are usually bought by rich businessmen looking for animals serving the purpose of 'Feng Shui' (bringing good luck on business; TPV unpublished data), but are much less frequently traded than the juvenile farmed turtles. This explains why juvenile turtles dominate the allochthonous sample available for trade on FB. Concerning the native species, the great majority of the traded individuals were certainly wild caught, and often

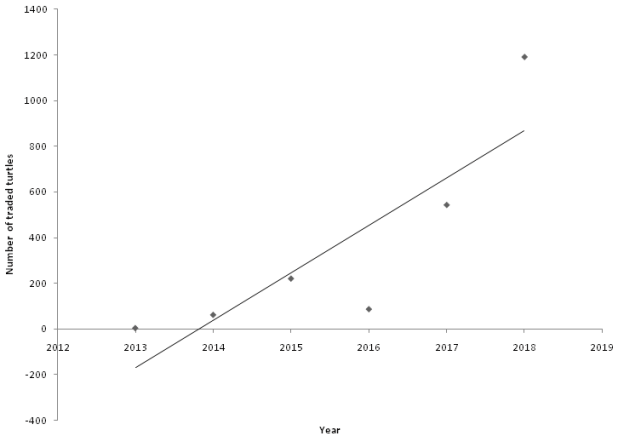

Figure 4. Correlation between year and number of native turtles of Vietnam traded on Facebook. For the statistical details, see the text.

Table 2. Number of turtle individuals native to Vietnam traded on Facebook in the period 2013-2018 by their age group. *Cuora* 'serata' is a hybrid between *C. galbinifrons* and *C. mouhotii*. The species are listed in alphabetical order.

Species	Number traded	% hatchling	% juvenile	% subadult	% adult
Amyda cartilaginea	10	100.0	0	0	0
Cuora amboinensis	66	27.2	4.5	18.3	50.0
Cuora bourreti	184	0	7.1	8.2	84.7
Cuora galbinifrons	209	0	3.4	7.7	88.5
Cuora mouhotii	91	0	18.7	54.9	26.4
Cuora picturata	71	0	28.2	2.8	69.0
Cuora 'serata'	1	0	0	0	100.0
Cyclemys oldhamii	2	0	100.0	0	0
Cyclemys pulchristriata	19	0	0	26.3	73.7
Geoemyda spengleri	197	0	0	0	100.0
Heosemys annandalii	154	32.5	0	0	67.5
Heosemys grandis	93	84.9	0	1.1	14.0
Indotestudo elongata	354	0	16.1	28.5	55.4
Malayemys subtrijuga	31	0	6.5	29.0	64.5
Manouria impressa	26	0	0	3.9	96.1
Mauremys annamensis	4	0	0	0	100.0
Mauremys mutica	263	94.3	5.7	0	0
Mauremys sinensis	253	90.9	2.4	0.8	5.9
Pelochelys cantori	2	0	0	0	100.0
Platysternon megacephalum	63	1.6	0	19.0	79.4
Sacalia quadriocellata	11	0	9.1	0	90.9
Siebenrockiella crassicollis	1	0	0	100.0	0

Table 3. List of turtle species fully protected by Vietnam national law.

Species	Decree 160/ NĐ-CP/2013	Decree 32/ NĐ-CP/2006
Amyda cartilaginea		
Cuora amboinensis		
Cuora bourreti		
Cuora galbinifrons	x	
Cuora mouhotii		
Cuora picturata		
Cuora 'serata'		
Cyclemys oldhamii		
Cyclemys pulchristriata		
Geoemyda spengleri		
Heosemys annandalii		x
Heosemys grandis		x
Indotestudo elongata		x
Malayemys subtrijuga		
Manouria impressa		x
Mauremys annamensis		x
Mauremys mutica		
Mauremys sinensis		
Pelochelys cantori	x	
Platysternon megacephalum		x
Sacalia quadriocellata		
Siebenrockiella crassicollis		

Table 4. Comparison of the price of turtles offered for sale on Facebook in the past (Le Dien Duc & Broad, 1995) and now (this study)

Species	1993 ($/kg)*	2018 ($/individual)**
Amyda cartilaginea	6.82	22.73
Cuora amboinensis	3.91	20.45
Cuora galbinifrons	3.91	19.09
Cuora mouhotii	1.82	22.73
Cyclemys oldhamii	1.82	17.27
Geoemyda spengleri	0.23	15.91
Heosemys grandis	1.50	25.00
Indotestudo elongata	1.82	113.64
Manouria impressa	2.27	45.45
Mauremys mutica	1.18	40.91
Platysternon megacephalum	1.82	81.82

of relatively big size likely because they are easier for hunters and hunting dogs to find in the wild. Thus, the shortage of juvenile individuals from native species is an evidence that FB trade of Vietnamese turtles is based on wild caught animals. This result also indirectly indicates that the breeding of many species at local farms might not very successful. By contrast, because captive breeding is more successful for *Mauremys mutica*, *Mauremys sinensis*, *Amyda cartilaginea* and *Heosemys grandis*, the juvenile and hatchling individuals dominate the traded turtles belonging to these four species.

The trade of native turtles

A total of 32 chelonian species (including five marine species) are naturally occurring in Vietnam (Turtle Taxonomy Working Group, 2017). Our study revealed that, through FB, 68.9 % (or 81.5 % if we exclude the marine species from the count) of the native chelonian species of Vietnam were traded online. Our estimates of the numbers of native turtles traded through FB are likely conservative, and more individuals would have been traded but escaped our monitoring. For instance, Linh et al. (2016) considered also Google trade exchanges (which we did not monitor), and their recorded numbers were therefore higher than ours for the same short study period in which our two respective studies overlapped (2013 to 2015).

As mentioned above, the native turtles mostly came from the wild, with individuals being caught not only in Vietnam but also in neighbouring countries such as Laos and Cambodia (Hendrie, 1998). In Vietnam, viable turtle populations can still be found in protected areas (e.g. Le, 2007), but rarely outside (Thong Pham Van & Leprince, unpublished data). Therefore, it is presumed that almost all the native turtles on trade come from protected areas due to the lack of attention from rangers and relevant agencies. Nowadays, poachers still operate in the surroundings of the protected areas (TPV et al., unpublished data), and they routinely enter the protected forest to hunt wildlife with hunting dogs. Le (2007) reported, for instance, that turtle trade around Cat Tien National Park was still high despite populations of six turtle species being viable (see also Morris et al., 2004). It is likely that turtle populations may have declined substantially inside protected areas, but sound data are lacking in this respect. This hypothesis is indirectly supported by the TPV's interviews of some poachers in 2017 and 2018, who said that they were able to collect bags of 10-20 kg of turtles per day in the 1980s-1990s, but that now they only can find one or two turtles per week.

The increase in the yearly number of FB traders throughout the years was essentially due to a change in the policy of the FB groups trading turtles in Vietnam. Indeed, in 2013 there were only 13 turtle traders actively selling online. However, due to conflict of interest, the administrators of the FB group "CLB nuôi rùa bảo tồn" decided to shift towards becoming a conservation group instead of a trading turtle group, and that was why some traders established their own group to be free to continue their online trade of turtles. Thus, the number of traders increased up to 142 in 2018.

Most sellers of turtles on FB are not the hunters. Indeed, the wild-caught turtles were bought by local traders from poachers and then sold to the provincial traders before joining the big traders, usually operating in the China's market. Although Vietnam's market of turtles is growing quickly, the overall number of turtles being kept within the country is certainly still very small compared to China. Thus, the online market is based on a few intermediate clients (Linh et al., 2016; TPV & Linh, unpublished data). The price of turtles traded in the traditional way was remarkably lower than those

advertised on FB: for example, the market price of *Geoemyda spengleri* is approximately $5/individual in the traditional way (TPV & Linh, unpublished data) whereas it is about $15.91 per individual on FB. This fact is also due to the fact that most FB clients are middle-class people in relatively good wealth.

The average turtle price has increased considerably in the last 15 years (Table 4; but notice that the data are not directly comparable because in 1993 it was a cost per kg and in 2018 it is a cost per individual), with a trend that was general across species. In this regard, *Indotestudo elongata* and *Platysternon megacephalum* were the species whose prices increased the most (Table 4). Exchange rates in 1993 were about 13,000 VND per US dollar, whereas currently is 22,000VND per US dollar. These differences in the exchange rate should also be considered when comparing the increases in price of the traded turtles. Either way, the considerable increase in turtle prices over the years was also noted by Linh et al. (2016), who showed how prices of six species increased considerably even within short time intervals, i.e. between 2013 and 2015. According to Linh et al. (2016), prices of turtles are not affected by weight but only by the size of the animals. We did not collect data in this regard, and thus we could not confirm this otherwise noteworthy remark.

Our study also documented that, although eight native species are fully protected by Vietnam National Laws Decree 160/NĐ-CP/2013 and Decree 32/NĐ-CP/2006 (Table 3), these species still appeared on online trade. Decree 32/NĐ-CP/2006, enforced by the Forest Protection Department (FPD) of the Ministry of Agriculture and Rural Development (MARD) of Vietnam, lists seven turtle species as protected. Six of these protected species, however, appeared regularly on FB. For instance, *Indotestudo elongata*, although fully protected by law, was one of the most commonly traded native species (n = 354 individuals).

Criminal Law modified in 2015 has served as the background to fine the wildlife traders involved in trading wildlife species listed in the Decree 160/2013/NĐ-CP and Decree 32/2006/NĐ-CP. For instance *Cuora galbinifrons* traders may be sentenced for up to three years in jail. Environment police are more equipped to gather evidence online, whereas forest rangers focus more on patrolling and monitoring violations in the field. Decree 160 was recently revised and submitted to the central government for approval. Four new turtle species have been proposed to be added in the revised version. Indeed, this law had substantially reduced the number of individual turtles traded on FB for the species it specifically protected (*Cuora galbinifrons*) and traders may be sentenced to jail for up to three years, thus certainly discouraging the trade. However, since only a few species were protected under this law (i.e. *Cuora galbinifrons*, *Cuora trifasciata*, *Mauremys annamensis*, *Pelochelys cantorii* and *Rafetus swinhoei*), this law is still irrelevant for the protection of all other species from trade. Therefore, lack of legislation still remains a big issue in native turtle conservation within the country, and the same is also true for the allochthonous species as there are no effective laws to control the turtle trade (Amanda et al., 2016).

In conclusion, our study revealed a rapidly rising trade of turtles, including threatened native species, via FB in Vietnam. Although the total number of traded individuals, as detected in this study, is much smaller than the number that was documented during the 1990s-2000s (Haitao et al., 2008), nonetheless the documented rise of the FB trade of turtles is worrying, especially because it concerns also some of the most threatened turtle species in the world (Stanford et al., 2018) and wild populations are already very much reduced (Hendrie, 2000). It is strongly recommended that Vietnamese police should quickly implement a rigorous control system for the online trade of turtles, in cooperation with rangers, as well as new regulations for online trading of wildlife in CITES and in the countries (Morgan & Chng, 2017) and awareness campaigns to the population (Linh et al., 2016).

We also agree with Amanda et al. (2016) that, also for Vietnamese species, it is imperative that (i) a multipronged approach should be used to combat the growing global turtle trade (for instance, with wild-caught individuals imported to China being now often sourced from South America and Africa as supply from South-east Asia decreases), and that (ii) alternative ways to meet end market demand should be studied in the long-term.

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Make the Adder Count: population trends from a citizen science survey of UK adders

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Concern has been growing about the status of UK adder populations, with expert opinion reporting widespread declines. Assessing the true scale of these declines, however, has been hampered by a lack of quantitative data. *Make the Adder Count* began in 2005 as a national surveillance programme collecting standardised counts of adders lying-out after emerging from hibernation. 260 sites have contributed data, confirming a significant decline, on average, across sites with small populations, while the few with large populations (<10 % of sites) are weakly increasing. If these trends continue, within 15–20 years, adders will be restricted to a few large population sites, significantly increasing the extinction risk for this priority species in the UK. Public pressure/disturbance was reported as the most frequent negative factor affecting sites, followed by habitat management and habitat fragmentation. Negative impacts from habitat management were reported almost as frequently as positive impacts, suggesting many management plans do not adequately consider the requirements of adders. The dataset also demonstrated earlier emergence among males, in warmer springs and at more northerly sites.

Key words: adders, population trends, habitat management, conservation, emergence survey, citizen science

INTRODUCTION

The adder, *Vipera berus*, was added to the UK Biodiversity Action Plan list of priority species in 2007. Although historically widespread (Taylor, 1963; Arnold, 1995), changes in status derived from biological recording data indicate adder numbers in the UK have been in decline since the 1930s (Cooke & Arnold, 1982). A questionnaire carried out by Cooke & Scorgie (1983) reported declines in three out of 12 Nature Conservancy Council regions, rising to six out of 12 regions in a follow-up assessment (Hilton-Brown & Oldham, 1991). Baker et al. (2004) evaluated population trends at specific sites, again via a questionnaire, and showed that more populations were judged to be decreasing than increasing, with population decreases particularly prevalent in the Midlands. A study combining historical records with adder habitat requirements and land cover data suggested the species had suffered a 39% range reduction by 2011 (Gleed-Owen & Langham, 2012).

The adder shares the same degree of legal protection as other widespread reptile species in the UK (e.g. grass snake, *Natrix helvetica*), however, aspects of its ecology mean that it may be less able to survive in an anthropogenic landscape and hence may deserve greater conservation prioritisation. The adder is confined to specific habitats, being found mostly in woodland, heathland and moorland (Swan & Oldham, 1993; Arnold, 1995). As a result, it demonstrates a patchy distribution over much of its range (Viitanen, 1967; Prestt, 1971; Madsen & Shine, 1992). Even within areas of suitable habitat, it is usually confined to habitat features or patches of warm micro-habitat, such that Gleed-Owen and Langham (2012) suggest adders may occupy as little as 29 % of the potentially suitable habitat in England, based on the species' habitat preferences. Adders are also relatively sedentary (e.g. compared with the grass snake), with small home ranges (Langton & Beckett, 1995), so that populations confined to specific localities can be effectively isolated. Moreover, many populations are small (fewer than 10 adults; Baker et al., 2004) and the combination of these factors means that adders are prone to inbreeding depression (Madsen et al., 1996) and local extinctions, with limited chances of recovery.

Despite growing concern for the status of UK adder populations and their high vulnerability to negative impacts from increases in human activity and land-use change, no co-ordinated programme for collecting data on adder numbers existed in the UK, prior to 2005. All previous assessments of adder status have been based on either expert opinion (Cooke & Scorgie, 1983; Hilton-

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Brown & Oldham, 1991; Baker et al., 2004) or analysis of ad-hoc records (Cooke & Arnold, 1982; Gleed-Owen & Langham, 2012). Recent initiatives such as Add an Adder (run by Amphibian and Reptile Conservation; ARC) and Record Pool (Amphibian and Reptile Groups of the UK and ARC) similarly collect ad-hoc sightings, while the only systematic survey of UK amphibians and reptiles – the National Amphibian and Reptile Recording Scheme – has yielded very little data on adders, with adders recorded in only 7 % (~22 squares) of the 310 1 km squares surveyed between 2007 – 2012 (Wilkinson & Arnell, 2013).

Assessing the true scale of adder declines has therefore been hampered by a lack of long-term, quantitative data. Make the Adder Count (MTAC) was set up in 2005 to change this, by providing the first long-term, national surveillance programme designed to collect standardised data on adder numbers across the UK. Adders hibernate over winter, regularly using the same hibernacula year after year and often hibernating communally (e.g. Prestt, 1971; Phelps, 2004), which concentrates populations in specific locations. After emerging from hibernation in spring, adult snakes (particularly males) bask for prolonged periods close to the hibernaculum (Prestt, 1971; Phelps, 2004), during which time they are detected relatively easily by surveyors. Experienced volunteer surveyors (those already involved in reptile fieldwork, rather than recruited from a wider public appeal) were encouraged to register known hibernacula with MTAC and submit spring counts of basking adders.

The aims of MTAC were threefold:

1. To test the potential of repeated spring counts by volunteer surveyors as a viable method for collecting quantitative data on adder populations.
2. To derive population trends for UK adders and assess the current factors affecting adder populations.
3. To locate hibernation sites to inform habitat management.

In this paper, we present results from the first 11 years of MTAC, which confirm, for the first time with standardised quantitative data, significant declines across the majority of adder sites. We also identify three key factors which must be addressed, if the adder is to have a viable future in the UK.

2. Methods

MTAC is a citizen science project that specifically targets experienced surveyors with knowledge of adder overwintering sites. Potential participants were contacted through a national network of local volunteer groups (the Amphibian and Reptile Groups of the UK) and an annual national conference (the Herpetofauna Workers Meeting). Many of the participants already monitored local adder populations, so MTAC aimed to standardise and collate count data that participants may have already been recording.

2.1 Survey Methodology

Sites were defined as a single hibernation site or aggregation area or, where hibernation sites were dispersed rather than aggregated (such that adders basked individually rather than communally), a repeatable survey transect. Surveyors made multiple visits to their sites between February and May. March–April was recommended as the optimum time window, but surveyors were asked to judge the date of site visits and appropriate weather conditions according to their own experience of the site, in recognition of geographic variation. A minimum of three visits was required, with five or six visits recommended. On each visit, surveyors recorded the number of adult snakes observed basking after emergence from hibernation (i.e. excluding juveniles, which were identified by their smaller body size and are rarely encountered during the chosen survey period). Although it is possible to identify individual adders via head markings (Sheldon & Bradley, 1989), thus enabling population size estimates via mark-recapture techniques, we considered collecting the necessary data too labour intensive for most volunteer surveyors and therefore limited data collection to recording number of adults observed (regardless of whether they have been previously encountered or not), in order to maximise participation. If it was possible to visually sex the adders without disturbing them, and if confident to do so, the surveyor was asked to record male and female adders separately (sexual dichromatism in adders is normally sufficient to distinguish between the sexes in adults; Beebee & Griffiths, 2000).

Surveyors were also asked to provide information about the site itself, including site area (i.e. the area of suitable adder habitat around the surveyed hibernation site/aggregation/transect on a two-point scale: 0–5 ha and more than 5 ha), connectivity (how well connected the surveyor considered the site was to other sites on a four-point scale: 1 = completely isolated by many kilometres, 2 = isolated from nearby sites by sub-optimal habitat, 3 = linked by corridors, 4 = part of a larger group of populations occupying more or less continuous habitat) and factors judged to affect the population. For the latter, 12 categories were given and participants could select any of these as having either a negative or positive effect. The factors were: public pressure through disturbance, habitat management, habitat fragmentation/isolation, neglect/succession, persecution, fire, predation, forestry operations, building development, agricultural changes, introduction (conservation) and introduction (development mitigation). An option to record factors that did not fall into these categories was also provided. The categories were based on those used in previous questionnaire studies (Cooke & Scorgie, 1983; Hilton-Brown & Oldham, 1991; Baker et al., 2004) to facilitate comparison.

2.2 Data Analysis

Data analysis was carried out using R version 3.4.3 (R Core Team 2017), with maps and nearest-neighbouring-site analysis generated using QGIS version 2.10.1 (QGIS Development Team 2015).

2.2.1 Population Trends

For each site j in each year i we used the peak count $k_{j,i}$ as an index of population size, where the peak count was

defined as the maximum number of adders recorded at site *j* on any one visit during year *i*. Throughout the survey season, adders are emerging, basking near the hibernaculum and then dispersing into the surrounding habitat, with the timing of emergence and length of this basking period not only subject to individual variation, but also affected by the individual's sex and breeding status (both of which are often unknown). This variation in detectability between individuals and for the same individual over time prevents the use of more complex statistical models which use repeat visits to disentangle detectability and abundance assuming constant detectability and closed populations (e.g. N-mixture models; Royle, 2004; Ward et al., 2017; Barker et al., 2018). The peak count therefore remains the most robust index of population size available under these conditions with the available data.

For each site, we calculated the mean peak count (K_j) across its entire time series and used this to split the sites into two groups: those with $K_j \leq K_{threshold}$, which we assume correspond to small population sites, and those with $K_j > K_{threshold}$, which we assume correspond to large population sites (see next section for determination of $K_{threshold}$). We analysed the population trends for these two groups separately.

Only sites with three or more years of data were included in the population trend analysis. Ideally, overall population trends would be modelled using site-level peak count data in a generalised linear model with a fixed site effect. However, such models only produced adequate fits for the large populations sites, which have relatively well-sampled and well-behaved time series, which are roughly consistent with linear trends. For the small population sites, linear models provided a poor fit to much of the site-level data, due in part to the nature of their population trends. Adopting a more complicated functional form for the time dependence (e.g. allowing for linear trends in some sites and exponential declines in others) was not feasible, given that many sites had only a few years of data and so had insufficient data to constrain multiple parameters. Consequently, we used the method described below, which accounts for variation in survey effort and determines comparable average population trends for the two groups, without imposing any functional forms at site level:

1. For each site, we calculated its normalised peak count per year as:

$$k_{norm,j,i} = \frac{k_{j,i}}{K_j}$$

This preserves the fractional change in population size.

2. For each year, we calculated the weighted mean normalised peak count (i.e. mean fractional change in population size) across all sites surveyed that year as:

$$K_{norm,i} = \frac{\sum_j w_{j,i} k_{norm,j,i}}{\sum_j w_{j,i}}$$

where $w_{j,i}$ is an individual weighting factor attributed to the normalised peak count from each of the sites surveyed in that year. The larger the number of visits that have gone into determining a peak count, the more confident we are that that peak count is representative of the population size at that site in that year. We therefore based the weighting factor for each peak count on the number of site visits (n_{visits}) used to determine that peak count. The weights were set as follows: w=1 for n_{visits}<3, w=2 for $3 \leq n_{visits} < 6$ and w=3 for $n_{visits} \geq 6$, i.e. for three sites with normalised peak counts 1.2, 2.4 and 3.5, derived from 4, 2 and 6 visits, respectively, we would calculate a mean normalised peak count of ((2 × 1.2) + (1 × 2.4) +(3 × 3.5))/(2 + 1 + 3) = 2.55.

3. We calculated the uncertainty (standard error) on each mean normalised peak count as:

$$\alpha_{K_{norm,i}} = \frac{\sigma_{k_{norm,j,i}}}{\sqrt{N_i}}$$

where the standard deviation of the normalised peak counts, $\sigma_{k_{norm,j,i}}$, was calculated including the weightings above and N_i is the number of sites surveyed in that year.

4. We fitted the following (generalised) linear model to assess the trend in the mean normalised peak count over time:

$$\text{glm}(K_{norm,i} \sim Y_i, \text{family = Gaussian, weights = } (1/\alpha_{K_{norm,i}}))$$

where Y_i is the year, we used a Gaussian error distribution since the model fitted the continuous mean normalised peak counts, and the weighting allowed the model more freedom where the uncertainty on the mean normalised peak count was large. A mean normalised peak count with uncertainty 2α therefore carried half as much weight in the model fit as a mean normalised peak count with uncertainty α.

Having used the method above to determine comparable average population trends for both the small and large population sites, we also applied a more sensitive site-level analysis to the large population sites, since these did show well-sampled and roughly linear time series at site level. For these large population sites, we fitted a generalised linear model of the form: glm($k_{j,i} \sim Y_i$ + *factor*($site_j$), family = Poisson (link = 'log'), weights = $w_{j,i}$), where we used the peak counts from individual sites with a fixed site effect, Poisson errors (since we now use the un-normalised discrete counts), and weights set by n_{visits} as described in step 2 above.

Where we identified declining population trends, we extrapolated the trend forward in time to estimate the number of years it would take for an average site to completely lose its adder population, assuming the populations continued to decline at their current rate, i.e. we calculated the year in which the mean normalised peak count was predicted to reach zero.

2.2.2 Separation into Large and Small Population Sites

We initially assumed $K_{threshold}$ =10 and used the population trend results to test the suitability of using $K_{threshold}$ =10 to separate the two groups. Calculating the average

population trends for small and large population sites, as described in section 2.2.1, using $K_{threshold}$ =10 revealed that the two groups show opposite average population trends. This suggests the optimum threshold at which to separate the two groups is that which produces the largest difference between their respective population trends. We assumed the large population sites would have above average mean peak counts. The average mean peak count across all sites included in the population trend analysis was 5.2. We therefore allowed the threshold to range in integer steps between $K_{threshold}$ =5 and $K_{threshold}$ =15 (which leaves only six sites above the threshold) and recalculated the population trends for the small and large population sites using each threshold, to determine the most suitable value for $K_{threshold}$.

2.2.3 Factors Affecting Sites

We compared the average number of negative factors per site, average number of positive factors per site and average connectivity score per site for the small and large population sites. Due to non-normality of all six datasets, this was done by comparing medians using the Mann-Whitney U-Test for unmatched samples.

2.2.4 Trends in Emergence Timing

Surveyors reported the date of their site visits, allowing us to investigate variation between years in the date on which the peak count occurs. We defined the date on which the peak count occurs as the 'peak day' and we assumed variation in the peak day reflects variation in emergence timing for the majority of individuals.

In order to obtain peak day estimates that were as accurate as possible, we applied strict data selection criteria for this analysis; only peak day estimates that were obtained from five or more visits before day 153 (corresponding to 1st June in a leap year or 31st May in a non-leap year) were included. When the peak number of animals was recorded on more than one day, we used the earliest occurrence as the peak day.

We expect emergence timing to depend on spring temperatures and also site location. We therefore used the UK mean spring temperature time series produced by the Met Office (where spring is defined as the time period from March – May; downloaded from https://www.metoffice.gov.uk/climate/uk/summaries/datasets#yearOrdered; accessed 15 December 2017) and we regressed peak day against year, mean spring temperature (°C), site latitude (described by each site's Ordnance Survey northing grid reference), site longitude (described by each site's Ordnance Survey easting grid reference) and the number of visits used to determine that peak day (to account for variation in surveyor effort) using a generalised linear model with a Poisson response and log link function, i.e. glm(PeakDay ~ Year + SiteOSNorthing + SiteOSEasting + MeanSpringTemp + No.ofVisits, family = Poisson (link = 'log')). A Poisson response was used to account for the fact the peak day data are discrete, truncated at zero and left skewed.

We also calculated the mean peak day in each year across all the sites surveyed in that year and regressed this against the mean northing and mean easting of the sites surveyed in a given year, mean spring temperature and year, while controlling for the number of sites surveyed per year. This was done using a generalised linear model with a Gaussian response (since the response variable is now the yearly mean rather than discrete days) and log link, i.e. glm(MeanPeakDay ~ Year + MeanOSNorthing + MeanOSEasting + MeanSpringTemp + No.ofSitesSurveyed, family = Gaussian (link = 'log')). We then used step-wise reduction, eliminating the variable with the largest P-value in turn, to identify a model for mean peak day where all predictors were significant.

3. Results

From 2005 to 2016, 181 surveyors provided information on 260 sites. Figure 1 shows the geographical locations of these sites. More than 60 % of the sites were described by their surveyors as being well connected to other adder populations (Fig. 2).

3.1 Population Trends

129 of the 260 sites had sufficient data (three or more years) to be included in the population trend analysis. Of these sites, 117 were classed as small population sites ($K_j \leq 10$) and 12 were classed as large population sites ($K_j > 10$).

Table 1 contains descriptive statistics for the peak counts obtained from all sites qualifying for population trend analysis and for the small and large population sites separately. One site showed substantially higher peak counts ($K_j = 94 \pm 5$) than any of the other large population sites (next highest mean peak count among the large population sites was $K_j = 32 \pm 1$), as can be seen by comparing the maximum peak counts in columns four and five of Table 1.

Figures 3a and b show the average populations trends for the small and large population sites, respectively, where the population trends were calculated as described by the steps in section 2.2.1.

The small population sites have shown a significant decline in their mean normalised peak count since the survey began (Fig. 3a). Fitting the generalised linear model described in step 4 of section 2.2.1, gave $K_{norm,i} = -(0.05\pm0.01)Y_i+(100\pm20)$, with $P<0.01$ and $R^2 = 0.655$. 12 out of the 117 small population sites recorded no adders for the last two or more years those sites were surveyed, suggesting that just over 10% of these sites may potentially have lost their adder populations since the monitoring programme began. Extrapolating the trend in mean normalised peak count forward in time to estimate the number of years it would take for an average small population site to completely lose its adder population, assuming these small population sites continue to decline at their current rate, gave a lower limit estimate of just 16 years using the unrounded fit values. This implies the average small adder population will be extinct by 2032. We emphasise this is a lower limit estimate, as we are assuming that adders are absent when surveyors return zero counts and detectability is almost certainly not 100 %.

Figure 3b shows the average population trend for

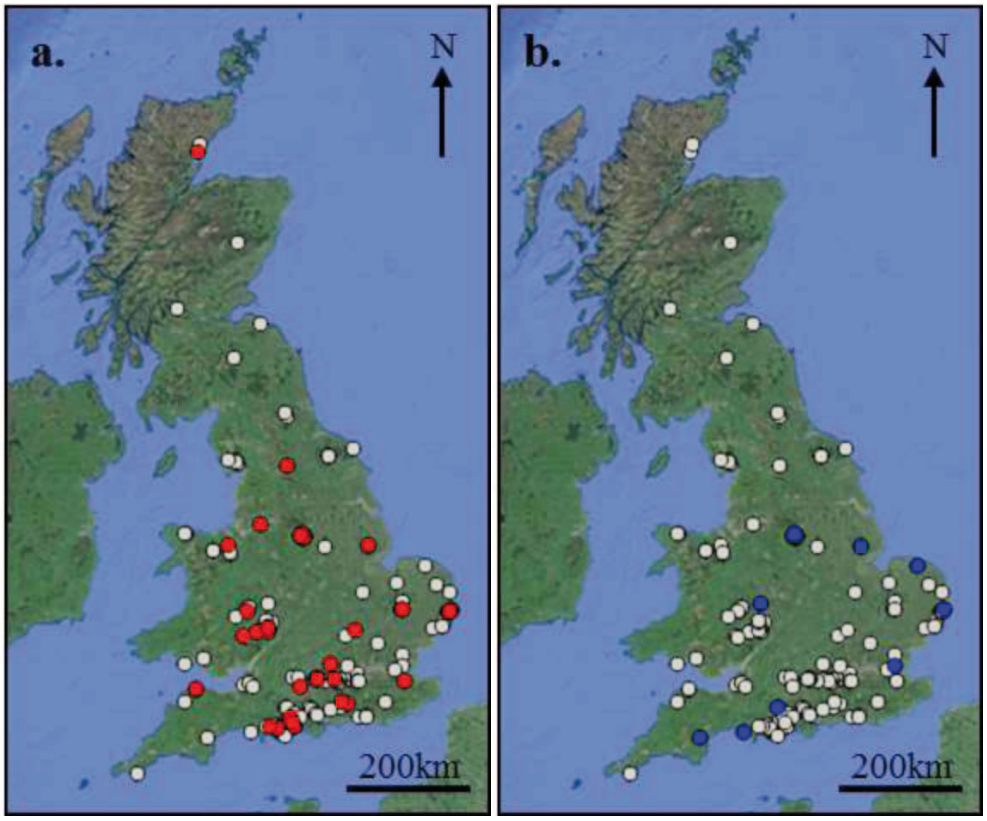

Figure 1. Geographical locations of survey sites. **a)** Red points show locations of small population sites included in the population trend analysis (117 sites with $K_j \leq 10$ and 3+ years of data) and grey points show all other MTAC sites. **b)** Blue points show locations of large population sites included in the population trend analysis (12 sites with $K_j > 10$ and 3+ years of data) and grey points show all other MTAC sites. Note that some sites appear overlaid on the map due to their close proximity. Base maps from Google Satellite.

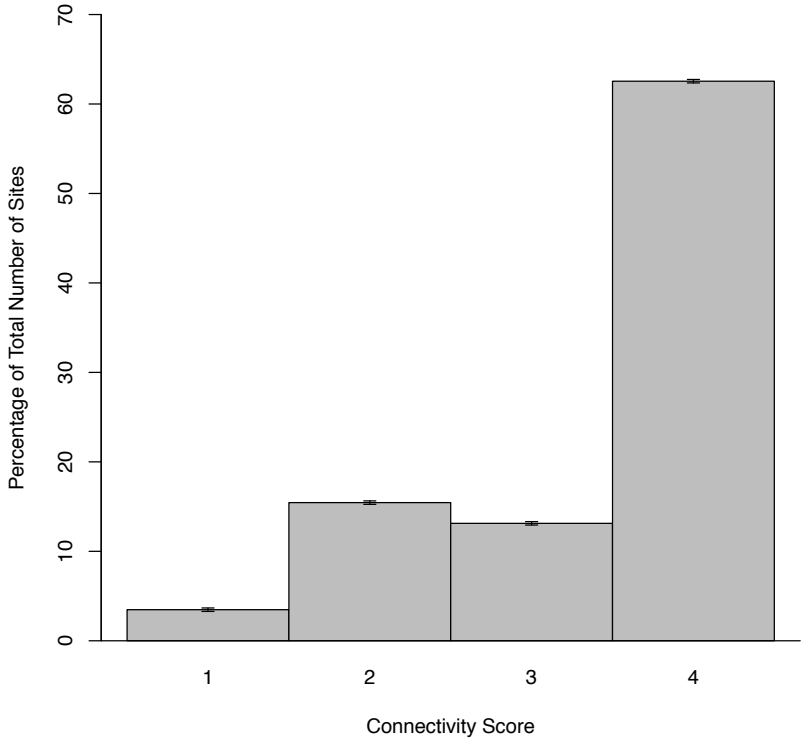

Figure 2. Connectivity scores reported for sites, where 1 = completely isolated by many kilometres, 2 = isolated from nearby sites by sub-optimal habitat, 3 = linked by corridors, 4 = part of a larger group of populations occupying more or less continuous habitat. Error bars show number of percentage points represented by a single survey site.

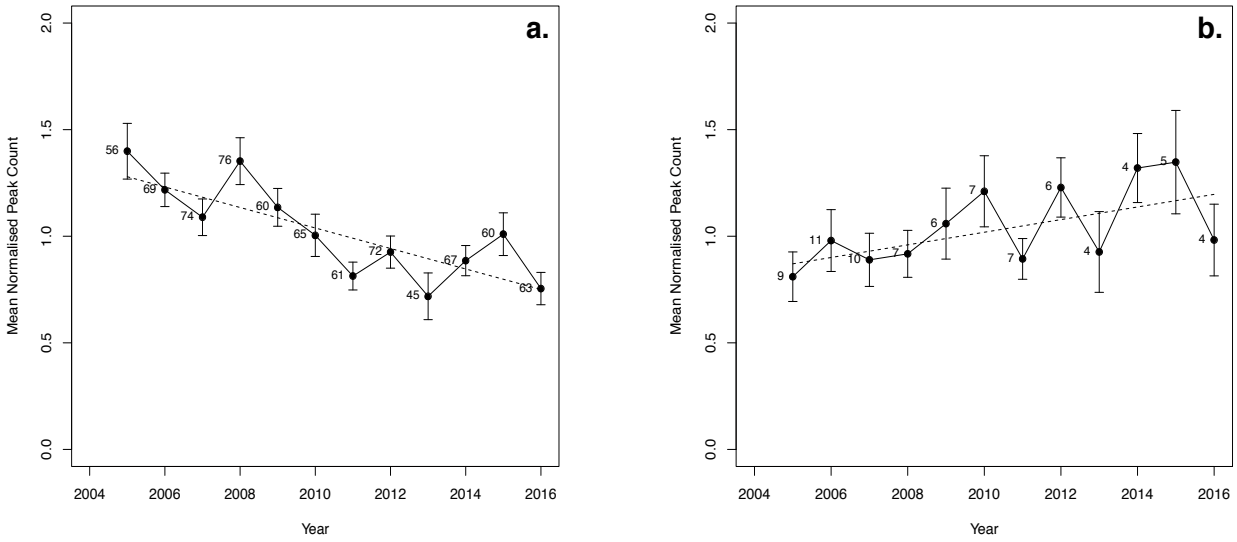

Figure 3. Average population trends for **a)** small population sites (117 sites with $K_j \leq 10$ and 3+ years of data) and **b)** large population sites (12 sites with $K_j > 10$ and 3+ years of data). Dashed lines show fits using the generalised linear model described in step 4 of section 2.2.1. Numbers adjacent to each data point show the number of normalised peak counts (i.e. the number of individual sites) contributing to the mean normalised peak count in that year. The number of sites contributing to each mean normalised peak count varies due to gaps in the time series from individual sites. Error bars show standard error on the mean, calculated as described in step 3 of section 2.2.1.

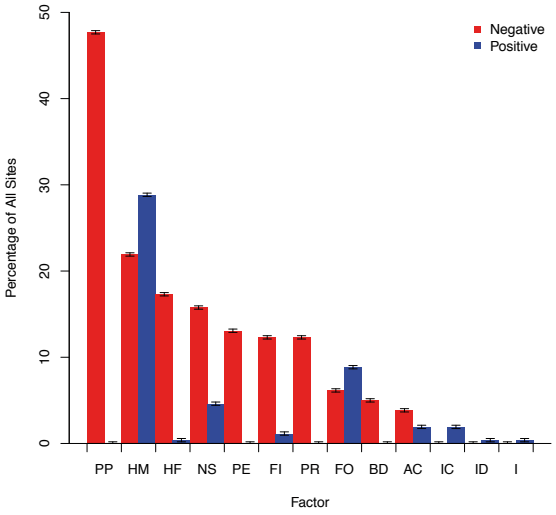

Figure 4. Percentage of all MTAC sites (260 sites) reported by surveyors to be positively and negatively affected by the following factors: PP = public pressure, HM = habitat management, HF = habitat fragmentation/isolation, NS = neglect/succession, PE = persecution, FI = fire, PR = predation, FO = forestry operations, BD = building development, AC = agricultural changes, IC = introduction (conservation), ID = introduction (development mitigation), I = introduction (unspecified). Error bars show number of percentage points represented by a single survey site.

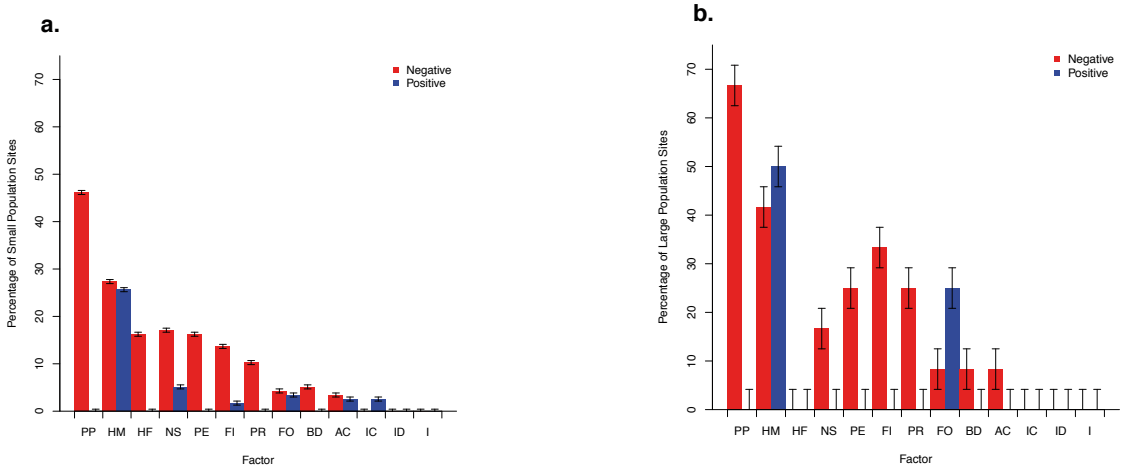

Figure 5. **a)** Factors affecting the small population sites included in the population trend analysis (117 sites with $K_j \leq 10$ and 3+ years of data). **b)** Factors affecting the large population sites included in the population trend analysis (12 sites with $K_j > 10$ and 3+ years of data). Factor abbreviations: PP = public pressure, HM = habitat management, HF = habitat fragmentation/isolation, NS = neglect/succession, PE = persecution, FI = fire, PR = predation, FO = forestry operations, BD = building development, AC = agricultural changes, IC = introduction (conservation), ID = introduction (development mitigation), I = introduction (unspecified). Error bars show number of percentage points represented by a single survey site in each case.

large population sites. In contrast, these have shown an increase in their mean normalised peak count. Fitting the generalised linear model described in step 4 of section 2.2.1, gave $K_{norm,i}$=(0.03±0.01)Y_i - (60±30), with P<0.05 and R^2=0.347. For these large population sites, the trend was weaker and the R^2 was lower. Since the large population sample was much smaller, we checked that the results were not unduly influenced by any one site (such as the outlying very large population site) by refitting the generalised linear model eliminating each site in turn from the dataset. We found the significance of the trend was not consistently robust to removal of individual sites from the model, although this was not due to any one site in particular, but rather due to a combination of the small sample size and the weakness of the trend. Fitting a more sensitive site-level model (glm($k_{j,i}$~Y_i + *factor*($site_j$), family = Poisson (link = 'log'), weights = $w_{j,i}$); see section 2.2.1) to these sites confirmed a significant positive trend ($k_{j,i} \propto (0.064 \pm 0.006)Y_i$), with significance P<0.01 which was consistently robust to removal of individual sites and showed a much improved R^2 of 0.768.

Finally, we tested the assumption that $K_{threshold}$ =10 was a suitable threshold at which to separate the sites into large and small populations (see section 2.2.2) and found that the most negative trend in the small population group was obtained using $K_{threshold}$ =6, while the most positive trend in the large population group was obtained using $K_{threshold}$ =12. Given that the median of these two extremes is $K_{threshold}$ =9, suggested that $K_{threshold}$ =10 was a reasonable threshold to use for this dataset.

3.2 Factors Affecting Sites

In order to assess potential causes of population trends, surveyors were asked to report the positive and negative factors they considered were affecting their sites. Figure 4 shows the positive and negative factors reported across all MTAC sites. The most frequently reported factors positively affecting adder populations were habitat management (28 % of sites), forestry operations (9 % of sites) and neglect/succession (5 % of sites). The most frequently reported negative factors were public pressure (48 % of sites), habitat management (22 % of sites) and habitat fragmentation/isolation (17 % of sites). Note that habitat management was reported as a negative factor almost as frequently as it was reported as a positive factor.

Figures 5a and b show separately the positive and negative factors reported for the small and large population sites included in the population trend analysis. Public pressure is still by far the greatest perceived threat in both cases, however there are notable differences in the reported impact of habitat management, forestry operations and habitat fragmentation. The small population sites reported habitat management and forestry operations as having both negative and positive effects, with slightly more sites reporting a negative effect. The large population sites similarly reported both negative and positive effects for these factors, but positive effects were reported more often than negative effects. None of the large population sites (which showed steady/increasing populations on average) reported habitat fragmentation as a factor, while 16 % of the small population sites cited habitat fragmentation as a negative factor.

There was no significant difference between the median number of negative factors reported per site between the small and large population sites (Mann-Whitney U-test; *W*=586, *P*=0.329) nor in the median number of positive factors reported per site between the small and large population sites (Mann-Whitney U-test; *W*=540, *P*=0.108). This suggested the declining small population sites were not simply facing more threats or experiencing fewer positive factors than the large population sites.

Table 2 compares the area classes, connectivity scores and conservation designations for the small and large population sites included in the population trend analysis. Caution should be used when comparing the percentages in Table 2, given the order of magnitude discrepancy in sample size between the small and large population sites. Nonetheless, there was no systematic difference between the area classes reported for the small and large population sites, with both showing roughly 15 % of sites ≤5 ha and 85 % larger than 5 ha. There was also no significant difference between the median connectivity scores for the small and large population sites (Mann-Whitney U-test; *W*=790, *P*=0.289). However, the large population sites did include a higher proportion of SSSI and NNR designations and all of the large population sites had at least one conservation designation (SSSI, NNR, ONR, CWS or NP), whereas 15 % of the small population sites had no conservation designation.

3.3 Trends in Emergence Timing

A total of 320 peak day estimates met the data selection criteria of five or more visits before day 153. Table 3 shows the results of regressing peak day against year, mean spring temperature, site latitude, site longitude and number of visits (as described in section 2.2.4). All four variables were found to be significant, with peak day generally becoming earlier over time and occurring earlier for higher mean spring temperatures and for more northerly and easterly sites. However, the R^2 for this model was low (0.135) with much residual variance, likely due to site-specific factors unaccounted for in the modelling, such as site aspect, site shading and local micro-climatic weather variations.

Averaging over the individual sites to obtain the mean peak day per year should remove some of this additional variance. Regressing mean peak day per year against the mean northing of the sites surveyed in a given year, mean spring temperature and controlling for number of sites surveyed per year gave a much improved R^2 of 0.882, with all three variables significant (Table 4). Neither year nor mean easting were found to be significant variables when fitting for mean peak day. The fitted coefficients implied a ~11% decrease in peak day for a 100 km increase in northing and ~5% decrease in peak day for a 1 degree increase in mean spring temperature. Using the mean peak day of 93 in 2005, these translate into real terms as ~11 day decrease in peak day for a 100 km increase in northing and ~5 day decrease in peak day for

Table 1. Descriptive statistics for the peak counts obtained from all sites qualifying for population trend analysis (129 sites with 3+ years of data), the subset of these which qualify as small population sites ($K_j \leq 10$; 117 sites), the subset which qualify as large population sites ($K_j > 10$; 12 sites), and the subset of large population sites excluding the site with the highest mean peak count ($K_j = 94 \pm 5$) which is substantially higher than the mean peak counts of the other large population sites.

Peak Count Statistic	All sites qualifying for population trend analysis	Small population sites	Large population sites	Large population sites excluding outlier
Minimum	0	0	1	1
1st Quartile	1	1	10	10
Median	3	3	14.5	14
Mean	5.66 ± 0.01	3.626 ± 0.004	25.2 ± 0.4	15.3 ± 0.1
3rd Quartile	6	5	23	18.75
Maximum	167	19	167	38
Total No. of Peak Counts	848	768	80	70

Table 2. Comparison of site characteristics for small population sites included in the population trend analysis (117 sites with $K_j \leq 10$ and 3+ years of data) with site characteristics for large population sites included in the population trend analysis (12 sites with $K_j > 10$ and 3+ years of data). Connectivity scores: 1 = completely isolated by many kilometres, 2 = isolated from nearby sites by sub-optimal habitat, 3 = linked by corridors, 4 = part of a larger group of populations occupying more or less continuous habitat. Conservation designations: SSSI = site of special scientific interest, NNR = national nature reserve, ONR = other nature reserve, CWS = community wildlife site, NP = national park, NCD = no conservation designation. Sites may have more than one conservation designation.

		Percentage (%) of small population sites	Percentage (%) of large population sites
Area Class	≤ 5 ha	13	17
	> 5 ha	84	83
	NA	3	0
Connectivity Score	1	3	0
	2	13	17
	3	9	25
	4	74	58
	NA	2	0
Conservation Designation	SSSI	62	83
	NNR	4	17
	ONR	16	17
	CWS	15	8
	NP	40	33
	NCD	15	0

Table 3. Results from fitting the generalised linear model PeakDay ~ Year + SiteOSNorthing + SiteOSEasting + MeanSpringTemp + No.ofVisits (family = Poisson, link = log) using site level data. Model $R^2 = 0.135$. Asterisks indicate statistically significant correlations, where * = P < 0.05, ** = P < 0.01 and *** = P < 0.001.

Variable	Estimate	Std. Error	Significance
Intercept	29	4	***
Year	-1.2×10^{-2}	0.2×10^{-2}	***
Site OS Northing	-1.7×10^{-7}	0.5×10^{-7}	***
Site OS Easting	-1.3×10^{-7}	0.6×10^{-7}	*
Mean Spring Temp.	-7.7×10^{-2}	1.0×10^{-2}	***
No. of Visits	7×10^{-3}	2×10^{-3}	***

a 1 degree increase in mean spring temperature.

Surveyors were asked, where possible, to record male and female adders separately and this allowed us to investigate differences between the peak day for males and the peak day for females. We applied the same strict selection criteria and only included peak day estimates obtained from five or more visits recording male and female adders separately before day 153. Not all surveys returned results for males and females separately, either because surveyors were not confident separating them or because the conditions on a given survey were not conducive to reliably distinguishing the sexes by sight and (often) at a distance. Consequently, the number of male peak day and female peak day estimates (238 and 131 estimates, respectively) were lower than the number of peak day estimates using the total number of animals. Figure 5 shows the resulting frequency distributions for male peak day estimates (blue) and female peak day estimates (red), where the estimates are binned on fortnightly intervals. Figure 5 shows that the male peak day distribution peaks a fortnight earlier than the female peak day distribution.

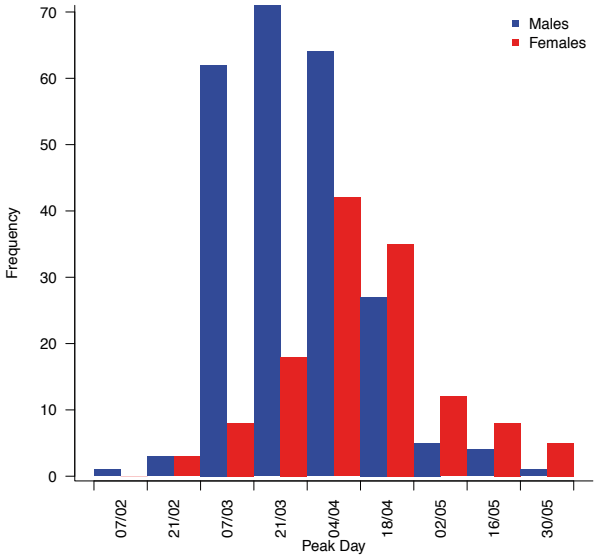

Figure 6. Frequency distributions for male peak day estimates (blue) and female peak day estimates (red), where the estimates are binned in fortnightly intervals. For each bin, the date (day/month) given is the midpoint of that bin.

4. Discussion

The population trend analysis shows that, on average, the small population sites have shown a significant decline in their peak counts over time ($K_{norm,i} \propto -(0.05 \pm 0.01) Y_i$), while the large population sites have shown a significant, but slightly weaker, increase over time ($K_{norm,i} \propto (0.03 \pm 0.01) Y_i$). This confirms earlier informed opinion that small populations are more prone to decline than large populations (Baker et al., 2004). More than 90 % of the sites included in the population trend analysis fell into the small (declining on average) population category, with only 12/129 sites being classed as large (increasing on average) population sites ($K_j > 10$). If these proportions and trends are representative of UK adder sites in general, then the results from MTAC suggest that adders may become increasingly restricted to a few large population sites, making the species increasingly vulnerable to extinction in the UK. The rate at which these small population sites are declining implies the average small adder population could be extinct by 2032.

The declines we have measured in UK adder populations are consistent with the wider picture of snake declines shown by Reading et al. (2010), who showed that 11 out of 17 snake populations spread across Europe, Nigeria and Australia have declined over a 20 year period. Saha et al. (2018) subsequently analysed data from the Living Planet database to show that such global declines are not confined to snakes, but shown by the entire reptile taxon. Both studies stressed the importance, not only of monitoring reptile declines, but also of collecting auxiliary data to identify potential causes, as we have attempted to do via MTAC.

We found no systematic difference between the area classes reported for the declining small population sites and the increasing large population sites. This suggests that the small population sites have sufficient area to support larger populations but habitat quality or some other factor is depressing the population, or that the wider site around them potentially contains other hibernacula which may or may not be included in MTAC. There was also no significant difference between the median connectivity scores for the small and large population sites. However, despite this, none of the large population sites reported habitat fragmentation as a negative factor, while 16 % of the small population sites did. The declines seen in the small population sites are consistent with inbreeding depression, as seen in an isolated Swedish adder population, where reduced genetic diversity, smaller brood sizes and reduced offspring viability (Madsen & Shine, 1996) have been linked to a population decline (Madsen et al., 1999). This suggests small population sizes and increased habitat

Table 4. Results from fitting the generalised linear model MeanPeakDay ~ MeanOSNorthing + MeanSpringTemp + No.ofSitesSurveyed (family = Gaussian, link = log) using data averaged over all sites surveyed in a given year. Model $R^2 = 0.882$. Asterisks indicate statistically significant correlations, where * = P < 0.05, ** = P < 0.01 and *** = P < 0.001.

Variable	Estimate	Std. Error	Significance
Intercept	5.2	0.2	***
Mean OS Northing	-1.3×10^{-6}	0.3×10^{-6}	**
Mean Spring Temp.	-6×10^{-2}	2×10^{-2}	**
No. of Sites Surveyed	2.4×10^{-3}	0.9×10^{-3}	*

fragmentation may be combining to promote inbreeding and further declines at some of these sites. Work is currently ongoing to quantify genetic diversity across a sample of UK sites and its role in relation to other factors (S. Ball, T. Garner & N. Hand, personal comm.).

There was no significant difference between the large and small population sites in the median number of negative factors reported per site, nor in the median number of positive factors reported per site. However, there were more positive than negative reports for habitat management and forestry operations for the large population sites, while for the small population sites, negative reports outweighed positive reports for both factors. The large population sites included a higher proportion of SSSI and NNR designations and all of the large population sites had at least one conservation designation (SSSI, NNR, ONR, CWS or NP), whereas 15 % of the small population sites had no conservation designation. This may have contributed towards more positive habitat management and forestry operation outcomes in the large population sites and the fact that neglect/succession was the third biggest negative factor reported for the small population sites. However, simply having a conservation designation clearly does not guarantee positive management impacts for adders. This conclusion is not unique to the UK; Reading et al. (2010) likewise found that eight snake populations had declined out of the 14 within their sample that were located within protected areas.

Across all the MTAC sites, by far the most frequently reported negative factor was public pressure (48 % of sites), followed by habitat management (22 % of sites) and habitat fragmentation/isolation (17 % of sites). Comparing these top three negative factors with those reported in previous studies (Cooke & Scorgie, 1983; Hilton-Brown & Oldham, 1991; Baker et al., 2004) indicates some notable changes in the perceived importance of factors over time. There has been a reduction in the perceived importance of agricultural changes (no longer in the top three in Baker et al., 2004 or MTAC), with high citation of agricultural changes in the earlier studies likely reflecting the intensification of agriculture during the 1970s–80s. Persecution also entered the top three in two out of the three previous studies, but is ranked fifth in MTAC. Habitat fragmentation is the third most cited negative factor in MTAC but was not even included as a category in the earlier studies and this probably reflects both an increase in the importance of this factor as well as increasing awareness of its significance. Negative reporting of public pressure and habitat management (the top two negative factors in MTAC) has increased dramatically across the four studies. Where public pressure was reported to MTAC, surveyors variously cited dog walking, mountain biking, photographers, vehicles, trampling of vegetation and collection of adders as causing problems at sites. There is very little quantitative data relating to the effects of disturbance on reptiles and the dramatic increase in the reporting of public pressure, such that nearly half of sites are now negatively affected by public pressure, suggests this factor needs urgent investigation, both to assess its impacts on adder populations and to identify potential measures to reduce these impacts.

The most frequently reported factors positively affecting adder populations were habitat management (28 % of sites), forestry operations (9 % of sites) and neglect/succession (5 % of sites). Where neglect/succession was cited as a positive factor, this was often for sites recovering after earlier aggressive habitat management. Adders have complex habitat requirements, using both open areas for basking and cover for hunting and travelling. Jofré et al. (2016) showed reptiles in conifer plantations prefer stands between 3 – 12 years old, suggesting that managing woodlands to produce a mosaic of small stands of different ages could increase the proportion of sites benefitting from forestry operations. While habitat management was the largest positive factor reported, the fraction of sites experiencing positive effects from habitat management (28 %) only just outweighed the proportion experiencing negative effects (22 %), i.e. where habitat management occurred, only in ≤56 % of cases was it considered to have a positive effect on adders. Examples of negative effects of habitat management given by the surveyors included overgrazing, heavy machinery/mechanical cutting, bracken clearance and managing specifically for another species with different habitat requirements (also noted by Sheldon, 2011). This suggests greater awareness is needed among land managers of adders' habitat requirements and of the threats posed by heavy machinery, particularly during hibernation and the spring emergence period. Advice on habitat management is available (Edgar et al., 2010) but our findings suggest that it requires better communication and/or implementation.

The date that adder counts peaked at the sites varied significantly over time and with spring temperature, site northing and site easting grid references. Peak counts were earlier under higher mean spring temperatures (~5 day decrease in peak day for a 1 degree increase in mean spring temperature) and occurred approximately 10 days earlier by the end of the eleven-year data collection period. Peak counts also occurred earlier at more easterly and northerly sites (~11 day decrease in peak day for a 100 km increase in northing). Earlier emergence for warmer temperatures is expected for ectotherms such as adders and has also been noted by Viitanen (1967) and Macartney et al. (1989). The advancement in peak day over time found in the site-level model fit may be a response to long-term climate warming, similar to the advancement in amphibian breeding behaviour in Britain (e.g. Beebee, 1995; Scott et al., 2008; Carroll et al., 2009), although we detected no significant warming trend in spring or winter temperatures during our (shorter) monitoring period. Alternatively, given the geographical trends in emergence timing, it may simply be an artefact of spatial variation in temporal coverage. It is unclear why adders should emerge earlier at more northerly sites; however this has been noted anecdotally by some surveyors. One possibility may be that lower temperatures and fewer total sunshine hours at northern sites may mean adders need to bask over a longer period to gain the same total amount of thermal energy.

Alternatively, adders at northern sites may simply be better adapted to lower temperatures so allowing them to emerge earlier. At least one invertebrate (glow worm, *Lampyris noctiluca*) shows a similar trend of earlier adult emergence at more northerly sites in the UK (Atkins et al., 2016; J.Tyler personal comm.). Although all four variables (year, mean spring temperature, northing and easting) were significant in the site-level model, there was still large residual variation in emergence timing across the sites and this likely reflects a number of site-specific factors unaccounted for in the modelling, such as site aspect, site shading and regional weather variations. Hibernacula with southerly aspects should warm faster, producing earlier emergence, while high site shading by trees and other vegetation may delay warming and emergence. Blouin-Demers et al. (2000) similarly observed large variation in emergence timing between different black rat snake hibernacula and found larger snakes emerge earlier, possibly due to lower predation risk. This suggests variations in predation risk and the size distributions of adders between hibernacula may also be a contributing factor. Surveyor activity (i.e. exact timing of visits) is another large and unavoidable source of variation, though we attempted to minimise this as much as possible by requiring five or more visits before the start of June and controlling for number of visits in the model.

The trends in emergence timing have important implications for habitat management. If hibernacula can be located, they can be avoided when carrying out habitat management using heavy machinery and operations can be timed to avoid the emergence period. The majority of peak counts were recorded from ~1st March onwards, however adders begin emerging several weeks before the peak count is reached, so a general cut-off of 1st February for management operations may be appropriate. However, managers should be aware that adders may emerge ~2–3 weeks earlier in a warm spring versus a cold spring (based on a 3 degree difference between the maximum and minimum mean spring temperatures during this study of 6° C and 9° C) and management operations at more northerly/easterly sites may need to be completed earlier.

We found that the peak counts of male adders occurred ~2 weeks earlier than the peak counts for female adders. This is consistent with the earlier emergence of males noted in other studies (Prestt, 1971; Phelps, 2008), with this early basking behaviour corresponding to a period of active spermiogenesis (Viitanen, 1967; Prestt, 1971; Herczeg et al., 2007). The fact our study included nearly twice as many peak day estimates for males as for females is a measure of the bias in the survey methodology towards detection of these early basking males in preference to the later emerging females. Sheldon (2011) found that non-breeding females not only emerge later than males but also disperse more quickly from hibernacula and that females in the Wyre Forest bred on average every three years, such that the number of females observed in spring counts may only represent the fraction of females that are breeding in any given year.

MTAC has demonstrated the potential of spring counts made by volunteer surveyors to collect quantitative data on adder populations, which can be used to derive population trends. To date, the scheme has relied on experienced surveyors. However, the skill level required is comparable to that required for other skilled citizen science surveys operating in the UK, such as the UK Breeding Bird Survey (organised by the British Trust for Ornithology; Baillie et al., 2006) and the National Bat Monitoring Programme (organised by the Bat Conservation Trust; Barlow et al., 2015), which both provide regional training to new volunteers to help them attain the required experience. If such training schemes can be developed for MTAC, this would greatly increase the volunteer pool available and the number of sites that can be monitored.

4.1 Caveats and Assumptions

We have assumed that changes in the number of adders recorded at a given site reflect genuine changes in population size and are not influenced by systematic changes in their detectability over time. Our analysis accounts for variation in survey effort, which strongly influences snake detectability (Ward et al., 2017), but other factors may also influence detectability. Detectability may increase over time if surveyors become more familiar with a site. However, since MTAC specifically targeted experienced surveyors with good existing knowledge of adder sites, we assume this effect is negligible. Habitat changes may systematically alter detectability at a site, e.g. if increasing vegetation reduces adder visibility over time. The fraction of sites negatively affected by neglect/succession (i.e. where increasing vegetation might potentially reduce adder detectability) was similar for both the large and small population sites, so changes in detectability are unlikely to explain the differing trends for large and small population sites.

We have assumed that detectability is comparable between sites, such that we can equate sites with large mean peak counts to large population sites and sites with small mean peak counts to small population sites. The fact that we derived a declining population trend for the subset of small population sites and an increasing population trend for the subset of large population sites suggests that this separation is plausible. It is possible that some of the small population sites were misclassified large population sites with low detectability. We currently have no information on the habitat types and vegetation densities at each site. If this data can be collected, then an improved population trend model incorporating this information to account for detectability differences between sites (and potentially over time) would improve population trend estimates and increase confidence in the division of sites into small and large populations.

We have assumed the measured declines at small population sites represent a genuine decrease in the number of adders, not that the adders have simply moved elsewhere, and that sites are independent. Adders are highly site faithful with relatively small home ranges (<5 ha; Langton & Beckett, 1995), but nonetheless, this independence assumption may not

be true for sites that are very close together. Just over 80 % of the small population sites and 50 % of the large population sites are within 1 km of another MTAC site, with a mean minimum distance of 2.4 km to the next nearest MTAC site for small population sites versus 13.6 km for the large population sites. We stress that these minimum distances are approximate, given that 58 % of sites supplied grid references at 100 m (three figure) resolution or less. These minimum distances are in good agreement with surveyors reporting the majority of sites to be well connected to other adder populations and suggest it is feasible that adders could move from one site to another, with this potentially more likely among the small population sites. However, since we derive average population trends, if adders move from one site to another, the decrease at one site should be offset by an increase at the other, assuming surveyors are aware of and surveying all hibernacula in the vicinity. The scheme's targeting of experienced surveyors with good prior knowledge of their sites increased the chances of this being the case and there were a few instances where surveyors stopped surveying a site that had gone to zero counts and started surveying another that was very nearby, suggesting this may occasionally happen.

Figure 1 shows that the geographical coverage of MTAC is currently strongly biased towards southern sites and this bias is further exacerbated by the data quantity restrictions required for inclusion in the population trend analysis. Although there are far fewer large population sites, figure 1 shows that these are reasonably well distributed across the geographical range of the small population sites. Work began in 2018 to recruit more surveyors and encourage resurveying of existing sites, in order to reduce this geographical bias and increase the confidence with which MTAC data can be used to represent UK adder population trends in general.

We have identified factors potentially affecting adder populations, however, as in previous studies (Cooke & Scorgie, 1983; Hilton-Brown & Oldham, 1991; Baker et al., 2004), these are the factors perceived by surveyors to be important. As such, there may be a bias towards those factors (e.g. public pressure) that leave clear signs visible to surveyors, while factors which are less visible (e.g. prey limitation, predation) may be underreported. Fluctuations in prey numbers have been shown to limit population size in an isolated adder population (Forsman & Lindell, 1997), while predation by pheasants may severely reduce reptile populations, particularly where pheasant densities are high (Rice, 2016), although much of the evidence on the impacts of pheasant predation remains anecdotal due to a lack of quantitative studies of sufficient size (Dimond et al., 2014). Changes in prey abundance are unlikely to be apparent to MTAC surveyors and the number of dead or injured adders encountered by surveyors may not accurately reflect predation pressure. We stress that detailed field studies are needed to truly assess the relative importance of such factors in relation to those highlighted in this study.

5. Conclusions

Make the Adder Count (MTAC) began in 2005 as a citizen science scheme designed specifically to gather the quantitative data needed to monitor adder population trends in the UK, to assess current threats to adder populations and to inform conservation actions. Surveyors were asked to make three or more counts each spring of adult adders lying out after emerging from hibernation, and to report the positive and negative factors they considered were affecting their adder sites. Between 2005 and 2016, 181 surveyors provided information on 260 sites. 129 of these sites contributed three or more years' worth of data and these were used to derive average population trends over time.

The data indicate a significant decline, on average, across sites with small adder populations ($K_j \leq 10$ individuals), while the relatively few sites with large adder populations (<10 % of sites) showed a significant increase over time. If these trends are representative of the UK as a whole, this suggests adders may become increasingly restricted to a few large population sites. The rate at which the small population sites are declining implies an average small adder population could be extinct by 2032, putting a timescale on this process of just 10–15 years. This restriction of adders to a few large population sites will greatly increase the extinction risk for this priority species in the UK.

Analysis of the factors reported to be affecting sites highlights three key factors which must be addressed, if we are to halt the observed decline in adder numbers in the UK:

1. Public pressure through disturbance (most frequently reported threat; 48 % of sites). The fact that public pressure was reported to negatively affect almost half of the 260 sites suggests this is an issue which needs urgent attention, to quantify its effects on adders and, if necessary, to identify effective means of reducing this pressure.
2. Habitat management (most frequently reported positive factor – 28 % of sites – and second most frequently reported negative factor – 22 % of sites). Greater effort must be made to raise awareness among land managers of the habitat requirements and activity patterns of adders. Currently around half of management operations negatively impact adders; if these negative impacts can be replaced by positive impacts, e.g. by protecting hibernacula and avoiding use of heavy machinery during active periods, this could contribute significantly towards halting population declines.
3. Habitat fragmentation (third most frequently reported negative factor; 17 % of sites). Just over 80 % of the small population sites were <1 km from another site and 16 % of them reported habitat fragmentation as a negative factor, while none of the large population sites were reportedly affected by habitat fragmentation. This suggests maintaining connectivity, especially between small populations, should be a priority.

MTAC has shown that spring counts by volunteer surveyors are a viable method for collecting quantitative data on adder populations, and not only can these data quantify population trends, but they can also provide

insights into adder behaviour and phenology. The current dataset demonstrates the earlier emergence of males and reveals that adder emergence occurs earlier, not only for warmer springs, but also for more northerly sites. The MTAC database provides an invaluable record of hibernacula locations across the UK and current effort now focuses on improving the geographical coverage of the survey, both by encouraging resurveying of existing sites and recruiting new volunteers. We hope that the declines shown by MTAC may motivate landowners and organisations to act to conserve adders and their habitat, that conservation interventions can be developed which address the three key threats identified in the survey, and that MTAC itself may grow and continue to provide the means to monitor the effectiveness of such interventions.

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