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Cryptic diversity in pygmy chameleons (Chamaeleonidae: *Rhampholeon*) of the Eastern Arc Mountains of Tanzania, with description of six new species

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Abstract. Previous molecular phylogenetic studies of pygmy chameleons have identified several cases of undescribed cryptic diversity of species, some of which have remained undescribed due to a lack of morphological information. Here, we combine descriptive morphology with principal component analysis, to quantify the overall morphological variation, and phylogenetic analysis to describe six new species of *Rhampholeon* from the Eastern Arc Mountains, including populations found in the Udzungwa, Rubeho, Nguru, Ukaguru, and Nguu Mountains. From our study we detected only limited morphometric variation between species. We distinguish the new species using genetics, combined with assessment of morphological features, and their geographical distribution. We highlight the threats to pygmy chameleons in East Africa from habitat change and exporting live specimens for the wildlife trade. Based on our understanding, we note a few species that we consider at risk of decline – mainly based on their narrow distribution and their apparent popularity in the export market. This study also further underlines the extraordinary biological value of the relatively small forest patches (less than 3000 km^2) of the Eastern Arc, which contain more species of chameleons than any other area in mainland Africa.

Keywords. Afromontane, biodiversity, East Africa, chameleons, new species, reptiles.

INTRODUCTION

Many of the best-known species radiations (e.g., Darwin's finches, Caribbean *Anolis*, African cichlids) are characterised by high species richness with substantial morphological disparity among species. However, species radiations can also be characterised by genetic diversification with little ecological or phenotypic divergence between species. Typically, these species are distributed allopatrically, but in ecologically similar areas (Gittenberger, 1991; Losos and Mahler, 2010). Chameleons in the genus *Rhampholeon* are an example of such a radiation (e.g., Branch et

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al., 2014). They are morphologically conservative, exhibiting limited phenotypic diversity, despite notable genetic divergence that has accumulated over millions of years in isolation (Branch et al., 2014; Hughes et al., 2018). Most *Rhampholeon* species occur in fragmented and ecologically isolated mountains in East Africa, where species have evolved in allopatry (Menegon et al., 2009; Branch et al., 2014; Hughes et al., 2018). The species essentially occur in ecologically similar forests on isolated mountains of the Eastern Arc Mountains, the Albertine Rift, and the sky islands of Mozambique and Malawi. Their phenotypic similarity however has resulted in difficulties diagnosing and identifying species using morphological characters (e.g., Menegon et al., 2002; Fisseha et al., 2013).

One particular radiation demonstrating morphological conservatism is the *Rhampholeon uluguruensis/moyeri* species complex from the Eastern Arc Mountains, which shows large phylogenetic diversity across its distribution (Fisseha et al., 2013). However, external morphological diversity appears to be low. For example, *Rhampholeon uluguruensis* from the Uluguru Mountains (Tilbury and Emmrich, 1996) was the first *Rhampholeon* found in the Eastern Arc Mountains to bear a soft, tuberculated rostral process. This feature is similar to that of *Rhampholeon nchisiensis* (Loveridge, 1953) from the Southern Highlands of Tanzania and Malawi, and of *Rhampholeon boulengeri* (Steindachner, 1911) from the montane forests of the Albertine Rift. Subsequently, the morphological analysis of *Rhampholeon* individuals from the Kihanga and Kitolomero valleys of the Uzungwa Scarp Nature Reserve bore strong resemblance to *R. uluguruensis* but were distinguished by the number of their interorbital scales and the number and arrangement of hemipenial papillae (Menegon et al., 2002). These differences became the basis for describing a new species, *R. moyeri* (Menegon et al., 2002).

Phylogenetic analyses that have incorporated wide geographic coverage across the Eastern Arc Mountains have shown highly divergent lineages within *R. moyeri* with distinct lineages endemic to single mountain blocks (Matthee et al., 2004; Fisseha et al., 2013). Furthermore, *R. uluguruensis,* restricted to the Uluguru Mountains, is deeply divergent from other members of the clade (Fisseha et al., 2013). Moreover, there are two highly divergent clades from the Uzungwa Scarp Nature Reserve populations, both currently assigned to *R. moyeri* suggesting that *R. moyeri* as currently understood is paraphyletic (Fisseha et al., 2013), with specimens in the type series assignable to both these clades. One clade is restricted to the Kitolomero valley and was provisionally designated as *R.* cf. *uluguruensis*, whereas the other clade is from Kihanga (the type locality for *R. moyeri*). In addition, two previously unknown but highly divergent clades were found from the Nguu and the Nguru Mountains, the latter of which is more closely related to *Rhampholeon beraduccii* (Mariaux and Tilbury, 2006) than to the species in the *R. uluguruensis/moyeri* complex. Despite substantial genetic differences shown in these studies, there are few identifiable and diagnosable morphological traits to separate lineages (e.g., Menegon et al., 2002; Mariaux et al., 2006; Fisseha et al., 2013; Branch et al., 2014).

Given the results from phylogenetic studies on *R. uluguruensis/moyeri* complex (e.g., Fisseha et al., 2013), the complex contains a number of unrecognised species. We combine existing data with new data from six candidate species of *Rhampholeon* from the Eastern Arc Mountains in a phylogenetic analysis (including Uzungwa Scarp Nature Reserve in the Udzungwa Mts; Mafwomero Forest Reserve in the Rubeho Mts; Mkingu Nature Reserve in the Nguru Mts; Mount Kanga, in the Nguru landscape; Mamiwa Kisara Forest Reserve in the Ukaguru Mts; and Nguu North and Kilindi Forest Reserves in the Nguu Mts). Using combined data, we provide taxonomic descriptions of six new species and outline, based on their distribution, the conservation status of these new species.

MATERIAL AND METHODS

Fieldwork

Chameleons were collected over several years from seven localities in the Eastern Arc Mountains (Table S1). Where possible, multiple individuals from each population were taken as specimens and fixed in buffered 2–4% formalin, then transferred into 70% ethanol or fixed directly in 80% ethanol for permanent conservation. A small piece of tissue (either liver or muscle) was collected before fixation and stored in 99% ethanol.

Morphometric analyses

Adults of *R. moyeri*, *R. uluguruensis* and each of the new species were measured using digital callipers to the nearest mm: Snout-Vent Length (SVL) – tip of the snout to the anterior edge of the cloaca; Tail Length (TL) – tip of tail to posterior edge of the cloaca; Head Length (HL) - from just behind the tip of the casque to the tip of the snout; Head Width (HW) – maximum width of head; Orbit Diameter (OD) – maximum horizontal width of orbit; Inter-orbital Distance (ID) – minimum width between orbits across crown (Branch et al., 2014); Parietal Crest to Snout (PCS) – distance from the middle of parietal crest to the tip of the snout from a sagittal view. All measurements were taken on the right side of the specimen.

Morphological variation between the species was examined using a multivariate approach in SPSS v.21. Given the small number of individuals per species in the dataset (eight species, total n = 56), the analysis was not partitioned by sex. Using log transformed original variables, a linear regression was run for each morphometric trait using a covariate (SVL) to remove the effect of body size. The resulting residuals were saved and input into a principal component analysis (PCA), to generate linear combinations of variables that explain overall morphological variation. Sampling adequacy for the PCA was assessed using a Kaiser-Mayer-Olkin test, while communalities were assessed to evaluate the contribution of each trait to the analysis (Tabachnick and Fidell, 1996). The varimax rotation of the component matrix was applied to maximize variation across multidimensional space. The first two principal components were extracted, and scores saved for each individual. Only the first PC had an eigenvalue greater than 1 (see Results), so an analysis of variance (ANOVA) was run for only PC1 with species as the fixed factor. Pairwise *ad hoc* Bonferroni tests were run for PC1 to examine pairs of species that differ for this PC.

Phylogenetic analyses

A phylogenetic analysis of the *R. uluguruensis/moyeri* complex was carried out using existing and new sequences from individuals from the type locality for *R. moyeri* (Kihanga) plus six additional localities in Tanzania (Kanga, Kitolomero, Nguru, Nguu, Rubeho, Ukaguru). The analysis included GenBank data from 18 additional described species of the 22 *Rhampholeon*, and all three species from the genus *Rieppeleon* as outgroup taxa (Table S1). DNA was extracted from the new samples using salt extraction (Aljanabi and Martinez, 1997), with PCR amplification, and cycle sequencing of two mitochondrial gene fragments following standard procedures: a 25 µl PCR reaction included 3 µl of 1 mM dNTPs, 3 µl of 25 mM $MgCl_2$, 0.2 µl of 10 pmol forward and reverse primer, 3 µl of Mg^{2+} free buffer solution, 0.1 µl (0.5U) Taq polymerase, and 1-2 µl of 25 ng/µl genomic DNA. Thermal cycling was run with initial denaturation for 4 min at 94 °C followed by: 35 cycles with denaturation for 30 s at 94 °C, annealing for 40 s at 55-57 °C, extension for 40 s at 72 °C, and final extension for 4 min at 72 °C. Primers used for amplification were ND2: L4437b (Macey et al., 1997a) and H5934 (Macey et al., 1997b), and 16S: L2510 and H3080 (Palumbi, 1996). PCR products were run on a 1% agarose gel and visualized under a UV light to verify amplification. Amplicons were sequenced directly using the forward primers at Macrogen (Seoul, Korea). Sequences were edited and aligned using Geneious software v 4.7 (Kearse et al., 2012). All new sequences have been deposited in GenBank (Table S1).

A Bayesian analysis of 1384 characters from the two mitochondrial genes (ND2: 923 bp and 16S: 461 bp) was used to investigate optimal tree space using MrBayes 3.2.2 (Huelsenbeck and Ronquist, 2001). To investigate which evolutionary model best fit the data, jModeltest was used (Posada, 2008). The AIC test specified the most complex model (GTR+I+G) for both markers. Therefore, two unlinked data partitions were created, each specifying six rate categories, including the gamma distribution and invariable sites, with uniform priors for all parameters. For 16S, 42 bases were excluded due to poor alignment. To ensure the results were robust, the MCMC was run twice in parallel for 20 million generations (four chains in each run), with trees sampled every 1000 generations. A 10% burn-in was examined (2 million generations, 2000 trees) in Tracer v1.6 (Rambaut et al., 2014) to check that the effective sample size (ESS) of all parameters met a threshold of 200 after burn-in. A 50% majority rule tree was constructed and nodes with ≥ 0.95 posterior probability considered supported.

In addition to the Bayesian analysis, a maximum likelihood (ML) search was run using RAxML HPC 7.2.8 (Stamatakis, 2006) on the CIPRES Science Gateway (www.phylo.org/sub_sections/portal/) for the combined dataset. The datasets were partitioned as in the Bayesian analysis, with a GTR+I+G model for all markers and rapid bootstrapping halted automatically (Stamatakis et al., 2008). This analysis was run three times to ensure that independent ML searches produced the same topologies. We considered nodes with a bootstrap value of ≥70% as supported.

Pairwise sequence divergence values (net p-distances) were estimated between species for both markers using MEGA v7 (Kumar et al., 2016). In addition, a barcoding approach was used to compare inter- and intra-specific sequence divergences, using SpeciesIdentifier v1.8 (Meier et al., 2006). Pairwise comparisons were generated for all *Rhampholeon* individuals in the phylogeny for the ND2 marker, and a frequency distribution of inter- and intraspecific comparisons was then made.

RESULTS

Morphological characterization of the R. uluguruensis/moyeri *complex*

Species in the *R. uluguruensis/moyeri* complex lack robust diagnostic characters; with the absence of any ossified horn, or crest; tubercles are small and often fragmented; tail is always very short; and the head lacks well

defined external features (Fig. 1). The qualitative descriptions of characters given here (e.g., number of tubercles, their position, etc.) often show intraspecific variation on par with interspecific variation. Therefore, these traits are not discussed as diagnostic characters but merely as morphological features characterizing the range of variation across and within these species. There are very few characters that show consistent differences between species (e.g., size and shape of the rostral process, presence/absence of inguinal and axillary pits, angular flexure of the snout or number of hemipenial papillae) and their description could be useful for species identification. In most of the cases there is comprehensive combination of morphological characters that distinguishes one species from the others of the complex.

Adult *Rhampholeon* species range from 35-110 mm in total length. All the species in the complex have short, non-prehensile tails, whereas in other species they can have relatively longer tails. Body colour is usually brown, grey, or green; although colour can lighten and darken, and green individuals can rapidly turn dark brown. *Rhampholeon* seem to have a smaller range of colour variation compared to chameleons in most other genera. There are usually 2 to 3 transverse lines on the flanks that can be dark brown to black, orange, green or blue in colour, larger, darker scattered spots of variable size are often present on the body and limb surface.

Body and limbs. Scales are sub-homogeneous to finely heterogeneous stellate, interlocked, small tubercles. Larger conical tubercles are scattered across body and limbs. Usually, three prominent enlarged cones are spaced along the upper flank, the first (and largest) is situated above the shoulder, the third is located above the sacrum and the second about 2/3 of the way along the flank. Dorsal crest is composed by an undifferentiated row of clustered tubercles in its first third and a clearer crenulation in its central and distal part. Crenu-

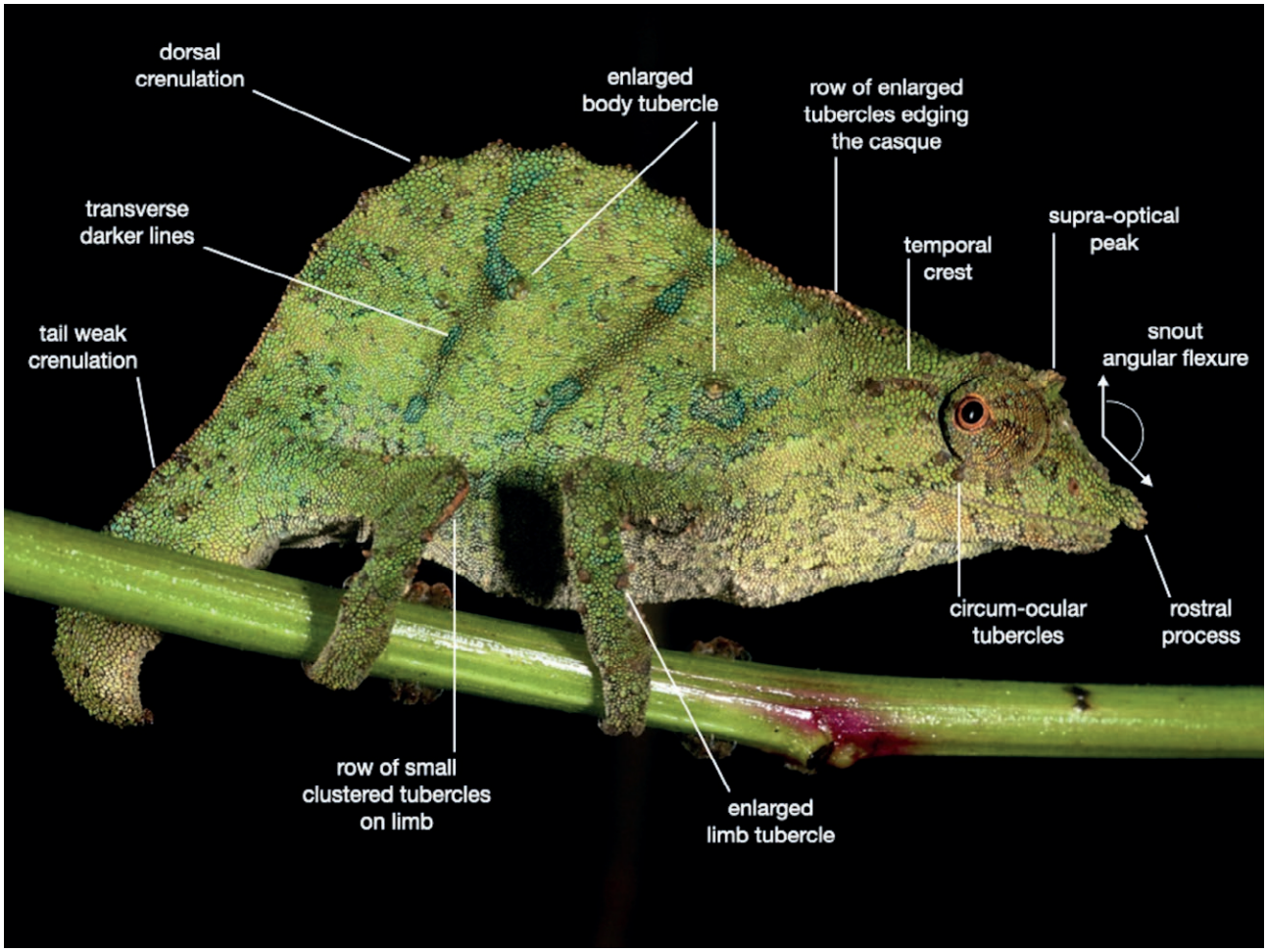

Fig. 1. Main features of external morphology used to describe and differentiate species in the *R. uluguruensis/moyeri* complex.

lation tubercles vary in shape and size from flat polygonal scales to more clearly conical ones. Crenulation may extend onto the tail, although this will be less conspicuous than the dorsal crenulation, or completely absent. There is no gular or ventral crest. There are enlarged spinous tubercles on the distal surface of the forelimbs and a prominent spine is often positioned anteriorly over the mid-radius. A longitudinal ridge of clustered and raised small tubercles, usually paler in colour on the limbs. Claws are always strongly bicuspid. The palms and soles have a smooth 'cobblestoned' appearance, with low palmar accessory spines present, usually close to base of the claws.

Head. Occiput flat. Temporal crest usually strong, formed by a ridge topped with rounded tubercles, lowest anteriorly and ending posteriorly in a large tubercle. Enlarged tubercles are present around the orbit. Parietal crest is usually weakly indicated by a row of round midline granules. Accessory rows of distinct granules may be present and, including the parietal granules form a Y or T shaped arrangement. The superior edge of the casque is edged in a row of enlarged subconical tubercles, which converge at the occiput. Above the eyes, a soft conical cluster of tubercles forms a supra-orbital peak, between the two peaks there is a V shaped row of slightly enlarged tubercles varying in number from 12 to 18. An accessory peaked cluster of tubercles forms distinct protuberances above nasal aperture. In all species in the *R. uluguruensis/moyeri* complex the two canthal ridges merge into the base of a soft, tuberculated rostral process which projects *ca.* 1 to 2 mm over the edge of the snout, in some individuals the rostral process is reduced to a short and pointy projection.

Hemipenes. The everted hemipenes are strongly club-shaped in all species. The bi-lobed distal ends bear two comparable large curved apical horns. Each of the horns possesses up to twelve thorn-like papillae, with the apical three usually arranged in a single row and the basal ones in two rows. The pedicel, truncus and apex cannot be differentiated because calyces and capitate structures are absent. The surface of the hemipenes is relatively smooth.

Morphometric analyses

Morphometric data from 56 individuals were input into the multivariate analyses (Table 1). The log transformed variables were size corrected and these residuals for each character were analysed using a PCA. The Kaiser-Mayer-Olkin test was high (KMO = 0.84), indicating adequate sampling for the PCA. The communality for eye diameter was low, indicating that its contribution to the overall variation in the dataset was negligible, so it was excluded from the analysis (Tabachnick and Fidell, 1996). With the two principal components (PCs) in the analysis, the remaining variables were close to, or higher than 0.70, so they were all retained in the analysis. However, only PC1 had an eigenvalue greater than 1.0 (Eigenvalue = 3.2) with PC2's eigenvalue at 0.65 (Table 2).

Given the small contribution of morphological variation represented by PC2, only PC1 was run for the ANOVA and pairwise comparisons. The ANOVA revealed significant differences between species ($F = 6.2$, $P < 0.001$), but the pairwise tests indicated that this is due to just two species (*R. nicolai* sp. nov. and *R. sabini* sp. nov.) both having higher values on PC1 than some of the other species (Fig. 2; Table S2), although they are not significantly different from each other. Given that PCS, HL and TL load highly on PC1, this would suggest that these traits are larger for any given body size in *R. nicolai* sp. nov. and *R. sabini* sp. nov. than for other species.

Phylogenetic analyses

Both the Bayesian and maximum likelihood topologies are consistent with other species level phylogenies for this genus (e.g., Branch et al., 2014). In addition, there is a well-supported clade corresponding with the *R. moyeri* complex, which is sister to *R. uluguruensis* (Fig. 3). Within this clade, *R. moyeri* from the type locality (Kihanga) is well-supported and distinct. There are five additional previously unrecognised lineages that represent candidate species from different mountains: Uzungwa Scarp Nature Reserve in the Udzungwa Mts; Mafwomero Forest Reserve in the Rubeho Mts; Mount Kanga, in the Nguru landscape; Mamiwa Kisara Forest Reserve and Mikuvi in the Ukaguru Mts; and Nguu North and Kilindi Forest Reserves in the Nguu Mts. Individuals from Mkingu Nature Reserve in the Nguru Mts are in a separate clade that includes species from central and east Africa (i.e., *R. boulengeri*, *R. hattinghi*, *R. acuminatus*, and *R. beraduccii*; Fig. 3).

The sequence divergence values for all the candidate species in the *R. uluguruensis/moyeri* complex were comparable to that found between other *Rhampholeon* species, ranging from 5.1–14.5% for ND2 and 4.4–12.7% for 16S (Table 3). In addition, the frequency distribution of inter- and intra-specific values for ND2 shows that the candidate species fall within the range of described species (Fig. S1).

Below we describe species which show high molecular sequence divergence and are allopatrically distributed. Despite the morphological conservatism, we also indicate, when possible, the best features that can be used as diagnosable morphological differences.

Table 1. Measurements (mm) of the 52 *Rhampholeon* specimens included for the morphometric analyses. Field ID numbers or voucher accession numbers (MTSN and MUSE = MUSE - Science Museum of Trento, MHNG = Muséum d'Histoire Naturelle, Geneva), sex, SVL (from tip of the snout to the anterior edge of the cloaca), TL (from tip of tail to posterior edge of the cloaca), HL (from just behind the tip of the casque to the tip of the snout), HW (maximum width of head), OD (maximum horizontal width of orbit), ID (minimum width between orbits across crown) and PCS (distance from the middle of parietal crest to the tip of the snout from a sagittal view).

ID number	Species	Sex	SVL	TL	HL	HW	OD	ID	PCS
MUSE 13745	*R. beraducci*	male	35.06	6.63	11.62	6.66	4.10	4.04	4.14
MUSE 13746	*R. beraducci*	male	35.06	6.80	12.12	6.87	3.99	3.98	4.90
MTSN 5379	*R. colemani* sp. nov.	male	37.58	7.04	11.44	7.78	3.93	4.00	5.01
MTSN 5380	*R. colemani* sp. nov.	female	41.50	6.18	12.29	7.07	4.27	4.02	5.38
MTSN 5381	*R. colemani* sp. nov.	female	35.48	6.10	10.51	6.20	4.08	3.80	4.99
MUSE 11033	*R. colemani* sp. nov.	female	29.03	5.44	10.05	5.37	3.53	3.04	4.64
MUSE 11029	*R. colemani* sp. nov.	male	30.13	6.72	9.57	5.96	3.59	2.77	4.31
MUSE 11031	*R. colemani* sp. nov.	male	29.04	4.75	9.01	4.90	3.52	3.06	4.61
MUSE 11032	*R. colemani* sp. nov.	female	29.45	5.35	9.89	5.64	3.62	3.06	4.61
MTSN 8542	*R. waynelotteri* sp. nov.	male	40.03	9.33	13.12	7.49	4.56	3.82	5.98
MTSN 8537	*R. waynelotteri* sp. nov.	male	43.06	9.50	13.70	8.02	4.80	4.12	6.11
MTSN 8540	*R. waynelotteri* sp. nov.	male	43.29	11.74	13.18	7.94	5.00	4.34	6.00
MTSN 8539	*R. waynelotteri* sp. nov.	female	41.80	9.28	14.27	7.78	4.73	4.03	6.23
MTSN 8541	*R. waynelotteri* sp. nov.	female	46.30	8.09	14.96	8.79	5.14	3.89	6.98
MTSN 5235	*R. moyeri*	male	44.89	11.50	13.81	7.57	4.58	4.78	6.13
MTSN 5372	*R. moyeri*	female	52.63	10.62	14.76	8.84	5.57	4.40	7.08
MTSN 5373	*R. moyeri*	female	50.67	11.24	15.21	8.53	4.84	5.45	6.84
MTSN 5374	*R. moyeri*	female	52.36	8.61	15.01	8.36	4.76	4.28	6.99
MTSN 5376	*R. moyeri*	male	37.85	7.57	11.41	6.32	4.12	4.38	5.31
MTSN 5377	*R. moyeri*	male	43.79	9.86	14.00	7.87	4.43	4.55	6.84
MTSN 5378	*R. moyeri*	male	40.09	8.65	13.83	7.87	4.45	4.37	6.57
MTSN 5592	*R. nicolai* sp. nov.	male	45.55	11.01	15.48	9.06	5.33	5.77	6.57
MTSN 5593	*R. nicolai* sp. nov.	female	33.83	6.24	11.46	6.75	3.66	3.77	5.00
MHNG 2624.47	*R. nicolai* sp. nov.	male	36.21	9.44	13.12	7.91	4.66	4.21	5.55
MHNG 2624.56	*R. nicolai* sp. nov.	male	43.10	12.46	14.02	9.70	5.24	4.76	6.44
MHNG 2624.49	*R. nicolai* sp. nov.	male	35.57	8.26	12.49	7.57	4.17	3.74	5.41
MHNG 2624.5	*R. nicolai* sp. nov.	male	34.32	8.49	13.09	8.05	4.51	4.49	5.78
MHNG 2624.86	*R. nicolai* sp. nov.	male	33.75	10.31	12.30	6.97	4.27	3.56	5.87
MHNG 2624.87	*R. nicolai* sp. nov.	male	27.84	9.26	10.46	6.39	3.83	3.47	4.96
MHNG 2624.48	*R. nicolai* sp. nov.	female	36.21	8.98	13.35	7.31	4.13	4.09	5.40
MHNG 2624.57	*R. nicolai* sp. nov.	female	39.09	7.49	14.00	8.21	5.00	4.65	6.19
MUSE 14034	*R. princeeai* sp. nov.	male	37.54	10.14	11.87	7.42	4.21	4.43	5.31
MUSE 14036	*R. princeeai* sp. nov.	male	35.36	10.18	12.05	7.02	4.15	4.84	5.65
MUSE 14033	*R. princeeai* sp. nov.	female	51.79	12.49	16.85	7.23	5.58	5.56	7.08
MUSE 14285 (MTSN 5537)	*R. princeeai* sp. nov.	male	34.04	9.47	11.04	5.71	4.13	4.42	5.41
MUSE 14286 (MTSN 5538)	*R. princeeai* sp. nov.	female	34.96	7.50	11.40	6.64	4.18	4.23	5.44
MTSN 5013	*R. rubeho* sp. nov.	male	41.12	11.12	14.76	8.17	5.00	4.53	6.47
MTSN 5012	*R. rubeho* sp. nov.	female	42.58	8.62	13.86	8.04	4.51	4.72	5.83
MTSN 5014	*R. rubeho* sp. nov.	female	40.23	8.71	13.14	6.95	4.09	4.20	5.68
MTSN 8895	*R. rubeho* sp. nov.	male	48.08	16.68	17.13	10.38	5.72	6.03	7.79
MTSN 8896	*R. rubeho* sp. nov.	male	38.79	11.26	13.49	7.50	4.60	4.46	5.83
MTSN 8899	*R. rubeho* sp. nov.	male	41.07	13.08	13.53	8.03	4.58	4.83	6.12
MTSN 8893	*R. rubeho* sp. nov.	female	42.94	12.29	14.71	8.18	4.67	5.46	6.36
MTSN 8898	*R. rubeho* sp. nov.	female	46.71	10.27	15.26	9.23	4.92	5.81	6.82
MTSN 5092	*R. sabini* sp. nov.	male	37.19	7.05	12.19	7.00	6.03	4.91	5.23
MTSN 5192	*R. sabini* sp. nov.	male	25.41	7.66	11.34	6.91	4.24	3.27	5.54

ID number	Species	Sex	SVL	TL	HL	HW	OD	ID	PCS
MTSN 5193	*R. sabini* sp. nov.	male	36.71	10.26	14.43	8.84	4.85	5.51	5.94
MTSN 5198	*R. sabini* sp. nov.	male	44.60	9.53	14.02	9.04	5.12	5.11	6.44
MTSN 5199	*R. sabini* sp. nov.	male	38.33	7.14	11.56	7.55	4.27	4.31	5.08
MTSN 5195	*R. sabini* sp. nov.	female	38.62	6.46	12.42	7.25	4.65	4.76	6.02
MTSN 5197	*R. sabini* sp. nov.	female	37.99	6.96	12.43	7.58	4.46	4.23	5.42
MTSN 9082	*R. sabini* sp. nov.	female	31.39	5.85	10.41	5.30	3.81	3.34	4.67
MHNG 2817.97	*R. uluguruensis*	male	42.72	10.82	13.17	7.47	4.42	4.14	5.86
MTSN 7764	*R. uluguruensis*	male	46.20	12.10	13.60	7.73	4.95	4.43	6.69
MTSN 7749	*R. uluguruensis*	female	43.10	7.34	12.56	7.91	4.49	4.03	5.71
MTSN 7776	*R. uluguruensis*	female	40.08	7.80	12.49	7.33	4.07	4.22	5.39
MTSN 7755	*R. uluguruensis*	male	45.49	12.28	13.90	7.64	5.06	4.19	6.16
MTSN 7757	*R. uluguruensis*	male	41.86	12.50	13.28	7.81	4.68	4.22	6.59

Table 2. Rotated principal component matrix for PC1 and PC2, with the loadings for each original variable on the principal components for nine species of *Rhampholeon*. Also given is the eigenvalue and % variation for each PC and the significance value from the analysis of variance (ANOVA). Note that the eigenvalue for PC2 is less than 1.

Trait	PC1	PC2
PCS: Parietal crest-snout tip distance	0.90	0.13
HL: Head length	0.73	0.52
TL: Tail length	0.70	0.28
HW: Head width	0.56	0.54
ED: Eye diameter	0.13	0.91
OD: Max horizontal orbit width	0.43	0.62
Eigenvalue	3.2	0.65
% variation	62	13
ANOVA	$P < 0.001$	NA

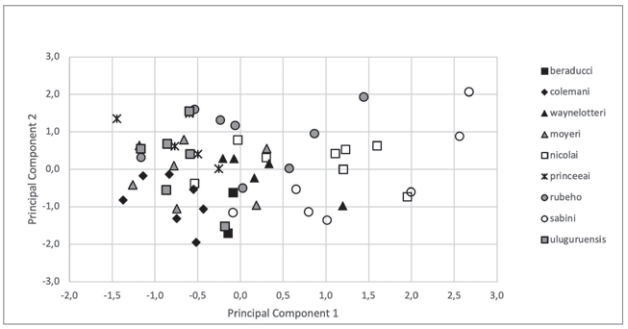

Fig. 2. Scatterplot of the principal component scores on PC1 and PC2 for nine species of *Rhampholeon*.

Species Descriptions

Rhampholeon uluguruensis Tilbury & Emmrich, 1996
Uluguru Pygmy Chameleon
Holotype: ZMB 48421

Synonym
Rhampholeon uluguruensis Tilbury & Emmrich, 1996
Rhampholeon uluguruensis Necas 1999: 284
Rhampholeon (Rhinodigitum) uluguruensis Matthee et al. 2004
Rhampholeon (Rhinodigitum) uluguruensis Tilbury 2010: 199
Rhampholeon (Rhinodigitum) uluguruensis Fisseha et al. 2013
Rhampholeon (Rhinodigitum) uluguruensis Glaw 2015
Rhampholeon (Rhinodigitum) uluguruensis — Spawls et al. 2018: 255

Original diagnosis (verbatim)
A small species with a maximum recorded total length (TL) of 50 mm (largest male 48.5 mm TL, and largest female 50 mm TL; Fig. 4). The tail is short, averaging 24.5% of the TL in males and 20.9% of the TL in females. It has a soft, tuberculated, rostral process and a background scalation of minute sub-homogeneous stellate granules. All specimens have axillary dermal pits, which may be shallow or deep, and in some specimens a mere indication of an inguinal pit is seen. Soles and palms are smooth, claws strongly bicuspid and low accessory palmar/plantar spines are present. Adult males and females have similar SVL. The hemipenis bears two curved apical horns, each with nine papillae comprising a single apical row of three, with six proximal papillae arranged in a double row of three. This combination of characters distinguishes this species from the others of the genus.

Amendments to the original diagnosis
Rhampholeon uluguruensis differs from other *Rhampholeon* for the ND2 gene by having the amino acid pheny-

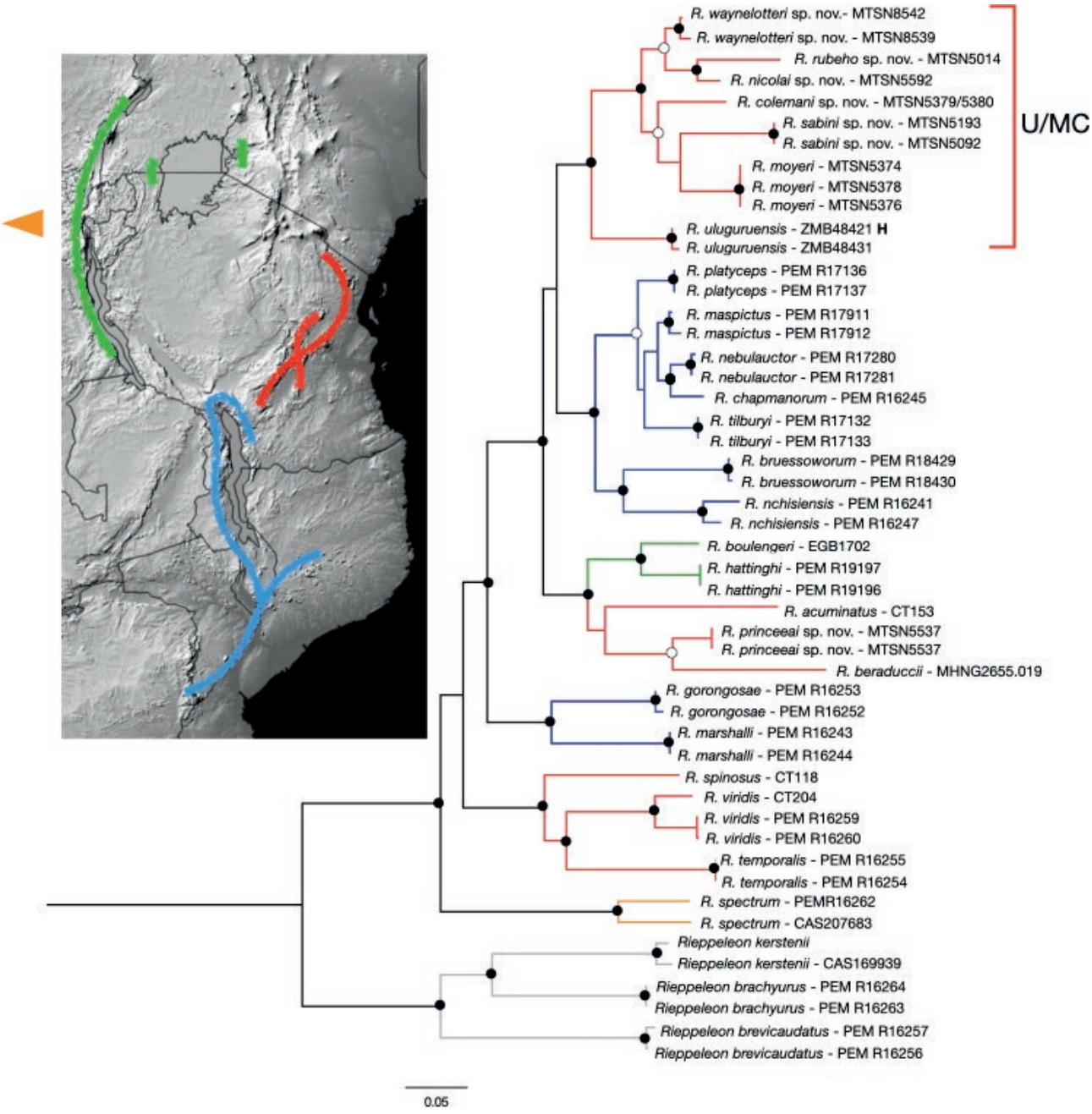

Fig. 3. Maximum likelihood topology for the *Rhampholeon uluguruensis/moyeri* complex (indicated by U/MC), and other members of the genus. Nodes supported by both maximum likelihood (≥70% bootstrap) and Bayesian (≥0.95 posterior probability) analyses are denoted with black circles, those supported by either maximum likelihood or Bayesian alone are denoted with white circles. Branches on the tree are colour coded to match the general distribution regions shown on the map (green: Albertine Rift; blue: The Southern Rift; red: The Eastern Arc Mountains). Orange arrow indicates that *R. spectrum* occurs outside the area represented in the map.

lalanine at codon position 466–468. For the 16S gene, it differs by having a G at position 896.

Distribution and remarks

Restricted to the Uluguru Mountains, including Uluguru Nature Reserve, and Mkungwe Forest Reserve.

Rhampholeon moyeri Menegon, Salvidio & Tilbury 2002
Moyer's Pygmy Chameleon
Holotype: MTSN 006TA

Synonyms

Rhampholeon moyeri Menegon, Salvidio & Tilbury 2002

Table 3. Uncorrected net p-distances for a) ND2 and b) 16S between species of *Rhampholeon* included in the phylogenetic analysis. Values within a species are indicated on the diagonal. Within-species values that were not estimated (only one sequence available for that taxon) are denoted by 'na'.

a)	*Rhampholeon*	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	*moyeri*	0.000																							
2	*princeeai*	0.145	na																						
3	*colemani*	0.111	0.118	na																					
4	*sabini*	0.121	0.000	0.093	na																				
5	*rubeho*	0.065	0.152	0.081	0.119	na																			
6	*waynelotteri*	0.069	0.129	0.079	0.107	0.056	0.013																		
7	*nicolai*	0.085	0.147	0.086	0.116	0.058	0.051	na																	
8	*boulengeri*	0.194	0.230	0.185	0.200	0.171	0.163	0.189	na																
9	*bruessoworum*	0.184	0.228	0.178	0.173	0.172	0.168	0.183	0.158	0.006															
10	*chapmanorum*	0.186	0.216	0.172	0.191	0.161	0.156	0.176	0.166	0.137	na														
11	*gorongosae*	0.218	0.217	0.195	0.214	0.197	0.186	0.200	0.167	0.196	0.191	0.008													
12	*hattinghi*	0.189	0.225	0.175	0.193	0.168	0.158	0.174	0.095	0.171	0.181	0.176	0.000												
13	*marshalli*	0.227	0.259	0.218	0.229	0.215	0.191	0.210	0.177	0.197	0.194	0.140	0.196	0.001											
14	*maspictus*	0.162	0.187	0.150	0.157	0.139	0.130	0.150	0.150	0.107	0.051	0.181	0.162	0.162	0.023										
15	*nchisiensis*	0.129	0.178	0.132	0.130	0.128	0.119	0.124	0.123	0.110	0.120	0.168	0.141	0.159	0.092	0.046									
16	*nebulauctor*	0.174	0.200	0.160	0.171	0.138	0.136	0.151	0.155	0.113	0.052	0.192	0.147	0.176	0.042	0.106	0.006								
17	*platyceps*	0.187	0.177	0.162	0.159	0.158	0.148	0.164	0.149	0.120	0.091	0.167	0.164	0.181	0.057	0.128	0.069	0.001							
18	*spectrum*	0.167	0.183	0.151	0.163	0.155	0.141	0.152	0.140	0.158	0.179	0.140	0.140	0.165	0.154	0.158	0.160	0.169	0.105						
19	*spinosus*	0.239	0.270	0.223	0.222	0.221	0.211	0.208	0.215	0.212	0.219	0.189	0.212	0.209	0.195	0.198	0.207	0.216	0.159	na					
20	*temporalis*	0.226	0.220	0.228	0.220	0.231	0.217	0.237	0.208	0.205	0.219	0.204	0.222	0.210	0.201	0.197	0.216	0.222	0.176	0.186	0.001				
21	*tilburyi*	0.180	0.186	0.170	0.183	0.160	0.143	0.159	0.149	0.146	0.101	0.192	0.169	0.195	0.063	0.129	0.065	0.100	0.166	0.215	0.220	0.000			
22	*uluguruensis*	0.135	0.166	0.115	0.120	0.124	0.123	0.142	0.173	0.162	0.171	0.179	0.157	0.193	0.146	0.140	0.161	0.142	0.166	0.192	0.206	0.163	0.007		
23	*viridis (North)*	0.204	0.235	0.204	0.198	0.195	0.188	0.203	0.196	0.183	0.218	0.187	0.205	0.204	0.194	0.190	0.205	0.202	0.140	0.166	0.161	0.216	0.206	na	
24	*viridis (South)*	0.209	0.239	0.188	0.209	0.195	0.171	0.191	0.191	0.201	0.231	0.181	0.196	0.205	0.192	0.207	0.207	0.216	0.163	0.165	0.175	0.224	0.202	0.070	0.002

b)	*Rhampholeon*	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	*moyeri*	0.000																							
2	*princeeai*	0.084	0.000																						
3	*colemani*	0.048	0.087	na																					
4	*sabini*	0.044	0.078	0.068	na																				
5	*rubeho*	0.119	0.104	0.109	0.127	na																			
6	*acuminatus*	0.102	0.071	0.100	0.094	0.134	na																		
7	*beraduccii*	0.111	0.046	0.101	0.110	0.130	0.085	na																	
8	*boulengeri*	0.075	0.067	0.080	0.076	0.121	0.073	0.080	na																
9	*bruessoworum*	0.081	0.080	0.086	0.083	0.125	0.089	0.111	0.083	0.000															
10	*chapmanorum*	0.053	0.067	0.060	0.068	0.106	0.085	0.094	0.075	0.065	na														

b)	*Rhampholeon*	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
11	*gorongosae*	0.092	0.087	0.097	0.101	0.107	0.098	0.106	0.106	0.091	0.075	0.002													
12	*hattinghi*	0.066	0.061	0.070	0.071	0.107	0.066	0.078	0.017	0.078	0.065	0.095	0.000												
13	*marshalli*	0.089	0.096	0.089	0.100	0.101	0.112	0.115	0.108	0.091	0.075	0.067	0.092	na											
14	*maspictus*	0.058	0.066	0.061	0.074	0.108	0.084	0.091	0.076	0.068	0.008	0.074	0.066	0.076	0.000										
15	*nchisiensis*	0.072	0.067	0.067	0.086	0.103	0.074	0.095	0.080	0.051	0.038	0.082	0.070	0.073	0.041	0.024									
16	*nebulauctor*	0.052	0.073	0.063	0.069	0.114	0.075	0.104	0.080	0.064	0.006	0.078	0.070	0.081	0.008	0.046	0.000								
17	*platyceps*	0.053	0.058	0.051	0.068	0.099	0.080	0.084	0.065	0.060	0.010	0.072	0.056	0.067	0.008	0.034	0.011	0.000							
18	*spectrum*	0.078	0.085	0.087	0.086	0.088	0.110	0.100	0.092	0.082	0.065	0.074	0.083	0.060	0.063	0.064	0.065	0.058	0.048						
19	*spinosus*	0.095	0.100	0.098	0.101	0.132	0.112	0.129	0.112	0.094	0.080	0.083	0.110	0.095	0.080	0.084	0.082	0.073	0.088	na					
20	*temporalis*	0.094	0.089	0.101	0.115	0.133	0.090	0.111	0.099	0.092	0.079	0.080	0.097	0.101	0.078	0.076	0.081	0.070	0.091	0.070	na				
21	*tilburyi*	0.060	0.060	0.058	0.068	0.106	0.085	0.087	0.070	0.068	0.017	0.075	0.059	0.079	0.013	0.043	0.017	0.012	0.067	0.080	0.075	0.000			
22	*uluguruensis*	0.060	0.058	0.055	0.071	0.101	0.078	0.089	0.065	0.055	0.043	0.075	0.061	0.072	0.042	0.041	0.041	0.034	0.063	0.085	0.077	0.043	0.002		
23	*viridis (North)*	0.110	0.112	0.115	0.115	0.120	0.130	0.141	0.117	0.101	0.092	0.102	0.111	0.102	0.088	0.095	0.093	0.085	0.085	0.088	0.080	0.090	0.100	na	
24	*viridis (South)*	0.101	0.108	0.104	0.115	0.113	0.126	0.132	0.106	0.093	0.082	0.094	0.107	0.094	0.080	0.083	0.087	0.075	0.078	0.083	0.070	0.087	0.091	0.024	0.002

Rhampholeon (Rhinodigitum) moyeri Matthee et al. 2004
Rhampholeon (Rhinodigitum) moyeri Tilbury 2010: 181
Rhampholeon (Rhinodigitum) uluguruensis Fisseha et al. 2013
Rhampholeon (Rhinodigitum) moyeri Glaw 2015
Rhampholeon (Rhinodigitum) moyeri Spawls et al. 2018: 252

Original diagnosis (verbatim)

A small chameleon with a maximum total length of 64 mm (maximum SVL = 51 mm) of which the tail comprises an average of 23% in males and 17% in females (Fig. 5). Adult males and females have similar SVL. A soft-tuberculated rostral dermal process and soft supraoptic peaks are present. Between 14 and 19 scales span the interorbital region between the bases of the peaks, with between 20 and 27 scales from peak to peak. The casque is flat posteriorly with a short indistinct parietal crest. The temporal crest is distinct. Deep axillary pits are present but there are no inguinal pits. Background body scalation is of fine sub-homogeneous stellate granules with scattered enlarged rounded tubercles. Claws are strongly bicuspid with low accessory palmar and plantar spines and smooth "cobblestoned" palms and soles. Hemipenis has two large incurving apical horns with prominent thorn-like papillae arranged in two proximal rows of four (occasionally five) papillae, followed by a single terminal row of four (occasionally five) papillae. The above characters distinguish *R. moyeri* from all the other known species of *Rhampholeon*.

Amendments to the original diagnosis

In our specimens, inguinal pits are sometimes faintly indicated, and the maximum number of hemipenial papillae is more than five but never exceeding ten. It also differs from other *Rhampholeon* for the ND2 gene by having the amino acid alanine at position 365–367, glutamine at 454–456, valine at position 475–477, valine at 478–480 and alanine at 619–621.

Distribution and remarks

Udzungwa Mountains. In the Uzungwa Scarp Nature Reserve, the species might occur sympatrically with *R. colemani* with some degree of elevational segregation between the taxa. Further research is required to properly assess the actual distribution of the two species within the Reserve. The individuals from the Kitolomero valley, smaller in size with males having up to 12 hemipenial papillae and originally included in the *R. moyeri* type series, belong to a distinct taxon (see *R. colemani* sp. nov. description).

Fig. 4. *Rhampholeon uluguruensis* from type locality, in life.

Rhampholeon beraducci Mariaux & Tilbury 2006
Beraducci's Pygmy Chameleon
Holotype: MHNG 2655.019

Synonyms
Rhampholeon (Rhinodigitum) beraduccii Mariaux & Tilbury 2006
Rhampholeon (Rhinodigitum) beraduccii Tilbury 2010: 167
Rhampholeon (Rhinodigitum) beraduccii Glaw 2015
Rhampholeon (Rhinodigitum) beraduccii Spawls et al. 2018: 250

Original diagnosis (verbatim)
Chamaeleonidae, *Rhampholeon* (*Rhinodigitum*). With the characters of the subgenus. A tiny brown chameleon with snout–vent length (SVL) 20.5–28 mm, maximum total

Fig. 5. *Rhampholeon moyeri* in life.

length (TL) 36 mm, and a very short tail, 19–22% of TL (Fig. 6). The smallest known *Rhampholeon* and the smallest chameleon from continental Africa. Head with a well-developed nasal process and short supra-optical peaks. Head flat with very slightly marked crests, temporal crest very weak. Dorsal keel weakly undulated. Body with sub-homogeneous granules, but conspicuous shoulder spine present. Deep axillary and inguinal pits present. Claws bicuspid with small accessory spines.

Amendments to the original diagnosis

Rhampholeon beraduccii has not been sequenced for the ND2 gene. For the 16S gene, it differs by having a G at positions 965 and 1008 and from all other species except *R. acuminatus* at position 1269 by having an A. It differs at position 1271 by having a G.

Distribution

Restricted to Sali Forest Reserve, in the Mahenge Mountains.

Fig. 6. *Rhampholeon beraduccii* from type locality, in life.

Fig. 7. *Rhampholeon acuminatus* from type locality, in life.

Rhampholeon acuminatus Mariaux & Tilbury 2006
Nguru Spiny Pygmy Chameleon
Holotype: MHNG 2645.001

Synonyms
Rhampholeon (Rhinodigitum) acuminatus Mariaux & Tilbury 2006
Rhampholeon (Rhinodigitum) acuminatus Tilbury 2010: 161
Rhampholeon (Rhinodigitum) acuminatus Glaw 2015
Rhampholeon (Rhinodigitum) acuminatus Spawls et al. 2018: 249

Original diagnosis (verbatim)
Chamaeleonidae, *Rhampholeon* (*Rhinodigitum*). With the characters of the subgenus. A small chameleon with SVL 47–57 mm (maximum TL 82 mm) and a tail 25–30% of TL (Fig. 7). Adults are unmistakable due to their large discoid and vertically flattened rostral process (up to 5 × 3 mm) projecting forward off the rostrum (Figs 6-7), spinous supra-orbital and other cranial projections, prominent casque, exaggerated dorsal crest and numerous spines on the body, limbs and tail. No axillary or inguinal pits. Claws bicuspid. Parietal peritoneum unpigmented.

Amendments to the original diagnosis
Rhampholeon acuminatus has not been sequenced for the ND2 gene. For the 16S gene, it differs by having an A at position 917 and from all other species except *R. beraduccii* at position 1269 by having an A.

Distribution
Restricted to Mkingu Nature Reserve in the Nguru mountains.

***Rhampholeon colemani* sp. nov.**
Uzungwa Scarp's Pygmy Chameleon
Holotype: Adult male in the MUSE, the Science Museum of Trento, MTSN 5379 (formerly MTSN 001TA) collected in the Uzungwa Scarp Nature Reserve on the 8 January 1999 by Michele Menegon and Sebastiano Salvidio and formerly included in the paratype series of *Rhampholeon moyeri* (Fig. 8 and Table S3).

Type locality
Kitolomero valley, a locality in the Uzungwa Scarp Nature Reserve at about 1200 m a.s.l. (-8.3975; 35.9786) Kilombero District, Morogoro Region of Tanzania.

Paratypes
Two adult females – MTSN 5380, MTSN 5381, collected in the Uzungwa Scarp Forest Reserve on 8 January 1999 by Michele Menegon and Sebastiano Salvidio were formerly included in the paratype series of *Rhampholeon moyeri*. Two additional females, MUSE 11032 and MUSE 11033, and two adult males, MUSE 11029 and MUSE 11031, collected at the same locality on 17 November 2013 by Michele Menegon, Elena Tonelli and Anna Sustersic.

Diagnosis
A very small chameleon (slightly larger in size than *R. beraduccii* which is the smallest chameleon in continental Africa), with a maximum total length of 44 mm (maximum SVL = 35.5 mm) of which the tail comprises an average of 19% in females and 24% in males. Adult females and males have similar SVL. Overall morphology similar to other species in the complex, differing by its smaller size (only *R. beraduccii*, with a max recorded size of 28 mm SVL and 36 mm total length, is smaller), a pronounced angular flexure of the snout (59° in *R. colemani*, 44° in *R.*

Fig. 8. *Rhampholeon colemani* from type locality, in life.

moyeri, the other species all <41°), by the ratio between tail length and orbital diameter, by having hemipenis with two long apical horns, curving inwardly toward the sulcal surface and each horn bearing up to 12 prominent thornlike papillae arranged in two proximal rows of 3 to 4, with an additional spine at base of the horn and with a row of 4 distal papillae. It also differs from other *Rhampholeon* for the ND2 gene by having the amino acid isoleucine at position 463–465, valine at position 577–579 and threonine at position 583–585 (Table S3), and by a known distribution restricted to a single locality in the sub-montane rainforest of the Uzungwa Scarp Nature Reserve.

Description of Holotype

Snout-vent 37.58 mm, tail 8.8 mm. Body habitus leaf like - typical of all other *Rhampholeon* (*Rhinodigitum*) species.

Head: occiput flat, parietal crest indistinct. The lateral crests are studded with several prominent tubercles. The two supra-orbital ridges are connected to each other by a series of 18 inter-orbital tubercles arranged in a shallow V across the top of the head with the last tubercle being the most prominent. A tuft of tubercles forms a small peak on the supra-orbital rim. The two peaks are connected between the orbits by an interorbital row of 19 scales counted from peak to peak. The canthal ridges are formed by a row of enlarged, relatively smooth tubercles which merge anteriorly on the tip of the snout and extend into a small soft tuberculated rostral process extending 1.2 mm off the tip of the snout. A distinct temporal crest arises from the mid post-orbital rim and consists of 5 tubercles on the right and 6 tubercles on the left, of which the most posterior is the largest. The nares open infero-posteriorly. There are no gular appendages or crests.
Body: the dorsal crest is crenulated over the anterior two-thirds of the dorsal keel, fading to smooth over the sacrum. The crenulations are formed by a cluster of mucronate tubercles sited above each dorsal spinous process. The tail is very weakly crenulate, more so over the distal third. A single deep pit is present in each axilla, but there is no trace of a pit in the inguinal region. The soles of the feet present a relatively smooth, non-spinous cobblestoned appearance. Low palmar spines are present at the bases of the digits. The claws are strongly bicuspid.
Scalation: body covered with a subhomogeneous background of small stellate edged tubercles. A more or less regular scattering of larger (2–4 times larger in diameter) subconical/rounded tubercles occurs on the sides of the body. A particularly large tubercle is sited just above the shoulder. The forearms have a scattering of enlarged spinous tubercles, two prominent spines along the radius. The belly is smooth with small homogeneous scales. The scales on the circular eyeball are relatively heterogeneous.
Hemipenis: stout and truncated. No calyces or capitate structures are present and sulcal lips smooth. Two long apical horns are present, curving inwardly toward the sulcal surface. Each horn is adorned with 10 to 12 prominent thornlike papillae arranged in two proximal rows of 3 to 4, with an additional spine at base of the horn and with a row of 4 distal papillae.

Distribution

The species is only known from the type locality, at 1200 m a.s.l., in the Uzungwa Scarp Nature Reserve, where it might occur sympatrically with *R. moyeri* with possible elevational segregation. Further research is needed to properly assess the distribution of the two species.

Etymology

Rhampholeon colemani is named in honour of Carter Coleman, who for more than 25 years has raised funds and campaigned for the conservation of Tanzania's forests. In 1991 he revived the Tanzania Forest Conservation Group based in Tanzania, and went on to establish the African Rainforest Conservancy in the USA and the African Rainforest Trust in the United Kingdom, organisations that remain dedicated to conserving Tanzania's high biodiversity forests

***Rhampholeon sabini* sp. nov.**
Nguu North Pygmy Chameleon

Holotype

Adult male in the MUSE, the Science Museum of Trento, MTSN 5092 collected in the Nguu North Forest Reserve at 1200 m a.s.l. on 22 March 2002 by Michele Menegon (Fig. 9 and Table S2).

Type locality

Nguu North Forest Reserve, 1200 m a.s.l. (-5.4803; 37.4753), Handeni District, Tanga Region, Tanzania.

Paratype

Four adult males – MTSN 5192, MTSN 5193, MTSN 5198, MTSN 5199, two adult females MTSN 5195, MTSN 5197, collected in Nguu North Forest Reserve, Nguu Mountains between 15–22 March 2002 by Michele Menegon and one adult female MTSN 9082, collected in Kilindi Forest Reserve on the 7 February 2008 by Michele Menegon.

Diagnosis

A small chameleon with a maximum total length of 54.13 mm (maximum SVL = 44.17 mm) of which the tail comprises an average of 18% in females and 26% in males. Adult females and males have similar SVL. Overall morphology is similar to other species in the complex, but in this species, the relative size of the head and tail is significantly larger than in other *Rhampholoen* species analysed, see Fig. 2 and related text. It also differs by a marked mitochondrial sequence divergence, more specifically for the ND2 gene by having the amino acid valine at position 460–462, leucine at position 487–489, proline at position 583–585, threonine at position 619–621, methionine at position 643–645, threonine at position 664–666 and threonine at position 667–669 (Table S3) and by its current distribution restricted to the sub-montane rainforest of Nguu and Kilindi forest reserves. Note that without locality or genetic information, preserved individuals of the

Fig. 9. *Rhampholeon sabini* from type locality, in life.

new species cannot be univocally distinguished on the ground of their morphology.

Description of Holotype

Snout-vent 37.19 mm, tail 7.05 mm. Body habitus leaf like - typical of all other *Rhampholeon* (*Rhinodigitum*) species.

Head: occiput flat, parietal crest formed by 5 enlarged tubercles. The lateral crests are studded with several prominent tubercles. No inter-orbital tubercles arranged in a shallow V across the top of the head. A tuft of tubercles forms a small peak on the supra-orbital rim. The two peaks are connected between the orbits by an interorbital row of 23 scales counted from peak to peak. The canthal ridges are formed by a row of enlarged, relatively smooth tubercles which merge anteriorly on the tip of the snout and extend into a small soft tuberculated rostral process extending 1.2 mm off the tip of the snout. An indistinct

temporal crest arises from the mid post-orbital rim and consists of some tubercles slightly larger than the surrounding ones of which the most posterior is the largest. The nares open infero-posteriorly. There are no gular appendages or crests.
Body: the dorsal crest is crenulated over the anterior two-thirds of the dorsal keel, fading to smooth over the sacrum. The crenulations are formed by a cluster of mucronate tubercles sited above each dorsal spinous process. The tail shows no crenulation.
A single deep pit is present in each axilla, but there is no trace of a pit in the inguinal region. The soles of the feet present a relatively smooth, non-spinous cobblestoned appearance. Low palmar spines are present at the bases of the digits. The claws are strongly bicuspid.
Scalation: body covered with a subhomogeneous background of small stellate edged tubercles. A more-or-less regular scattering of larger (2–4 times larger in diameter) subconical/rounded tubercles occurs on the sides of the body. A particularly large tubercle is sited just above the shoulder. The forearms have a scattering of enlarged spinous tubercles, two prominent spines along the radius. The belly is smooth with small homogeneous scales. The scales on the circular eyeball are relatively heterogeneous.
Hemipenis: stout and truncated. No calyces or capitate structures are present and sulcal lips smooth. Two long apical horns are present, curving inwardly toward the sulcal surface. Each horn is adorned with 7 to 8 prominent thornlike papillae arranged in two proximal rows of two, with an additional spine at base of the horn and with a row of three distal papillae.

Distribution
The species is known to occur in the Nguu North and Kilindi Forest Reserves only, Handeni district, Tanga Region, Tanzania.

Etymology
The species name is a patronym for Andy Sabin who provides financial support to many organizations and is actively engaged with community and environmental programs around the world. As an extension of his lifelong fascination with reptiles and amphibians and dedication to environmental education.

***Rhampholeon rubeho* sp. nov**
Rubeho's Pigmy Chameleon

Holotype
Adult male in the MUSE, the Science Museum of Trento, MTSN 5013 collected in the Mafwomero Forest Reserve on 22 March 2002 by Michele Menegon, Andrew Perkin and Lucinda Lawson (Fig. 10 and Table S2).

Type locality
Mafwomero Forest Reserve in the Rubeho Mountains, at 1970 m a.s.l. (-6.8981; 36.5653), Mpwapwa District, Dodoma Region of Tanzania.

Paratypes
Two adult females MTSN 5012, MTSN 5014, collected in the Mafwomero Forest Reserve on 8 January 1999 by Michele Menegon, Andrew Perkin and Lucinda Lawson.

Additional material
Three adult males – MTSN 8895, MTSN 8896, MTSN 8899 and two adult females – MTSN 8893, MTSN 8898 collected in Ilole Forest Reserve by Michele Menegon on 21 September 2006.

Diagnosis
A small chameleon with a maximum total length of 63 mm (maximum SVL = 46.8 mm) of which the tail comprises an average of 24% in females and 30% in males. Adult females and males have similar SVL. Overall morphology is similar to the other species in the complex, but it differs by a marked mitochondrial sequence divergence from other *Rhampholeon* more specifically for the ND2 gene by having the amino acid valine at position 421–423 and leucine at 469–471. For the 16S gene, it differs by having a T at position 1080, T at position 1123 and T at position 1125 (Table S3) and by its' restricted distribution in forest fragments of the Rubeho Mts. Without locality or genetic information, preserved individuals of the new species cannot be univocally distinguished on the basis of their morphology.

Description of Holotype
Snout-vent 41.12 mm, tail 11.12 mm. Body habitus leaf like - typical of all other *Rhampholeon* (*Rhinodigitum*) species.

Head: occiput flat, parietal crest formed by 5 enlarged tubercles. The lateral crests are studded with several prominent tubercles. The two supra-orbital ridges are connected to each other by a series of 18 inter-orbital tubercles arranged in a shallow V across the top of the head with the last tubercle being the most prominent. A tuft of tubercles forms a small peak on the supra-orbital rim. The two peaks are connected between the orbits by an interorbital row of 19 scales counted from peak to peak. Posteriorly to the interorbital row there's an additional, less distinct, one. The canthal ridges are formed

Fig. 10. *Rhampholeon rubeho* from type locality, in life.

by a row of enlarged, relatively smooth tubercles which merge anteriorly on the tip of the snout and extend into a small soft tuberculated rostral process extending 1 mm off the tip of the snout. A distinct temporal crest arises from the mid post-orbital rim and consists of 7 tubercles on both sides, of which the most posterior is the largest. The nares open infero-posteriorly. There are no gular appendages or crests.

Body: the dorsal crest is crenulated over the anterior two-thirds of the dorsal keel, fading to smooth over the sacrum. The crenulations are formed by a cluster of mucronate tubercles sited above each dorsal spinous process. The tail shows no crenulation. A single deep pit is present in each axilla, but there is no trace of a pit in the inguinal region. The soles of the feet present a relatively smooth, non-spinous cobblestoned appearance. Low

palmar spines are present at the bases of the digits. The claws are strongly bicuspid.
Scalation: body covered with a subhomogeneous background of small stellate edged tubercles. A more-or-less regular scattering of larger (2–4 times larger in diameter) subconical/rounded tubercles occurs on the sides of the body. A particularly large tubercle is sited just above the shoulder. The forearms have a scattering of enlarged spinous tubercles, two prominent spines along the radius. The belly is smooth with small homogeneous scales. The scales on the circular eyeball are relatively heterogeneous.
Hemipenis: stout and truncated. No calyces or capitate structures are present and sulcal lips smooth. Two long apical horns are present, curving inwardly toward the sulcal surface. Each horn is adorned with 8 to 9 prominent thornlike papillae arranged in 3 basal ones and a row of 5 to 6 distal papillae.

Distribution
This species is known from Mafwomero Forest Reserve. However, there is an isolated population of *Rhampholeon* in the Ilole Forest Reserve, on the southern end of the Rubeho massif, about 50 km south from Mafwomero Forest Reserve. Individuals from the Ilole population were not included in the phylogenetic analysis, so we tentatively assign this population to *R. rubeho*. Inclusion in a phylogenetic analysis is needed to assess its taxonomic position.

Etymology
The species is named after the mountain block (Rubeho) where the type series was collected and where it is considered to be restricted. The specific epithet is considered to be a noun in apposition.

***Rhampholeon nicolai* sp. nov**
Nicola's Pigmy Chameleon

Holotype
Adult male in the MUSE, the Science Museum of Trento, MTSN 5592 collected in the Mamiwa Kisara North Forest Reserve on the 25th of January 2004 by Michele Menegon (Fig. 11 and Table S2).

Type locality
Mamiwa Kisara North Forest Reserve in the Ukaguru Mountains, at 1970 m a.s.l. (-6.3716; 36.9248), Gairo District, Morogoro Region of Tanzania.

Paratypes
One adult female – MTSN 5593 from same locality as the holotype. Four adult males – MHNG 2624.47, MHNG 2624.56, MHNG 2624.49, MHNG 2624.50 and two adult females – MHNG 2624.48, MHNG 2624.57 collected by Jean Mariaux in Ikwamba Forest Reserve (-6.3453; 36.9770) on 4–5th May 2002.

Diagnosis
A small chameleon with a maximum total length of 60.1 mm (maximum SVL = 47.44 mm) of which the tail comprises an average of 22% in females and 29% in males. Adult females and males have similar SVL. Overall morphology is similar to other species in the complex, but in this species, the relative size of the head and tail is significantly larger than in the other *Rhampholeon* species analysed, see Fig. 2 and related text. It also differs from other *Rhampholeon* for the ND2 gene by having the methionine codon ATC at position 445–447, and the amino acid threonine at position 454–456 (Table S3) and by its restricted distribution to the forest fragments in the Ukaguru Mts. Without locality or genetic information, preserved individuals of the new species cannot be unequivocally distinguished on the ground of their morphology alone.

Description of Holotype
Snout-vent 45.55 mm, tail 11.01 mm. Body habitus leaf like - typical of all other *Rhampholeon* (*Rhinodigitum*) species.

Head: occiput flat, parietal crest indicated by a few low tubercles. The lateral crests are studded with several prominent tubercles. No inter-orbital tubercles arranged in a shallow V across the top of the head. A tuft of flat roundish tubercles forms a small peak on the supra-orbital rim, and additional cluster of enlarged tubercles for a second less raised peak on the orbital rim, posterior to the main one. The two main peaks are connected between the orbits by an interorbital row of 18 scales counted from peak to peak. The canthal ridges are formed by a row of enlarged, relatively smooth tubercles which merge anteriorly on the tip of the snout and extend into a small soft tuberculated rostral process extending 2 mm off the tip of the snout. A distinct Y shaped temporal crest arises from the mid post-orbital rim and consists of 12 tubercles on the right and 13 tubercles on the left, of which the most posterior is the largest. The nares open infero-posteriorly. There are no gular appendages or crests.
Body: the dorsal crest is crenulated over the anterior two-thirds of the dorsal keel, fading to smooth over the sacrum. The crenulations are formed by a cluster of mucronate tubercles sited above each dorsal spinous process. The tail is very weakly crenulate, more so over the

Fig. 11. *Rhampholeon nicolai* from type locality.

distal third. A single deep pit is present in each axilla, but there is no trace of a pit in the inguinal region. The soles of the feet present a relatively smooth, non-spinous cobblestoned appearance. Low palmar spines are present at the bases of the digits. The claws are strongly bicuspid.
Scalation: body covered with a subhomogeneous background of small stellate edged tubercles. A more or less regular scattering of larger (2–4 times larger in diameter) subconical/rounded tubercles occurs on the sides of the body. A particularly large tubercle is sited just above the shoulder. The forearms have a scattering of enlarged spinous tubercles, two prominent spines along the radius. The belly is smooth with small homogeneous scales. The scales on the circular eyeball are relatively heterogeneous.
Hemipenis: stout and truncated. No calyces or capitate structures are present and sulcal lips smooth. Two long

apical horns are present, curving inwardly toward the sulcal surface. Each horn is adorned with 7 to 8 prominent thornlike papillae arranged in 3 basal ones and a row of 4 to 5 distal papillae.

Distribution

Rhampholeon nicolai occurs in the Mamiwa Kisara North and Mamiwa Kisara South forest reserves, which are contiguous in the Ukaguru mountains. In addition, specimens were recorded from Ikwamba Forest Reserve, adjacent to Mamiwa-Kisara North Forest Reserve. *Rhampholeon* individuals from Mikuvi forest have been recorded, and we tentatively assign this population to *R. nicolai*. However, these specimens will need to be properly evaluated to verify the taxonomic status of this population.

Etymology

This species is named after Nicola Colangelo, an entrepreneur and industrialist who worked in Tanzania for most of his life. He supported conservation initiatives and organisations, and before many others argued for the sustainable utilisation of natural resources. He was passionate about conservation for its intrinsic importance but also for a sustainable development of economies, such as low impact tourism activities.

***Rhampholeon waynelotteri* sp. nov**

Wayne's Pygmy Chameleon

Holotype

Adult male in the MUSE, the Science Museum of Trento, MTSN 8542, collected in the Kanga Forest Reserve on the 22 March 2004 by Michele Menegon (Fig. 12 and Table S2).

Type locality

Kanga Forest Reserve in the Nguru Mountains landscape, at 1280 m a.s.l., (-5.9168; 37.7056), Mvomero District, Morogoro Region of Tanzania.

Paratypes

Four adult males – MTSN 8537, MTSN 8538, MTSN 8540, MTSN 8543 and two adult females – MTSN 8539, MTSN 8541 with the same locality as holotype.

Diagnosis

A small chameleon with a maximum total length of 55 mm (maximum SVL = 46.2 mm) of which the tail comprises an average of 21% in females and 26% in males. Adult females and males have similar SVL. Overall morphology is similar to other species in the complex. It differs from other *Rhampholeon* for the ND2 gene by having the amino acid histidine at position 436–438, threonine at position 445–447 and leucine at position 478–489 (Table S3) and by the restricted distribution in the forest of Mt. Kanga and Mkingu Nature reserve. In Mkingu, it is sympatric with *R. acuminatus* from which it can be easily distinguished by the lack of a large, discoid and vertically flattened rostral process and supra-orbital and cranial spinous projections, and with *R. princeeai* sp. nov. from which it is distinguished by the lack of a triangular, platform like rostral process. Aside from the two sympatric species, without locality or genetic information, preserved individuals of the new species cannot be univocally distinguished on the ground of their morphology

Description of Holotype

Snout-vent 40.03 mm, tail 9.33 mm. Body habitus leaf like - typical of all other *Rhampholeon* (*Rhinodigitum*) species.

Head: occiput flat, parietal crest indicated by a few low tubercles. No inter-orbital tubercles arranged in a shallow V across the top of the head. A tuft of tubercles forms a small peak on the supra-orbital rim. The two peaks are connected between the orbits by an interorbital row of 18 scales counted from peak to peak. The canthal ridges are formed by a row of enlarged, relatively smooth tubercles which merge anteriorly on the tip of the snout and extend into a small soft tuberculated rostral process extending 1 mm off the tip of the snout. A distinct temporal crest arises from the mid post-orbital rim and consists of 5 tubercles on the right and 4 tubercles on the left, of which the most posterior is the largest. The nares open infero-posteriorly. There are no gular appendages or crests.

Body: the dorsal crest is crenulated over the anterior two-thirds of the dorsal keel, fading to smooth over the sacrum. The crenulations are formed by a cluster of mucronate tubercles sited above each dorsal spinous process. The tail is very weakly crenulate, more so over the distal third. A single deep pit is present in each axilla, but there is no trace of a pit in the inguinal region. The soles of the feet present a relatively smooth, non-spinous cobblestoned appearance. Low palmar spines are present at the bases of the digits. The claws are strongly bicuspid.

Scalation: body covered with a subhomogeneous background of small stellate edged tubercles. A more-or-less regular scattering of larger (2-4 times larger in diameter) subconical/rounded tubercles occurs on the sides of the body. A particularly large tubercle is sited just above the shoulder. The forearms have a scattering of enlarged spinous tubercles, two prominent spines along the radius. The belly is smooth with small homogeneous scales. The

Fig. 12. *Rhampholeon waynelotteri* from type locality (top) and from Mkingu Nature Reserve (bottom left and right), in life.

scales on the circular eyeball are relatively heterogeneous. Hemipenis: not fully everted.

Distribution

Known only from Kanga and Nguru mountains. The population from Nguru, which has not been included in the phylogenetic analysis, is separated from the one in Kanga by a lowland corridor of about 8 km wide. On the basis of their geographic proximity and morphological similarities, we tentatively assign the Nguru population to this species. Further phylogenetic analysis is needed to confirm its taxonomic placement.

Etymology

This species is named after and dedicated to Wayne Lotter, in recognition to his ground-breaking work in developing a holistic and strategic intelligence-based approach to anti-poaching. He helped successfully reverse the high

rates of elephant poaching in Tanzania, during 2010's. Unfortunately, he died on 16 August 2017 bravely fighting for the cause he was most passionate about. The specific epithet is patronym in the genitive masculine singular.

***Rhampholeon princeeai* sp. nov**
Princeeai's Pygmy Chameleon

Holotype
Adult male in the MUSE, the Science Museum of Trento, MUSE 14034 (field tag MW 06838) collected in the Nguru South Forest Reserve, now part of Mkingu Nature Reserve, on 13 January 2008 by David Gower, Simon Loader, Hendrik Mueller and Mark Wilkinson (Fig. 13 and Table S2).

Type locality
Mkingu Nature Reserve in the Nguru Mountains, at 1870 m a.s.l. (-6.0655; 37.4907), Mvomero District, Morogoro Region of Tanzania.

Paratypes
One adult male – MUSE 14036 (field tag MW 06914), one adult female, MUSE 14033 (field tag MW 06837) and one juvenile – MUSE 14035 (field tag MW0 06839) same locality as the holotype. One adult male – MTSN 5537 and one adult female – MTSN 5538 (field tags respectively KMH 35703 and KMH 35704) collected by members of Frontier Tanzania on 7 September 2002.

Diagnosis
A small chameleon with a maximum total length of 46 mm (maximum SVL = 35.5 mm) of which the tail comprises an average of 28% in females and 29% in males. Adult females and males have similar SVL. Despite an overall morphology similar to other species in the complex, *R. princeeai* can be distinguished from all the other *Rhampholeon* by the shape and structure of the rostral process, which is expanded at the base, forming a scaly triangular, platform formed by 9 transverse and 11 longitudinal scales and by having a shallow pit in the inguinal region. It also differentiated by a marked mitochondrial sequence divergence, more specifically for the 16S gene by having a T at position 891, C at 1021, G at 1075, C at 1080, A at 1125, C at 1126 and T at 1135 (Table S3), and its' known distribution restricted to the high elevation areas of the Mkingu Nature Reserve.

Description of Holotype
Snout-vent 37.54 mm, tail 10.14 mm. Body habitus leaf like - typical of all other *Rhampholeon* (*Rhinodigitum*) species.
Head: occiput slightly elevated, a Y shaped parietal crest indicated by 3 short series of elevated, polygonal tubercles. The lateral crests are studded with several prominent tubercles and form a tuft of tubercles over the occiput. A tuft of conical, pointed tubercles forms a small peak on the supra-orbital rim. The two peaks are connected between the orbits by an interorbital row of 22 scales counted from peak to peak. The canthal ridges are formed by a row of enlarged, conical tubercles which merge anteriorly on the tip of the snout and extend into a triangular, tuberculated rostral process extending 1.3 mm off the tip of the snout with the base almost as wide as the length. A distinct temporal crest arises from the mid post-orbital rim and consists of 6 tubercles of which the most posterior is the largest. The nares open infero-posteriorly. There are no gular appendages or crests.
Body: the dorsal crest is crenulated over the anterior two-thirds of the dorsal keel, fading to smooth over the sacrum. The crenulations are formed by a cluster of mucronate tubercles sited above each dorsal spinous process. The tail shows no crenulation. A single deep pit is present in each axilla, a shallower pit is present in the inguinal region. The soles of the feet present a relatively smooth, non-spinous cobblestoned appearance. Low palmar spines are present at the bases of the digits. The claws are strongly bicuspid.
Scalation: body covered with a subhomogeneous background of small stellate edged tubercles. A more or less regular scattering of larger (2–4 times larger in diameter) subconical/rounded tubercles occurs on the sides of the body. A particularly large tubercle is sited just above the shoulder. The forearms have a scattering of enlarged spinous tubercles, two prominent spines along the radius. The belly is smooth with small homogeneous scales. The scales on the circular eyeball are relatively heterogeneous.
Hemipenis: not everted.

Distribution
Rhampholeon princeeai is only known from the type locality, presumably restricted to the montane forest above 1800 m a.s.l. Within the nature reserve, *R. princeeai* is syntopic with *R. acuminatus* and occurs sympatrically with the Nguru population of *R. waynelotteri*.

Etymology
The species is named after Richard Williams, better known by his stage name Prince Ea, an American activist and inspirational spoken word artist, poet, rapper and filmmaker. From 2014 he has shifted his focus from music to creating motivational and inspirational spoken word films and content covering a wide range

Fig. 13. *Rhampholeon princeeai* from Mkingu Nature Reserve, in life.

of topics such as deforestation and the reckless destruction of our environment for which we are all responsible. The specific epithet is considered to be a noun in apposition.

DISCUSSION

Morphological and phylogenetic diversity

The genus *Rhampholeon* is a morphologically conservative group, and species delimitations have relied heavily on molecular phylogenetic evidence (e.g., Fisseha et al., 2013). Our new phylogeny suggests that there are

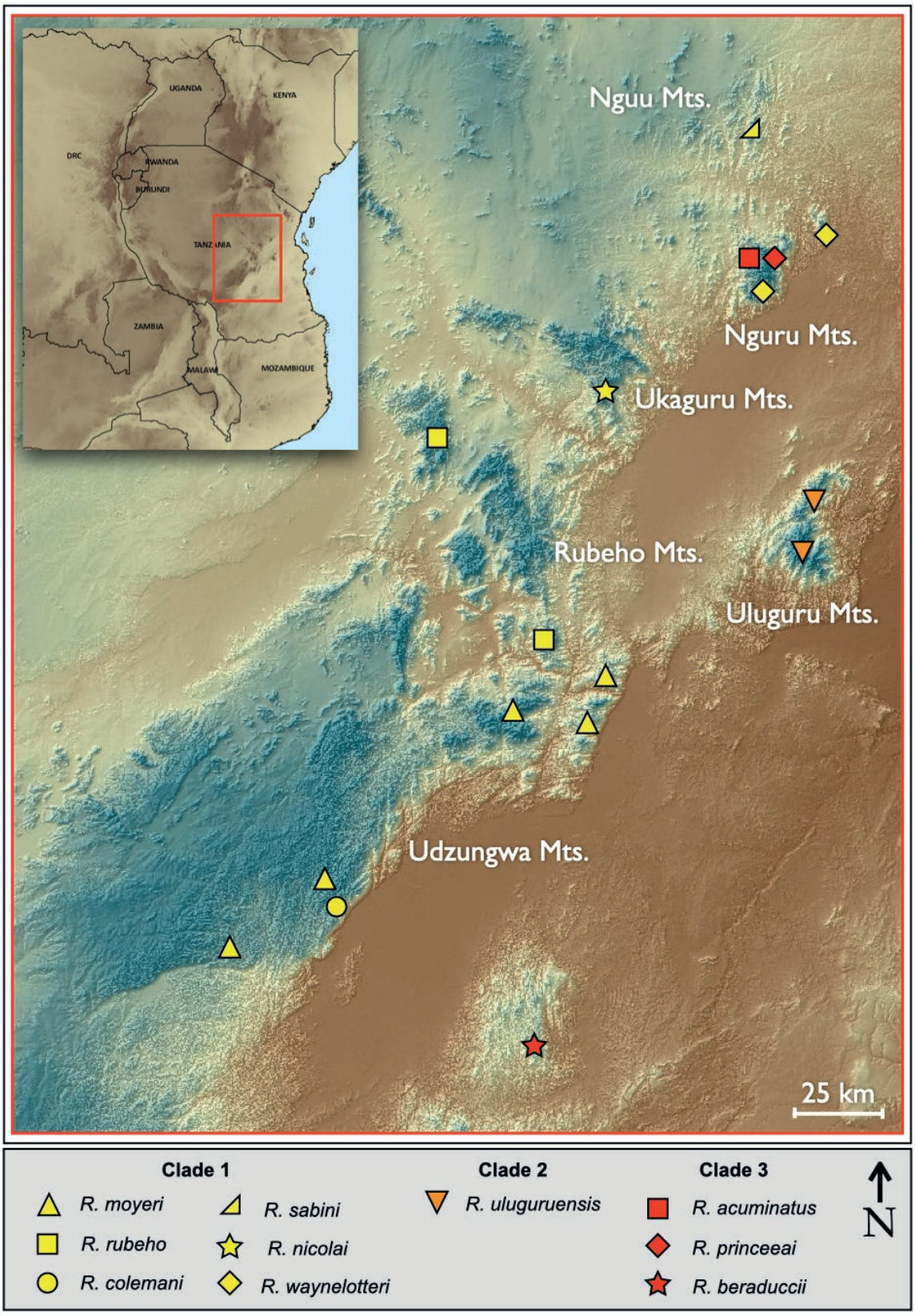

Fig. 14. Distribution of *Rhampholeon* species across the Eastern Arc Mountains of Tanzania. Inset map shows the study area.

at least seven species within the *Rhampholeon uluguruensis/moyeri* complex, of which six are undescribed. This conclusion is supported by the comparative barcoding approaches, where species divergences among these new species, ranging from 5 to 16%, show a difference comparable to other described *Rhampholeon* species (see Fig. S1). In *R. colemani* and *R. princeeai*, the clear genetic differences are supported by discrete morphological differences. The large phylogenetic differences are in contrast with the limited morphometric differences exhibited between most species. When morphological differences are lacking for species recognition, genetic and distributional information can be key characteristics for assisting in diagnosing species.

Our integrative taxonomic approach is broadly aligned with the General Lineage Concept (GLC) whereby species are considered as independently evolving metapopulation lineages (de Queiroz, 1998; 2007). Under the GLC, species distinctions are based on multiple criteria, such as pre- and post-zygotic isolation, monophyly, ecology and morphology. However, the GLC does not require all criteria to be fully met but does entail evaluating multiple lines of evidence to determine the distinction of a species. As outlined, we evaluated genetic and morphological data, providing a test of two lines of evidence. All the species have a similar ecological niche, inhabiting a leaf litter microhabitat in Afrotemperate forest. Despite this, they are geographically isolated by barriers of unsuitable habitat (savanna) which species are unable to inhabit or cross. Given the isolation of forests has been in place for a substantial amount of time (e.g., Tolley et al., 2013; Loader et al., 2014), we speculate that these species have likely been on separate evolutionary trajectories but largely nonchanging ecologically and phenotypically. In addition to morphology and species ecology, this is further supported by divergence estimates, which indicates that *R. uluguruensis* and the *R. moyeri* complex diverged more than 20 million years ago (Tolley et al., 2013).

The description of these six new species of pygmy chameleons increases the number of *Rhampholeon* to twenty-six. Although this is a substantial increase in species number, there is no doubt that additional *Rhampholeon* species remain to be described given that much of central and eastern Africa is poorly surveyed (Tolley et al., 2016). The number of described *Rhampholeon* has doubled in just the last 15 years, most of which are endemic to specific mountain chains in the Eastern Arc of Tanzania. At present, nearly every Tanzanian mountain chain (excluding Malundwe) has an endemic *Rhampholeon* species.

Conservation status

Chameleons have a high proportion of threatened species according to the IUCN Red List (see also Tolley et al., 2016). This high proportion of threatened species is linked to their habitats being increasingly impacted by human activities (e.g., Hall et al., 2009; Tolley et al., 2016; see Global Forest Watch). Although habitat loss, including fire, is the primary trigger for the elevated extinction risk in this group (Jenkins et al., 2013; Tolley et al., 2016), many species are also threatened by illegal or unsustainable harvesting for the pet trade (Jenkins et al., 2013). However, our understanding of the factors contributing to declines of individual species is poor. Our limited knowledge of species ecology and the absence of any information on population demographic trends prevents assessment of population declines or trends.

As is the case with many reptiles, the evaluation of chameleon species for their IUCN Red List status is based primarily on the extent of habitat loss within their inferred distributions. In order to infer these distributions, the spatial distribution of recorded localities is used, and for poorly sampled species, the estimation of distribution is difficult and could result in under-estimates of range size. The degree to which the inferred ranges overlap with protected areas as well as land cover change (e.g., habitat loss) allows for an assessment of extinction risk. However, the impact of habitat loss on chameleon populations (e.g., demographic decline trends) has not been assessed for any species. Clearly, a priority would be to monitor the population demographics of threatened species to better understand chameleon demographic trends given their susceptibility to environmental change and, at the same time, investigate the real supply of chameleons on the international market. For example, at reptile trade fairs in Europe, it is not uncommon to find various species of pygmy chameleons of the genus *Rhampholeon* for sale. However, given that *Rhampholeon* was only recently included on the Convention on International Trade in Endangered Species (CITES) list in 2017, some of these individuals could have entered the pet trade prior to their listing and be of captive bred stock. Thus, it is possible that wild caught and illegally exported individuals could be laundered as captive bred from animals that were in trade prior to the CITES listing.

The genus *Rhampholeon* includes a number of species with small, and fragmented ranges, with more than 30% of species considered threatened with extinction (https://www.iucnredlist.org/). Based on our field experience in East Africa, *Rhampholeon* species do not occur outside intact forest areas, and are restricted to the remaining, small, closed canopy forest fragments. The loss of forest

is the primary factor for their high threat status. For the new species described here, we anticipate that most are at risk of extinction given that they have small distributions that have been impacted by habitat loss. Among them, *R. sabini, R. colemani* and *R. waynelotteri* mainly occur in submontane forests, where deforestation is particularly acute, unlike the higher elevations, e.g., >1500m (Hamunyela et al., 2020). In particular, *R. colemani* has a very small range (about 36 km^2) in a single forest patch. Although the entire distribution is within the Uzungwa Scarp Nature Reserve, an area that is officially protected, the forest habitat has declined in quality due to anthropogenic impacts that are continuing at present. It is unlikely that this species can be considered secure in that protected area. More broadly, other *Rhampholeon* that occur in Tanzania are also at risk of extinction, particularly *R. spinosus* (Endangered) and *R. acuminatus* (Critically Endangered) due to their highly fragmented and small distributions, that are impacted by habitat loss and have limited protection (in the Usambara and Nguru Mountains, respectively). While the high elevation montane forest region of the Eastern Arc has undergone less habitat loss than lower elevation forests, increasing deforestation levels are evident more recently (Hamunyela et al., 2020). For example, from 2001 to 2020, Mkingu Nature Reserve and Kanga Forest Reserve combined lost 1180 ha of humid primary forest and the total area of humid primary forest in decreased by 5.2% in this time period (data from Global Forest Watch, 2021). These increased impacts are likely to intensify the threats to high elevation *Rhampholeon* populations and species.

Without effective forest conservation measures, market-driven agricultural development is likely to trigger an expansion of cropland at the expense of forests to meet the increased demand for the agricultural products promoted, with negative impact on biodiversity, carbon sequestration and water services (Hamunyela et al., 2020). Based on recent estimations there are approximately 296,000 ha of closed canopy natural forest left in the Eastern Arc Mountains, that is more highly fragmented than previously estimated (Koskikala et al., 2020). In general, forest fragmentation causes multiple impacts for ecological resilience of tropical forests through direct habitat loss, reduction of fragment size, increase in edge effects, and spatial isolation, while fragmented forests are also prone to further deforestation (Koskikala et al., 2020). From a conservation perspective, small natural forest fragments are considered less important compared to large and intact natural forest patches (Pimm et al., 2013). However, small fragments are likely to harbor irreplaceable unique or rare biodiversity values compared to an area of the same size in intact landscape (Tulloch et al., 2016). In Tanzania, commercial and subsistence agriculture is the main driver of deforestation, and aside from protected areas, there is no clear policy limiting the conversion of forests to agricultural land, which would require a greater inter-sectoral coordination between the agriculture, livestock, land, energy and forest sectors (Doggart et al., 2020).

Our knowledge of the species diversity of *Rhampholeon* has improved greatly, particularly for Tanzania, but we still know very little about their basic ecology and life history. Furthermore, their geographic distributions are poorly known as most species are known from just a few records from even fewer localities. It is unclear if *Rhampholeon* species from Tanzania are significantly buffered from extinction risk, despite many occurring in protected areas. For example, *R. acuminatus* from the officially protected Nguru Mountains is considered Critically Endangered suggesting that formal protection of the habitat has not safeguarded this species from extinction. An improved quantitative assessment of demographic trends for these threatened species is needed to understand whether protected areas are functioning as adequate buffers for these species. Overall, there is concern regarding the long-term survival of East African pygmy chameleons but limited data on their biology, distribution and population prevents empirically based solutions to mitigate declines and their future survival.

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SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at <http://www-9.unipv.it/webshi/appendix/index.html> manuscript number 12978

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Preliminary genetic characterisation of Southern Smooth Snake *Coronella girondica* (Serpentes, Colubridae) populations in Italy, with some considerations on their alpine distribution

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Abstract. The Southern smooth snake, *Coronella girondica*, is a small-sized colubrid found in Northwest Africa and Southwest Europe. Mitochondrial DNA-based studies showed that the species can be split into five clades: two from Northwest Africa (one Moroccan and one Tunisian-Algerian) and three from Europe (one in the south-west of the Iberian Peninsula, one in the south-east of Spain and one in the rest of the European range). With regards to Italy, to date, only two samples have been analysed both from the Province of Pisa, Tuscany, pointing at that fact that genetic characterisation of Italian populations is still lacking. Accordingly, we have increased the sampling coverage with 19 new samples from northern and central regions of Italy, including two populations, apparently disconnected from the rest of the known range, and analysed their phylogenetic relationships using a portion of the mitochondrial cytochrome b gene. Our results confirm the general phylogenetic arrangement detected in previous studies; specifically for Italian populations, no variability emerged from the Apennine populations, and a slight differentiation could be shown for the Alpine and subalpine ones. This pattern can be explained assuming past spread and recent isolation of *C. girondica* relict populations in the Alpine region, likely during the Last Glacial Maximum. Later, during the Holocene, the Italian Alps and the Po Plain went through various climatic variations and high anthropization which may have influenced *C. girondica* distribution through expansion and contraction processes.

Keywords. *Coronella girondica*, Italy, distribution, relict populations.

INTRODUCTION

The Southern Smooth Snake *Coronella girondica* (Daudin, 1803) is a small-sized Colubridae with a Mediterranean chorotype (West-Mediterranean, Sindaco et al., 2013). The species is present in Northwest Africa (north-central Morocco, northern Algeria and northwestern Tunisia) and Southwest Europe (Portugal, Spain, Southern France and Peninsular Italy), where it usually lives in dry and stony Mediterranean scrub habitats (Sindaco et al., 2013; Geniez, 2018; Di Nicola et al., 2021).

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With regards to Italy, the secretive and mainly crepuscular habits of the species (Ferri and Morimando, 2004; Razzetti and Bernini, 2011) hampered the full description of its distribution framework for a long time (Razzetti and Bonini, 2006) and for some areas its presence has even only been recently confirmed (Razzetti et al., 2000; Capula et al., 2010; Rugiero et al., 2018; Di Nicola et al., 2020; Ferri and Soccini, 2020; Iversen et al., 2020).

The species, originally described by Daudin (1803) as *Coluber girondicus*, is currently considered monotypic (Razzetti and Bernini, 2011; Santos and Pleguezuelos, 2015; Sindaco and Razzetti, 2021). Subsequently, *Rhinechis amaliae* (Boettger, 1881) was described from Moroccan specimens, on the basis of a single morphological character relating to the rostral zone (Santos and Pleguezuelos, 2015). However, further morphological investigations did not provide support for the validity of this taxon (Saint Girons, 1956; Domergue, 1962; Santos and Pleguezuelos, 2003). More recently, Santos et al. (2012a) performed a molecular study across the distribution range of the whole species using analysis of mitochondrial genes and highlighted the presence of three major clades: one from Northwest Africa, one from the south-east of Spain and the latter occurring in the rest part of the European range. According to their calibration, the divergence among the clades occurred around 1.4-2.0 Ma, roughly coinciding with the Plio-Pleistocene transition. Within two out of the three major clades, further differentiation was detected into five clades (see Santos et al., 2012b): two from Northwest Africa (one Moroccan and one Tunisian-Algerian) and three from Europe (one in the south-west of the Iberian Peninsula, one in the south-east of Spain and one in the rest of Europe).

In Italy, *C. girondica* is distributed in the north-western part of the country and extends south to the Gargano peninsula (Northern Apulia) and southern Lazio, including the region of Molise (Capula et al., 2010; Razzetti and Bernini, 2011; Rugiero et al., 2018; Di Nicola et al., 2021). Recently, two apparently isolated populations have been found in western Lombardy (Di Nicola et al., 2020) and around Lake Garda, in an area that is transitional between the Trentino-Alto Adige, Veneto and Lombardy regions (Ferri and Soccini, 2020; Iversen et al., 2020). These isolated populations are out of the geographical range of the other populations by around 70 km east and 160 km north (Fig. 1, 2).

With regards to genetic characterisation of the species in Italy, only two samples have been analysed, both from the province of Pisa (GenBank accession numbers JQ837570 and JQ837571). The present work therefore describes, for the first time, the intraspecific relationships between Italian populations of *C. girondica* including some recently confirmed observations from Northern Italy (Di Nicola et al., 2020; Ferri and Soccini, 2020; Iversen et al., 2020).

MATERIALS AND METHODS

Given the lack of adequate sample coverage within the Italian range of the species, a total of 19 new samples were collected from seven regions of Northern and Central Italy (Table 1; Fig. 1), including five samples from the isolated populations, respectively western Lombardy (n = 1) and Trentino-Alto Adige (n = 4). Small tissue fragments (tail tips, pieces of ventral scales) were removed and preserved in ethanol 96% from individuals that were found dead (most of the samples) or live specimens. For live specimens, a non-invasive scale clipping of ventral scales was performed. *Coronella girondica* was sampled by nocturnal active search only at two separate localities of Arco (Iversen et al., 2020) and Somma Lombardo (Di Nicola et al., 2020), while in other cases samples were recovered from dead specimen as roadkills, domestic cats or human persecution (Table 1).

In order to infer the phylogenetic relationships between Italian samples and other populations, we selected the mitochondrial DNA cytochrome b (cyt b) gene, a marker often used to infer intra-specific diversity on many vertebrates and invertebrates species, including snakes (Carranza et al., 2006; Mezzasalma et al., 2015; Faraone et al., 2020b). Approximately 20 mg of tissue was used to extract total DNA as described in Tagliavia et al. (2016). Genomic DNA was used as a template for PCR amplification with primers CB1(F) (5'CCATCCAACATCTCAGCATGATGAAA3') and CB2(R) (5'CCCTCAGAATGATATTTGTCCTCA3') (Carranza et al., 1999). DNA bands of the expected size (~300 bps) were obtained and then sequenced with the primer CB1(F) (BMR Genomics, Padua, Italy).

The resulting sequences were each around 255 nucleotides long and were analysed and manually proof-read with the DNA sequencing software CHROMAS v. 2.6.6 (Technelysium Pty. Ltd. 1998, Queensland, Australia). The coding gene fragments of cyt b were translated into amino acids to assess the lack of premature stop codons. Later, using CLUSTAL W (Larkin et al., 2007) with default parameters, the sequences from Italian samples generated in this study were aligned with homologous sequence downloaded from GenBank (Carranza et al., 2004; Santos et al., 2008, 2012a; Carvalho et al., 2017). Four species belonging to Colubridae and Psammophiidae families were used as outgroups (Carranza et al., 2006; Santos et al., 2012a; Faraone et al., 2020a, b).

Table 1. Italian samples and observation details of *Coronella girondica* used in the present study. The numbers and letters reported respectively in the first and second column are referred to localities shown in Fig. 1 and Fig. 2. The haplotype code is shown in brackets after the GenBank accession number. Samples marked with an asterisk were previously published by Santos et al. (2012a).

Fig. 1	Fig. 2	Year	Locality	N	E	Observer/Reference	Accession number
1	C	2019	Arco, Trentino Alto Adige	45.9238°	10.9433°	Iversen et al., 2020	OK573460 (H2)
2	C	2020	Arco, Trentino Alto Adige	45.9238°	10.9433°	Iversen et al., 2020	OK573463 (H2)
3	C	2020	Arco, Trentino Alto Adige	45.9238°	10.9433°	Iversen et al., 2020	OK573462 (H2)
4	C	2021	Arco, Trentino Alto Adige	45.9238°	10.9433°	Iversen et al., 2020	OK573461 (H2)
5	G	2020	Somma Lombardo, Lombardy	45.6713°	8.6833°	Di Nicola et al., 2020	OK573464 (H2)
6		2020	Cassinelle, Piedmont	44.5760°	8.5616°	Cavanna S. pers. obs.	OK573465 (H3)
7		2020	Isola del Cantone, Liguria	44.6432°	8.9664°	De Cresi U. pers. obs.	OK573473 (H1)
8		2018	Albenga, Liguria	44.0970°	8.2129°	Graglia M. pers. obs.	OK573469 (H1)
9		2018	Albenga, Liguria	44.0970°	8.2129°	Graglia M. pers. obs.	OK573468 (H1)
10		2019	Albenga, Liguria	44.0970°	8.2129°	Graglia M. pers. obs.	OK573467 (H1)
11		2020	Peagna, Liguria	44.0989°	8.2013°	Graglia M. pers. obs.	OK573472 (H1)
12		2020	Peagna, Liguria	44.0989°	8.2013°	Graglia M. pers. obs.	OK573471 (H1)
13		2020	Peagna, Liguria	44.0989°	8.2013°	Graglia M. pers. obs.	OK573470 (H1)
14		2020	Aurigo, Liguria	43.9953°	7.9209°	Fecchio L. pers. obs.	OK573466 (H1)
15		2021	Vigolzone, Emilia Romagna	44.9110°	9.6879°	Gereschi V., Mazzotta M. pers. obs.	OK573478 (H4)
16		2020	Foreste casentinesi, Emilia Romagna	43.8874°	11.8939°	Molinari G. pers. obs.	OK573475 (H1)
17		2021	Foreste casentinesi, Emilia Romagna	43.8874°	11.8939°	Molinari G. pers. obs.	OK573476 (H1)
18		N/A	S. Giuliano Terme, Tuscany*	43.7579°	10.4434°	Santos et al., 2012a	JQ837570 (H1)
19		N/A	S. Giuliano Terme, Tuscany*	43.7579°	10.4434°	Santos et al., 2012a	JQ837571 (H1)
20		2020	Piombino, Tuscany	42.9268°	10.5310°	Banchi R. pers. obs.	OK573474 (H1)
21		2020	Capestrano, Abruzzo	42.2867°	13,7942°	D'Amico M. pers. obs.	OK573477 (H1)
	A	2011	Rivoli Veronese, Veneto	45.5872°	10.8215°	Campagnari M. pers. obs.	
	A	2013	Caprino Veronese, Veneto	45.5898°	10.8215°	Campagnari M. pers. obs.	
	B	2020	Avio, Trentino Alto Adige	45.7383°	10.9433°	Secchi M. pers. obs.	
	D	2019	Pietramurata, Trentino Alto Adige	46.0268°	10.9420°	Iversen et al., 2020	
	E	2020	Limone sul Garda, Lombardy	45.8108°	10.7866°	Di Nicola et al., 2020	
	F	2020	Toscolano Maderno, Lombardy	45.6666°	10.6166°	Ferri & Soccini, 2020	
	H	2021	Sostegno, Piedmont	45.6658°	8.2852°	Zonari A. pers. obs.	
	I	2015	Zubiena, Piedmont	45.4833°	8.0333°	Ciracì A. pers. obs.	

The phylogenetic analysis was performed with Maximum Likelihood (ML) under the Akaike Information Criterion using the "Smart Model Selection" (SMS) (Lefort et al., 2017), implemented in PHYML v. 3 (Guindon et al., 2010). Jukes-Cantor (JC) (Jukes and Cantor, 1969) was the most appropriate evolutionary model (-Log likelihood value 1283.58), with a 0.32 gamma estimate of invariable sites and a 1.00 discrete approximation of the gamma distribution. The same model was obtained by using both all available sequences and by previously collapsing the sequences into haplotypes. Node support was estimated by bootstrap (Felsenstein, 1985) with 1,000 replicates and the MEGA X software (Tamura et al., 2021) was used to implement the ML tree. The unrooted minimum spanning network were obtained using the median-joining algorithm (Bandelt et al., 1999) implemented in PopART (http://popart.otago.ac.nz/) (Leigh and Bryant, 2015).

With the aim of consolidating current knowledge of *C. girondica* distribution in Northern Italy, bibliographical data from Razzetti and Bonini (2006) were recorded. Furthermore, unpublished observations from the northern Po River area were collected between 2011 and 2021 from authors' field observations and online records. All observations that were not reported by Razzetti and Bonini (2006) were considered new and, subsequently, compared with previous unconfirmed findings. In addition, Citizen Science (see Haklay et al., 2021) was also critical to the generation of distributional datasets through collaborative efforts between herpethologists around Italy and users of a Facebook group managed by two of the authors (MRDN and FPF) "Identificazione

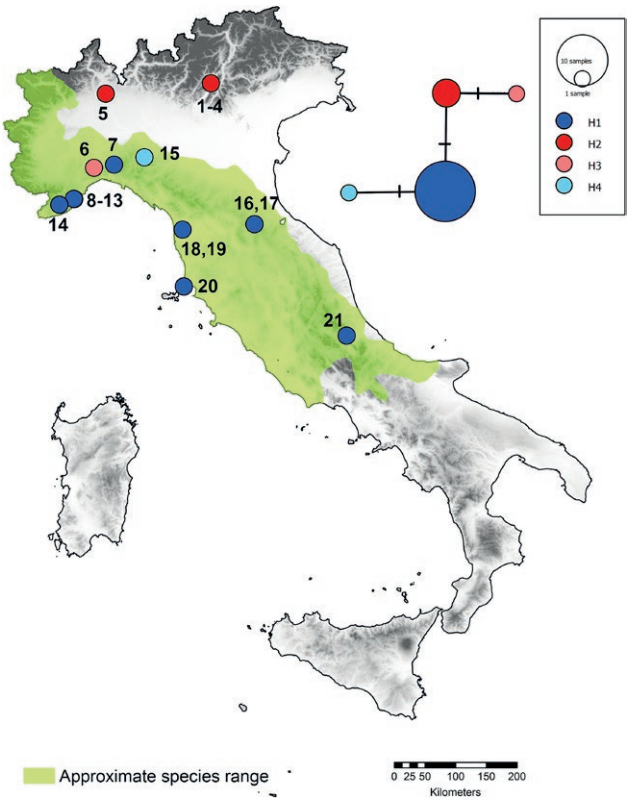

Fig. 1. Minumum spanning network based on the *Coronella girondica* cytochrome b fragment in Italy, and geographic distribution of the haplotypes. Circle sizes are proportional to haplotype frequency, and each black bars represents a mutational step. Samples are numbered as in Table 1. The green area in the map represents the approximate range of *C. girondica* in Italy.

Anfibi e Rettili". The users of the Facebook group provided locations for known European amphibians and reptiles and provided further distributional sites and samples for *C. girondica* found dead in various Italian regions. For each observation recorded through social networks, the authors meticulously checked the original files, the species identification and the location provided by the users through field survey of the coordinates. The new observations were mapped using Adobe Photoshop CC (©1990-2018 Adobe Systems incorporated, Release 19.1.5), together with those available from recent literature (Razzetti and Bonini, 2006; Di Nicola et al., 2020; Iversen et al., 2020; Ferri and Soccini, 2020; Di Nicola et al., 2021).

RESULTS

Overall, 94 sequences of 255 bp total length were analysed including the outgroups and the results confirm the same overall phylogenetic arrangement previously shown by Santos et al. (2012a) with five major clades (Fig. 3). All the 21 Italian samples (GenBank accession numbers JQ837570, JQ837571 and OK573460-78) fall within 'Clade 5' (sensu Santos et al., 2012a) which also includes sequences from France and most of Iberian Peninsula (Fig. 3). However, the monophily of 'Clade 5' is not adequately supported (bootstrap = 65%) on the basis of the cyt b fragment analysed here and it is considered as an haplogroup.

We found four cyt b haplotypes amongst the Italian *C. girondica* populations, differing by 1-2 mutation steps (Fig. 1). Most of the samples (n: 14) shared the same haplotype (H1), which is also shared with samples from Spain (JQ837574, JQ837587, JQ837589, JQ837607, JQ837610, JQ837635) and Portugal (JQ837569, JQ837582, JQ837595, JQ837634) (Fig. 3). In contrast, the sample from Emilia Romagna (OK573478) share the haplotype H4 with a sample from Ciudad Real, Spain (JQ837591). The following two unpublished haplotypes were detected among the northernmost Italian samples: H2 shared by all the five samples of the recently confirmed separated localities in western Lombardy (OK573464) and Trentino-Alto Adige (OK573460-63) and H3 from southern Piedmont (OK573465). H2 and H3 were shown to belong to the same haplogroup (Figs. 1, 3).

With the exclusion of the observations recently reported in the literature (Iversen et al., 2020; Di Nicola et al., 2020; Ferri and Soccini, 2020), three new localities (B, I, H) that were not recently confirmed (Razzetti and Bonini, 2006) have been registered, see Table 1 and Fig. 2 for details. Observation B resulted from a live adult snake observed on a low wall near Avio (province of Trento, Trentino-Alto Adige) just east of Lake Garda, as part of a cluster which includes observations recently published by Iversen et al. (2020) and Ferri and Soccini (2020) (Table 1, Fig. 2). Observations I and H corresponded to a live and a road-killed snake respectively and were both recorded in the Biella province (Piedmont) falling within the geographic range already known in the north-western regions of the Alps (Razzetti and Bonini, 2006) and the point G, a separated locality recently reported in Lombardy by Di Nicola et al. (2020) (Fig. 2).

DISCUSSION

The results presented here confirm a low genetic diversity for *C. girondica* within the cluster 'Clade 5', as shown by Santos et al. (2012a). For this haplogroup, rapid expansion process from south to north has been hypothesised to be likely derived from climatic warming events and followed by a bottleneck effect (Santos et al., 2012a). This pattern of recent expansion from the Iberian Penin-

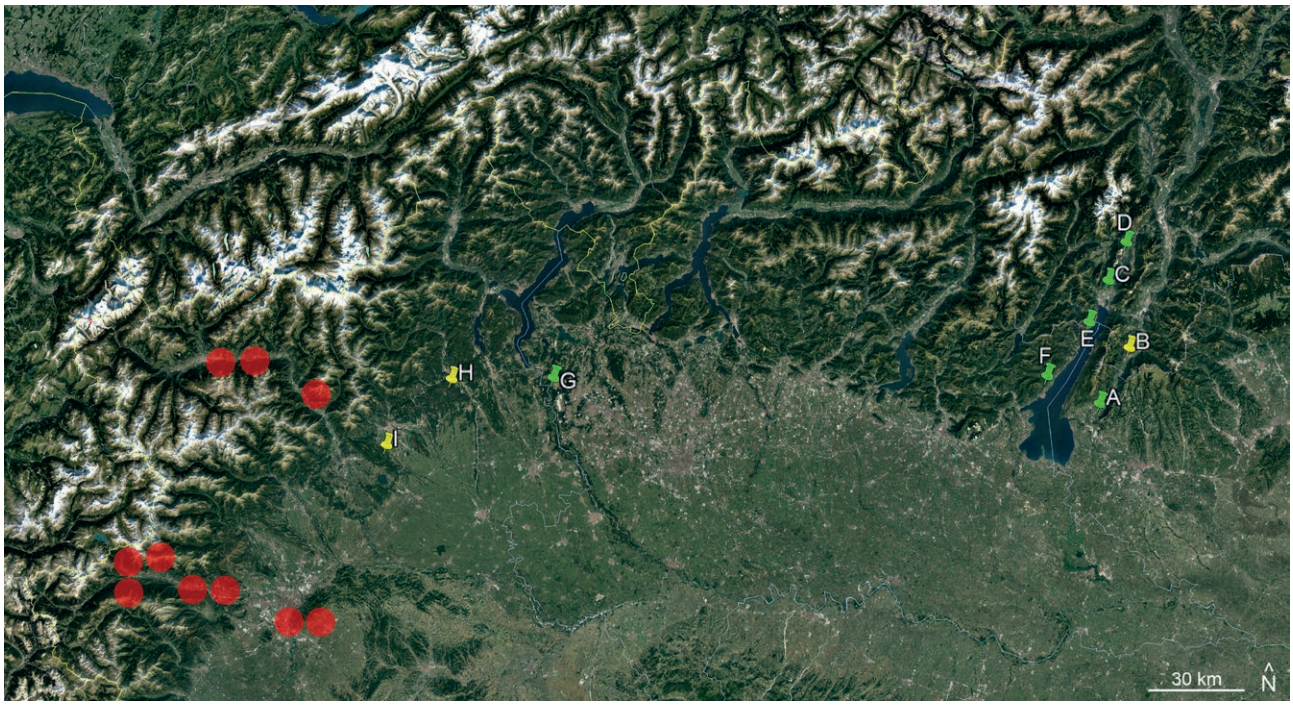

Fig. 2. Distribution of *Coronella girondica* in the Italian Alps north of the Po river. Red circle are the areas reported up to Razzetti and Bonini (2006), green landmarks represent the observations published after 2006 (Di Nicola et al., 2020; Iversen et al., 2020; Ferri and Soccini, 2020), yellow landmarks are the unpublished findings. The details of each point are shown in Table 1.

sula towards the north-east has also been hypothesised for other snake species such as *Natrix maura* (Guicking et al., 2002).

The genetic structure that emerges from the Italian *C. girondica* samples shows a lack of mitochondrial variability in the Apennine cyt b samples and a slight differentiation in Alpine and subalpine samples (Fig. 1, 3), which highlight that the endemic haplotypes H2 and H3 belong to the same cluster. This mtDNA pattern partially matches with that from other species of the Italian herpetofauna (Canestrelli et al., 2007; Canestrelli and Nascetti, 2008; Salvi et al., 2013; Chiocchio et al., 2021), including the Barred grass snake *Natrix helvetica* (Schultze et al., 2019) and confirms the margin of the Northern Apennines as a suture area of the Italian Peninsula (Hewitt, 2011; Chiocchio et al., 2021). This is compatible with recent spreading and isolation of *C. girondica* in Northern Italy, likely during the last glacial maximums, which allowed a slight haplotype differentiation. Subsequently, the Italian Alps and the Po Plain went through various climatic fluctuations and extensive human impact during the Holocene (Colombaroli et al., 2010; Nussbaumer et al., 2011; Joannin et al., 2013). These factors may have modulated the distribution of *C. girondica* with expansion and contraction processes.

The H4 haplotype, found in a single Italian sample (OK573478) collected in Emilia Romagna (Table 1, Figs. 1, 3), is identical to JQ837591 from Ciudad Real, central Spain (Fig. 3). This observation is similar to other cases reported for *Natrix natrix* and *N. helvetica* (Kindler et al., 2017; Schultze et al., 2019, 2020) and could be indicative of human translocation events. However, this hypothesis still needs further investigation through testing additional markers.

Recent Italian findings of *C. girondica* north of the Po River can be grouped into two main clusters. A Northwestern Alpine cluster includes some Alpine valleys of Northern Piedmont and Aosta Valley, and it is apparently separated from both the neighbouring French and Italian populations (Razzetti and Bonini, 2006; Sillero et al., 2014). An Eastern Alpine cluster is located around Lake Garda, and falls within the territories of Lombardy, Trentino-Alto Adige and Veneto (Iversen et al., 2020; Di Nicola et al., 2020; Ferri and Soccini, 2020). The populations around Lake Garda have been reported from the literature with many detailed records (De Betta, 1857; Dalla Torre, 1912) and later attested only by a few museum specimens dated up to 1977 (see Iversen et al., 2020). Other more recent information has been reported by Lorenzi and Bruno (2006) which, however, do not

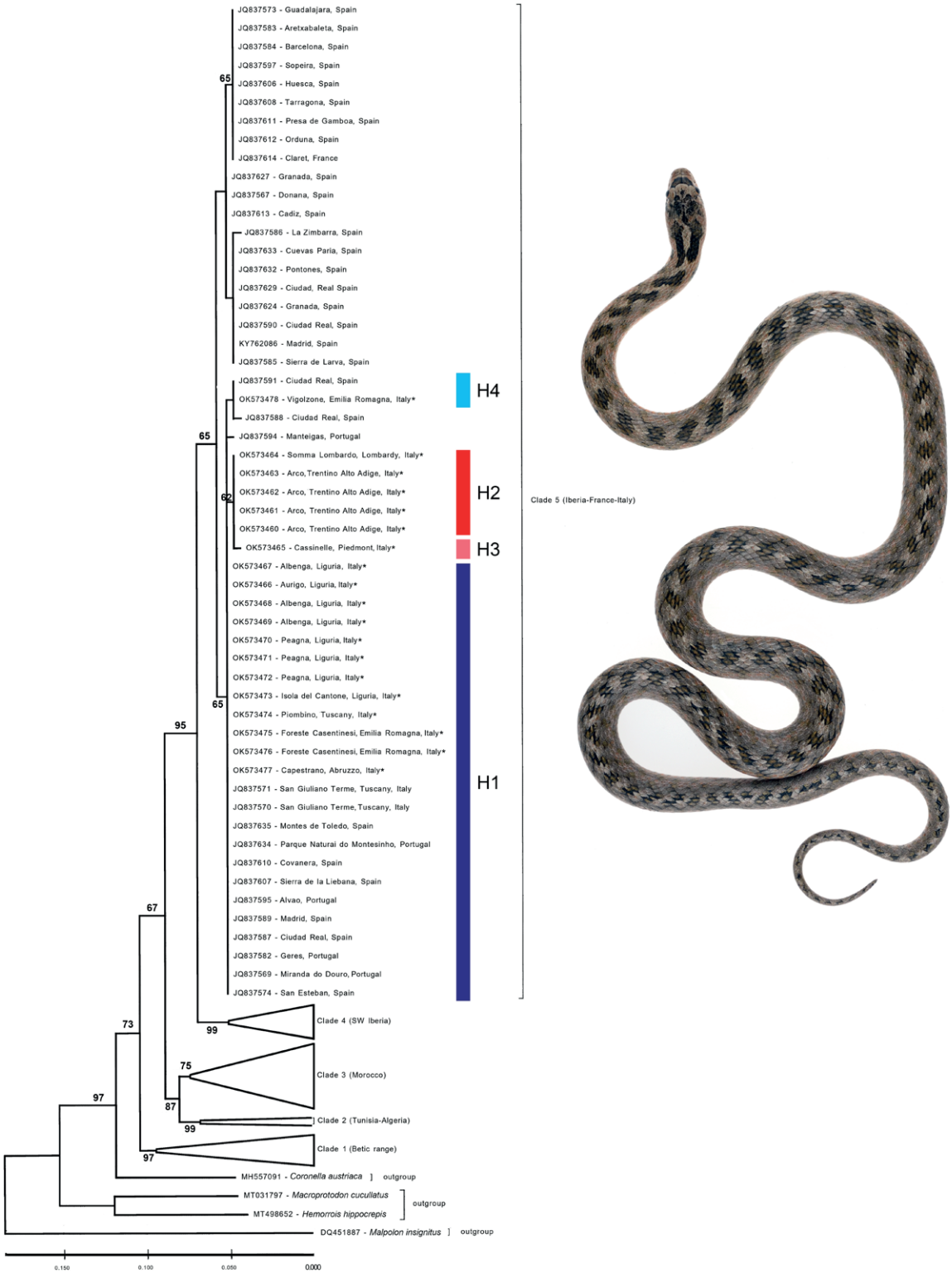

Fig. 3. Maximum Likelihood (ML) tree of *Coronella girondica* inferred from the mitochondrial cytochrome b gene, with a detail on the haplotypes of the 'Clade 5' (sensu Santos et al., 2012a) cluster. The haplotypes that occur in Italy are marked with the same colors used in Fig. 1. The numbers at nodes are ML bootstrap percentages. With the exception of the unpublished samples (see Table 1) all the others are taken from literature (Carranza et al., 2004, 2006; Santos et al., 2008, 2012a, b; Carvalho et al., 2017; Faraone et al., 2020a, b).

clearly contextualise their personal observations. The scarcity of findings during the twentieth century has led zoologists to consider this population close to extinction (Razzetti and Bonini, 2006) or probably extinct (Razzetti and Bernini, 2011). Recently, new observations led to the confirmation of the presence of *C. girondica* in Veneto (Novarini et al., 2017; Iversen et al., 2020), Trentino-Alto Adige (Iversen et al., 2020) and Lombardy (Di Nicola et al., 2020; Ferri and Soccini, 2020). New observations around Lake Garda largely confirmed previous records provided by Dalla Torre (1912), including the more recent point "B" (Fig. 2), which falls within a locality highlighted by the author between 1896 and 1900.

The observations "I" and "H" are located between the Northwestern cluster and point "G", recently reported in Lombardy by Di Nicola et al. (2020) (Table 1, Fig. 2). Point "I" is an unpublished locality, while "H" surprisingly confirms a site with a single specimen (MSNM RE 1439) preserved in the Civic Natural History Museum of Milan (see also Andreone and Sindaco, 2002), dated back to 1926. The geographic position of points "H" and "I" (Fig. 2) suggests that the North-western cluster and point "G" could be connected by small, scattered and low-density populations that may be detected by increasing the environmental monitoring of the species.

As indicated by Bombi et al. (2009), with the exclusion of the Maritime Alps and the Northern Apennines, Northern Italy has very low suitability values for *C. girondica* since only a few scattered sub-optimal patches in the area north of the Po River have been found. The results obtained here confirm this scenario, since *C. girondica* has a clearly fragmented distribution in the Alpine and subalpine areas. Furthermore, this species is mostly found in habitats within sub-Mediterranean climates, often characterised by xerophilous faunistic and vegetational communities which are very specific compared to other habitats and surrounding territories (La Greca, 1956; Gratani and Varone, 2003; Agabiti et al., 2005).

In conclusion, the results generated in this study suggest that the fragmented distribution of *C. girondica* north of the Po river can be mainly attributed to relict populations, based on the following points: (a) the finding of endemic haplotypes, compatible with a recent separation occurred during the last glacial events; (b) the few and scattered observations, highly localised in small patches with xeric Mediterranean features, suitable for the species; (c) current records largely match with historical records which, on the other hand, indicate few Alpine areas without recent confirmation; (d) the post-glacial history of Northern Italy is characterised by changes in ecosystems caused by climatic fluctuations and a strong human impact, and this could have caused the expansion and contraction of *C. girondica* as a thermophilic snake. This hypothesis will be further investigated through more extensive sampling in the field and the analysis of a greater number of loci in order to further detail the genetic structure of the Italian *C. girondica* populations. A greater fieldwork effort will be necessary, especially on the Western Alps, where our results could indicate a probably incomplete *C. girondica* distribution, which may be slightly less fragmented than previously thought.

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Species diversity and distribution of amphibians and reptiles in Sardinia, Italy

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Abstract. Although distribution databases are a dynamic tool, continuously updated, it is important to take "snapshots" of the species distribution over time to promptly identify potential conservation issues. With this work, we provide an update of the distribution of amphibians and reptiles in Sardinia and satellite islands. Data derive from both direct field observations (carried out since 2005 until July 2022) and literature, accounting for over 7000 records: 1416 records of 11 species of amphibians and 5600 records of 18 species of reptiles. Distribution maps (on 10 × 10 km UTM grid) of 29 species are provided in supplementary materials as well as the updated list of the amphibians and reptiles occurring in the circum-Sardinian islands. Most of the meshes were characterized by the presence of 1-3 amphibian species (73%) and 6-8 or 9-11 reptile species (32% with 6-8 species, 30% with 9-11 species). Species abundance was favoured by environmental heterogeneity, and mostly varied in relation to elevation range and edge density.

Keywords. Sardinia, amphibians, reptiles, islands, endemics, micro-insular herpetofauna, distribution maps.

INTRODUCTION

Sardinia is the second-largest island in the Mediterranean and, together with Corsica, with which it shares its paleo-origin, it is one of the most relevant biodiversity hotspots in the Mediterranean (Blondel et al., 2010).

Due to its long isolation (24-20 Mya), the complex geological history, the geographical position, the climatic and historical events, Sardinia is home to numerous endemic herpetological species, eight amphibians and five reptiles, some of which derive from ancestors present on the Sardinian-Corsican microplate before its detachment from the main European plate (Alvarez, 1972; Lanza, 1983; Carmignani et al., 1995, 2001, 2016; Corti et al., 1999; Speranza et al., 2002; Rodríguez et al., 2017). Sardinia is home to 11 amphibian and 18 reptile species (SHI, 1996; Bassu et al., 2010). The current herpetological composition of the Island can be mainly referred to a) the Messinian salinity crisis which occurred in the Miocene (~5 Mya) when important climatic variations occurred with consequent impact on flora and fauna, b) sea level oscillations due to the alternation of recurrent glacial and interglacial periods that have repeatedly separated and connected the island with Corsica and with the continent, c) the arrival of man (Corti et al., 1999; Duggen et al., 2003; Senczuk et al., 2019).

In the "Provisional atlas of Italian amphibians and reptiles" (*Atlante provvisorio degli Anfibi e Rettili italiani*,

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as part of the atlas project of the Italian Society of Herpetology, SHI, 1996) preliminary distribution maps of the Sardinian species were reproduced, subsequently published with some updates in the "Atlas of Italian amphibians and reptiles" (Sindaco et al., 2006). In the last three decades, the scientific interest in Sardinian herpetological species has intensified. In addition to some updates on the species distribution, an increasing number of articles have been produced focusing on phylogeography, ecology and conservation which have also contributed to provide data on the distribution of amphibians and reptiles on the Island (Corti et al., 2000, 2010; Vasconcelos et al., 2006; Van der Meijden et al., 2009; Salvi et al., 2010, 2011, 2017; Salvi and Bombi, 2010; Vamberger et al., 2011; De Pous et al, 2012; Fritz et al., 2012; Bombi and Vignoli, 2014; Biaggini et al., 2016; Rodríguez et al., 2017; Cossu et al., 2018; Ficetola et al., 2018; Lunghi et al., 2020; Mulargia et al., 2018; Sillero et al., 2018; Bellati et al., 2019; Senczuk et al., 2019; further references are given as supplementary material L1). At the same time, recent paleontological investigations (see Zoboli et al., 2019, 2022, and literature therein) are providing interesting baseline information testifying for the presence of taxa that are present in Sardinia since a relatively deep past (as green toads, *Emys orbicularis*, *Testudo hermanni*, *Natrix*) or went locally extirpated (as *Speleomantes*, *Discoglossus*, *Salamandrina, Mauremys*, giant tortoises, softshell turtles, worm lizards, agamid lizards, *Timon*, *Vipera*) or even globally extinct ('*Tomistoma*' *calaritanus*, *Trachyaspis lardyi*, *Testudo pecorinii*, pleurodiran turtles, *Sardophis elaphoides*).

With this work, we aim to provide updated distribution data collected from literature and direct field observations, together with a critical comment on the diversity of the Sardinian herpetofauna. Although distribution databases are a dynamic tool constantly updated, we still believe it is important to take "snapshots" of the distribution of the various species from time to time to promptly identify potential critical issue and intervene with appropriate conservation measures.

A particular focus was also made on the fauna of satellite islands, with an updated list of amphibians and reptiles of the circum-Sardinian islands that actively contribute to the herpetological diversity of Sardinia.

MATERIAL AND METHODS

Study site and data source, maps

Sardinia is located in the western Mediterranean and is one of the largest Italian regions. The island has an area that slightly exceeds 24,000 km^2 and is characterized by a diversified territory consisting of plains, plateaus, hills, and mountains, as well as an extensive and varied geomorphological coastline and numerous satellite islands, islets, and rocks.

The data on the distribution of amphibians and reptiles derive from both literature review and direct observations in the field carried out since 2005 until July 2022. Surveys have been carried out at different altitudes and visiting different types of natural and anthropogenic habitats, both by day and by night. Each data has been georeferenced with a satellite radio navigation device (Global Positioning System-GPS), or has been attributed to a toponym reported by the IGM maps (*Istituto Geografico Militare*). All data are stored in the database of the Sardinian Section of the Italian Society of Herpetology (SHI) "*tilighelta*". The dataset was enriched with bibliographic (e.g., Corti et al., 2000; Bassu et al., 2008, 2010, 2013; Salvi & Bombi, 2010; De Pous et al., 2012; Cossu et al., 2018; Mulargia et al., 2018) and with museum records (MZUF).

For the elaboration of species distribution maps, we used the UTM (Universal Transverse Mercator Projection, Coordinate Reference System WGS84 / UTM zone 32N) grid (10 × 10 km), dividing the island into 312 meshes. Bibliographic data without exact coordinates were reported in the respective UTM mesh. Each map shows data prior to 2010 and new data recorded from 2010 until July 2022. Other categories represented in the maps for some species are: a) doubtful records; b) single sporadic observation, referred to single individuals found out of the species range; c) multiple sporadic observations, when more than one individual was observed - simultaneously or over time – out of the species range (e.g., translocated *Testudo* spp.). The species distribution maps (see supplementary material) were produced using QGIS 3.14.16-Pi (QGIS.org, 2022).

Study species

The complete list of amphibian and reptile species inhabiting Sardinia is given in Table 1, where the endemic species are also indicated; Table S1 (supplementary material) reports the updated list of the herpetofauna of the circum-Sardinian islands. The species nomenclature follows Speybroeck et al. (2020). The presence of *Zamenis lineatus/longissimus* in Sardinia is currently debated (Razzetti and Zanghellini, 2006) and therefore here not reported. Introduced species with a relatively wide distribution are reported (e.g., *Trachemys*), while those recorded only through sporadic encounters of single individuals (e.g., *Mauremys*) are not. Due to ongoing studies on the presence of different *Pelophylax* species, all the observations related to the species of this genus are reported in a single map. However, in Table 1 they are all listed.

Table 1. List of amphibians and reptiles of Sardinia. The endemic species are marked as follow: EEE = exclusively endemic to Sardinia; EE = Endemic to Sardinia and Corsica; E = Endemic to the Central-Western-Mediterranean.

Amphibia	
Euproctus platycephalus (Gravenhorst, 1829)	EEE
Speleomantes flavus (Stefani, 1969)	EEE
Speleomantes genei (Temminck & Schlegel, 1838)	EEE
Speleomantes imperialis (Stefani, 1969)	EEE
Speleomantes sarrabusensis Lanza, Leo, Forti, Cimmaruta, Caputo & Nascetti, 2001	EEE
Speleomantes supramontis (Lanza, Nascetti & Bullini, 1986)	EEE
Bufo bufo (Linnaeus, 1758)	
Bufotes viridis balearicus (Boettger, 1880)	
Discoglossus sardus Tschudi, 1837	E
Hyla sarda (De Betta, 1857)	E
Pelophylax bedriagae (Camerano, 1882)	
Pelophylax bergeri (Günther, 1986)	
Pelophylax kurtmuelleri (Gayda, 1940)	
Reptilia	
Emys orbicularis (Linnaeus, 1758)	
Trachemys scripta (Thunberg in Schoepff, 1792)	
Testudo hermanni Gmelin, 1789	
Testudo graeca Linnaeus, 1758	
Testudo marginata Schoepff, 1792	
Euleptes europaea (Gené, 1839)	E
Hemidactylus turcicus (Linnaeus, 1758)	
Tarentola mauritanica (Linnaeus, 1758)	
Algyroides fitzingeri (Wiegmann, 1834)	EE
Archaeolacerta bedriagae (Camerano, 1885)	EE
Podarcis siculus (Rafinesque, 1810)	
Podarcis tiliguerta (Gmelin, 1789)	EE
Chalcides chalcides (Linnaeus, 1758)	
Chalcides ocellatus (Forskål, 1775)	
Hemorrhois hippocrepis (Linnaeus, 1758)	
Hierophis viridiflavus (Lacépède, 1789)	
Natrix helvetica cetti Gené, 1839	EE
Natrix maura (Linnaeus, 1758)	

Data analyses

Analyses were performed excluding sporadic observation (mainly related to *Testudo*), doubtful observations, and *Trachemys* spp. as an alien species that in recent times spread on the islands.

For each UTM mesh, we extrapolated the following environmental variables: number of Corine Land Cover classes, classified at level 3 ($NCLC_3$; Kosztra et al., 2019); index of environmental heterogeneity increasing with $NCLC_3$ and number of land use polygons (HETER = $NCLC_3$ × N polygons / mesh surface); index of edge density (ED = perimeter/surface calculated on land uses' polygons; we considered the mean value per mesh); maximum elevation (Elev); elevation range (ΔElev); abundance of wetlands (WET, the relative surface occupied, in a UTM mesh, by polygons belonging to the CLC classes Wetlands and Waterbodies). For each UTM mesh, we also extrapolated the number of all species (NTOT); endemic species (N_ETOT, *Discoglossus sardus, Euproctus platycephalus, Speleomantes* spp., *Hyla sarda* among amphibians; *Algyroides fitzingeri*, *Archaeolacerta bedriagae*, *Euleptes europaea*, *Natrix helvetica cetti*, *Podarcis tiliguerta* among reptiles); amphibian species (NAmph); endemic amphibian species (N_EAmph); reptile species (NRept); endemic reptile species (N_ERept). Even if the total number of species was correlated with those of amphibians and reptiles (considering all species and the endemic ones; tested with Pearson correlation, see Results), we performed analyses on all the six categories of species abundance, in order not to miss possible meaningful differences.

To test if the species abundance per UTM mesh varied depending on the above-listed environmental variables ($NCLC_3$, HETER, ED, Elev, ΔElev, WET), we used generalized linear models (GLZ) with, in turn, NTOT, N_ETOT, NAmph, N_EAmph, NRept, N_ERept, as dependent variable, with Poisson error distribution. We performed stepwise regression, and we selected the best-fit model according to the Akaike Information Criterion (we selected the models with the lowest AIC; Burnham and Anderson, 2002).

RESULTS

Sardinia falls inside 312 grid meshes, seven of which occupied by a very small terrestrial surface (<1 ha to about 37 ha). We analysed 7016 records: 1416 records of 11 species of amphibians and 5600 records of 18 species of reptiles. Most of the meshes were characterized by the presence of 1-3 amphibian species (73%; Figure 1) and 6-8 or 9-11 reptile species (32% with 6-8 species, 30% with 9-11 species; Figure 2). Distribution maps (on 10 × 10 km UTM grid) of the 29 species are provided as supplementary materials.

NTOT was correlated with NAmph ($N = 312$, $r = 0.697$, $P < 0.001$) and NRept ($r = 0.961$, $P < 0.001$); N_ETOT with N_EAmph ($r = 0.772$, $P < 0.001$) and N_ERept ($r = 0.870$, $P < 0.001$). Results of the analysis of the pattern of species abundance per UTM mesh (model selection and following GLZs) are shown in Table 2 and 3. NTOT decreased in those meshes with higher maximum elevation but, at the same time, was favoured by increas-

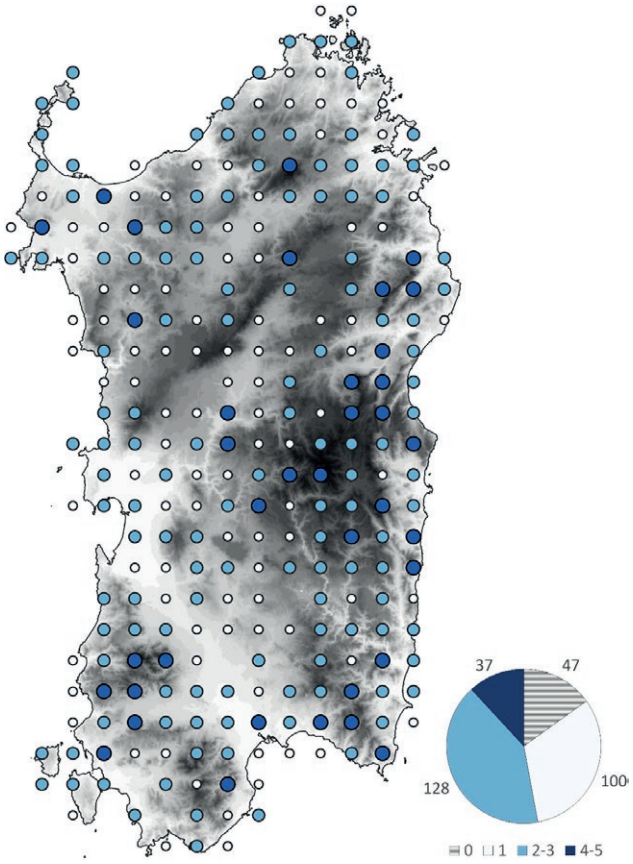

Fig. 1. Number of amphibian species in UTM 10 × 10 km grid meshes. Dots in the map indicate the presence of 1 (white small dot), 2-3 (small dot, in light blue), 4-5 (big dot, in dark blue) species. The pie chart summarizes the number of meshes hosting different ranges of species abundance, including those meshes with no species.

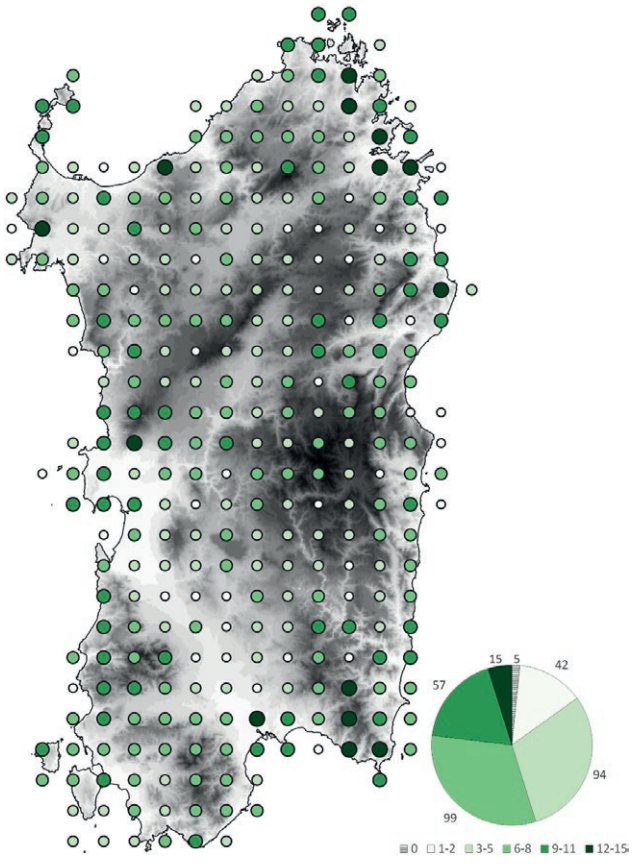

Fig. 2. Number of reptile species in meshes UTM 10 × 10 km grid. Dots in the map indicate the presence of 1-2 (small white dot), 3-5 (small light green dot), 6-8 (medium light green dot), 9-11 (medium dark green dot), 12-15 (dark green dot) species. The pie chart summarizes the number of meshes hosting different ranges of species abundance, including those meshes with no species.

Table 2. Akaike Information Criterion (AIC) in the selection of the best model explaining the pattern of abundance of all amphibian and reptile species (NTOT, NAmph, NRept) and of only endemic species (N_{ETOT}, N_EAmph, N_ERept) per UTM mesh, considering the following predictors: number of land cover classes ($NCLC_3$), index of environmental heterogeneity (HETER), index of edge density (ED), maximum elevation (Elev), elevation range (ΔElev), abundance of wetlands (WET); w = Akaike weight of the best model; w_1/w_2 = Akaike weight ratios between the first and second ranking models.

Response variable	Predictors	AIC	w	w_1/w_2
NTOT	HETER; Elev; ΔElev; $NCLC_3$; ED	1667.340	0.326	1.310
N_ETOT	HETER; ΔElev; ED	1158.529	0.139	1.014
NRept	HETER; Elev; ΔElev; $NCLC_3$; ED	1525.052	0.315	1.721
N_ERept	HETER; ΔElev; $NCLC_3$; ED	955.4630	0.283	2.594
NAmph	HETER; ΔElev; ED; WET	795.2846	0.188	1.141
N_EAmph	ΔElev; ED; WET	800.9570	0.259	1.830

ing elevation range, number of land uses and edge density. Not surprisingly, given the numerical preponderance of reptiles on the total amount of data, the predictors selected when considering all reptiles were the same selected for NTOT, with the small difference that, among those with a significant effect, there was the index of heterogeneity instead of $NCLC_3$. On the contrary, considering all amphibians, the selected predictors with a signif-

Table 3. GLZ testing the effects of the environmental variables selected by models in Table 2 on the abundance of all species (NTOT, NAmph, NRept) and of only endemic species (N_E, N_EAmph, N_ERept) per UTM mesh.

Response var.	Predictors	df	Estimates	Wald - Stat.	P
NTOT	Intercept	1	0.931	57.444	0.000
	HETER	1	0.006	2.896	0.089
	Elev	1	-0.001	18.820	0.000
	ΔElev	1	0.001	20.167	0.000
	$NCLC_3$	1	0.017	5.297	0.021
	ED	1	0.018	52.524	0.000
N_ETOT	Intercept	1	-0.222	1.263	0.261
	HETER	1	-0.000	0.000	0.999
	ΔElev	1	0.001	54.109	0.000
	ED	1	0.019	26.551	0.000
NRept	Intercept	1	0.840	39.018	0.000
	HETER	1	0.008	4.263	0.039
	Elev	1	-0.001	20.727	0.000
	ΔElev	1	0.001	14.647	0.000
	$NCLC_3$	1	0.015	3.089	0.079
	ED	1	0.017	38.575	0.000
N_ERept	Intercept	1	-0.321	1.852	0.174
	HETER	1	0.002	0.200	0.654
	ΔElev	1	0.001	24.799	0.000
	$NCLC_3$	1	-0.033	12.875	0.000
	ED	1	0.018	4.563	0.033
NAmph	Intercept	1	-0.739	4.879	0.027
	HETER	1	0.025	2.526	0.112
	ΔElev	1	0.001	17.858	0.000
	ED	1	0.0178	7.973	0.005
	WET	1	-0.2488	5.259	0.027
N_EAmph	Intercept	1	-1,238	16.129	0.000
	ΔElev	1	0.018	46.501	0.000
	ED	1	0.001	15.233	0.000
	WET	1	0.019	0.712	0.399

icant effect on the abundance of species per mesh were ΔElev, ED, and the relative abundance of wetlands.

Focusing on endemic species, the abundance of all species was significantly influenced by ΔElev and ED (HETER was selected, but it had no significant effects) (Table 2 and 3). $NCLC_3$ was added to these predictors when analysing endemic reptiles, and WET when analysing endemic amphibians, but without a significant effect (Table 2 and 3).

DISCUSSION

The maps we obtained in this work represent an important improvement on the distribution of amphibians and reptiles in Sardinia. Compared to previous publications (Sindaco et al., 2006; Bassu et al., 2008, 2010, 2013), the area surveyed for each species has been widely implemented (percentage increase of UTM meshes compared to Sindaco et al., 2006: e.g., *Euproctus platycephalus* 142%, *Speleomantes* spp. 0-115%, *Bufotes viridis balearicus* 632%, *Discoglossus sardus* 130%, *Hyla sarda* 379%; *Euleptes europaea* 228%, *Hemidactylus turcicus* 226%, *Emys orbicularis* 518%, *Testudo hermanni* 370%, *Algyroides fitzingeri* 121%, *Archaeolacerta bedriagae* 58%, *Podarcis tiliguerta* 142%, *Chalcides ocellatus* 105%, *Hierophis viridiflavus* 130%, *Natrix helvetica* 54%). By examining the distribution of the single species, it is to be noted that almost all the endemic species are missing in the plains of Nurra and Campidano (NW and SW Sardinia, respectively). Only in a few places, some of the endemic species occur in these regions. In particular, the distribution of the endemic lizards *Algyroides fitzingeri* and *Podarcis tiliguerta* very rarely includes wetlands and intensively cultivated plains where they have been observed only in "edge" contexts, while *Archaeolacerta bedriagae*, being a rupicolous species, is found exclusively in rocky habitats, from sea level to high altitudes (Sindaco et al., 2010). Approximately the same applies to *Euleptes europaea*, a tiny gecko also widely distributed in micro-insular systems. Only *Hyla sarda*, among endemic species, being particularly linked to lentic waters, has settled in these two aforementioned plains.

Among the amphibians and in particular among Urodela, the endemic and/or sub-endemic species, such as the endemic Sardinian brook newt, *Euproctus platycephalus*, and the cave salamanders, *Speleomantes flavus, S. genei, S. imperialis, S. sarrabusensis, S. supramontis* (the ranges of these last five species do not overlap), are distributed on the main island exclusively in hilly and mountain environments. It is interesting to note that four species of Testudines live in Sardinia, one Emydidae and three Testudinidae: the native freshwater European pond terrapin, *Emys orbicularis* and *Testudo hermanni*, whose presence on the island seems to date back to the Early Pleistocene (Biello et al., 2021; Zoboli et al., 2022) and, *T. graeca* and *T. marginata*. The populations of these last two species settle in distinct areas of the island despite *T. marginata*, whose large size often makes this species a preferred target of illegal collection and translocation, is the most easily observed in areas far from its primary Sardinian range. As for snakes, four species inhabit the island. The distribution of the endemic *Natrix helvetica cetti* (Schultze et al. 2020), a relatively elusive subspe-

cies, follows the distribution pattern of the other endemic taxa, according to his rupicolous habits and avoidance for plains (Vanni & Cimmaruta, 2010; Lunghi et al., 2019). *Hierophis viridiflavus*, is certainly the most widespread snake found on the Island whereas *Hemorrhois hippocrepis*, whose presence in the past has been reported in much of south-western Sardinia (Bruno and Hotz, 1976), seems to have restricted its range to such an extent that, in the last decade, it has been reported only for the city of Cagliari and its surroundings. Contrary to what is known for this species, considered rather xerophilous (Zuffi, 2006), the Sardinian population of *H. hippocrepis* lives near wetlands, in agricultural habitats and in urban areas.

As for the green frogs, *Pelophylax* spp., further research is needed to draw a clear picture of the distribution of the different taxa on the island, given that *P. kurtmuelleri*, *P.* cf. *bedriagae* and *P. bergeri* populations have been detected (Bellati et al., 2018). The introduced *P. kurtmuelleri* and *P.* cf. *bedriagae* can be considered naturalized following Bellati et al. (2019). The latter species is found in both northern and southern Sardinia. The settlement of the introduced green frogs may be favoured by vacant niches, even though the particularly dry climate could limit their expansion (Bellati et al., 2017, 2018, 2019).

When analysing how the number of species varies in relation to several environmental variables, the importance of elevation range and edge density in determining the abundance of herpetofauna species emerges. Indeed, increasing the elevation range usually entails a higher habitat diversity, and edge habitats (including, for instance, ecotones and riparian boundaries) are well known key elements for the herpetofauna.

The comparison between the factors influencing the abundance of all species and of those influencing the abundance of endemic species only reveals further interesting insights. For instance, the total number of species decreases in mountainous areas (that is those included in meshes with higher maximum elevation), while this was not a limiting factor for endemic species, many of which are also found at high altitudes.

When focusing on all amphibians, the relative abundance of wetlands was among the factors influencing species abundance, whereas it was not selected as significant factor when analyzing the endemic species, *Hyla sarda*, *Discoglossus sardus*, and *Speleomantes* spp. Indeed, these amphibians often spawn in minor water bodies, not included in the CLC classification as "Wetlands" and "Waterbodies", or in underground environments as in the case of *Speleomantes*.

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SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at <http://www-9.unipv.it/webshi/appendix/index.html> manuscript number 13627

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The Italian wall lizard, *Podarcis siculus campestris*, unexpected presence on Gorgona Island (Tuscan Archipelago)

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Abstract. We here report the unexpected presence of the Italian wall lizard (*Podarcis siculus campestris*) on Gorgona Island, in the Parco Nazionale Arcipelago Toscano (Tuscan Archipelago, Tyrrhenian Sea, Tuscany, Central Italy). Field observations were carried out in 2020 confirming its presence on the island, where it had never been reported before. We recorded 37 GPS points of the species in three major areas of Gorgona (with 50 lizard records) and about 180 visual counts regarding all age classes (newborns, juveniles and adults). The species was found in the urban area (site of state prison) and in two grassy and bushed areas, around and along olive tree plantations. Seven individuals were captured and their tails were used to assess the sequence variation of the mitochondrial *CYB* gene. Biometrical parameters were also evaluated for six of these individuals. We detected three distinct *CYB* haplotypes that were compared to *Podarcis siculus CYB* sequences available in public databases. They resulted identical or phylogenetically closest to those found in mainland Tuscany. One haplotype, found in three specimens, was identical to one previously detected at Orti Bottagone (WWF Oasis in Piombino), while the other two haplotypes were most similar to haplotypes reported in the Giannella peninsula and Pisa, respectively.

Keywords. Introduced species, *Podarcis siculus campestris*, Gorgona Island, Tuscan Archipelago, mtDNA *CYB* sequences.

INTRODUCTION

The Italian wall lizard *Podarcis siculus* (Rafinesque-Schmaltz 1810) is a Mediterranean species endemic to the Italian peninsula, Sardinia, Sicily, Corsica, coastal Slovenia and Croatia, and the majority of small islets of the Adriatic and Tyrrhenian Seas (Corti, 2006). It is also present, as an allochthonous taxon, in several European and non-European countries (Crnobrnja Isailovic et al., 2009; Corti et al., 2011; Silva-Rocha et al., 2012, 2014; Adamopoulou, 2015; Mizsei et al., 2016; Ribeiro and Sá-Sousa, 2018; Clemens and Allain, 2021). According to Crnobrnja Isailovic et al. (2009) "generally it is an invasive that can displace native populations of other species in its invasive range (the southern part of its range and in the areas where it has been introduced)". Recent data on introduced populations found that the species shows a marked resilience (Burke et al., 2002), and efficient adaptation patterns (Kapsalas et al., 2016). In some cases, the species showed clear-cut ecological plasticity in adapting to a new environment, changing some anatomical and physiological traits (Herrel et al., 2008). Locally,

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the species is supposed to drive the extinction of indigenous lizards (Ribeiro and Sá-Sousa, 2018). As far as we were aware, eradication projects proved successful results only in Greece (Adamopolou and Pafilis, 2019). Thus, *P. siculus* still represents worldwide a serious threat to autochthonous species, particularly due to the facility with which it can be transferred from its native area into a new environment (Adamopoulou, 2015; Silva-Rocha et al., 2012; Mizsei et al., 2016; Clemens and Allain, 2021).

In Italy, it is widespread from the North to the South, being common in coastal and hilly areas of Northern and Central Italy, while in the South it can reach higher altitudes (Corti et al., 2011). Recent unpublished data report new introduced *Podarcis siculus* individuals in some areas in Northern Italy (province of Trento), via olive trees transfer from central Italy (K. Tabarelli de Fatis pers. comm.). The species is naturally present in Tuscany and in its insular environments, as reported by the latest regional atlas (Vanni and Nistri, 2006). Regarding the seven larger islands of the Tuscan Archipelago, it has been reported as naturally present in Capraia, Elba (with three small populations), Montecristo, Giglio and Giannutri, and it was likely introduced in Pianosa (Vanni and Nistri, 2006). However, the species was never reported from Gorgona island. Previous repeated survey sessions, whose results were published in 2006 and in 2011 (Corti, 2006; Corti et al., 2011) did not find the species on the island, thus suggesting that this unexpected presence should be a very recent introduction.

From a phylogeographic and phylogenetic point of view, several papers have evaluated the distribution and the genetic variation of this taxon (Podnar et al., 2005; Senczuk et al., 2017), also when regarding allochthonous populations (Silva-Rocha et al., 2012, 2014). Considering the above scenario regarding the dispersal ability of the species in novel places and its ecological plasticity, we have aimed at assessing the population distribution of *P. siculus campestris* on Gorgona, within the framework of a larger project granted by the "Parco Nazionale Arcipelago Toscano" on Habitats Directive species occurring in the Tuscan Archipelago, and the mitochondrial DNA (mtDNA) variation (*CYB* gene) of some individuals from the island, with the overarching goal to obtain preliminary results concerning the geographical origin of the female founders.

MATERIALS AND METHODS

Study area and sampling

The study area is Gorgona Island (43.429008°N, 9.899226°E), the northernmost island of the Tuscan Archipelago, about 34 km westward from the Italian coast. It is a rocky island with a very small surface (2.1 km^2) and a perimeter of about 7 km (Fig. 1A). The herpetological assemblage of the island lacks amphibians, due to the absence of freshwater areas, while known reptile species are the wall lizard (*Podarcis muralis*), the Moorish gecko (*Tarentola mauritanica*), the Turkish gecko (*Hemidactylus turcicus*) and the Whip snake (*Hierophis viridiflavus*) (Vanni and Nistri, 2006), species that are still present and abundant according to recent unpublished surveys (C. Corti, pers. comm.; M.A.L. Zuffi and M. Boschetti pers. obs.). Sampling surveys were limited to *Podarcis* species and were carried out from mid to end of summer 2020, on three different occasions, on the 29th of July, 29th and 30th of September. We selected three different transects, A = 1,678 m, B = 2,073 m, and C = 2,240 m (Fig. 1B). According to the recommendation concerning the monitoring of relative small-sized lizards (Sacchi and Scali, 2016; Sindaco et al., 2016), we adopted a visual census, that shall be repeated in the following years to establish the relative abundance of the target species and other reptile species for the above-mentioned project. We counted all the lizards observed along each transect and, every 10-12 m, we marked the lizards presence with a GPSMAP® 62 series GPS. We therefore provided *i*) a distribution mediated by the GPS recording and *ii*) an overall count of observed animals along the whole transect. According to the capture feasibility, in some areas along the different transects, we also captured seven individuals of *P. siculus* by noosing. From the captured individuals (six out of seven, one escaped before measurements) we recorded multiple morphometrics and body size (body mass, snout to vent length, SVL, head length, width and height, as in Kaliontzopoulou et al., 2007; Table 1), determined sex and ontogenetic stage (male, female, juvenile) and collected the tail tip to assess the variation of the mitochondrial *CYB* gene. From its external appearance, the species shows the typical continental *P. siculus campestris* dorsal pattern, with two green parietal bands (Fig. 2). Being on this island allochthonous and likely invasive, after the measurements the captured individuals were transferred to the museum lab, maintained alive in terraria for further analyses and comparisons, waiting for the Ministry's approval for euthanasia.

Samples analysed for CYB gene variation

Seven *P. siculus* specimens collected in Gorgona were analysed for the sequence variation of the *CYB g*ene at the University of Pavia. DNA was extracted from either tails or tail re-growths stored in 95% ethanol. The majority of the *CYB* gene (at least 924 bp, from np 14,357 to np 15,280) was determined for all specimens.

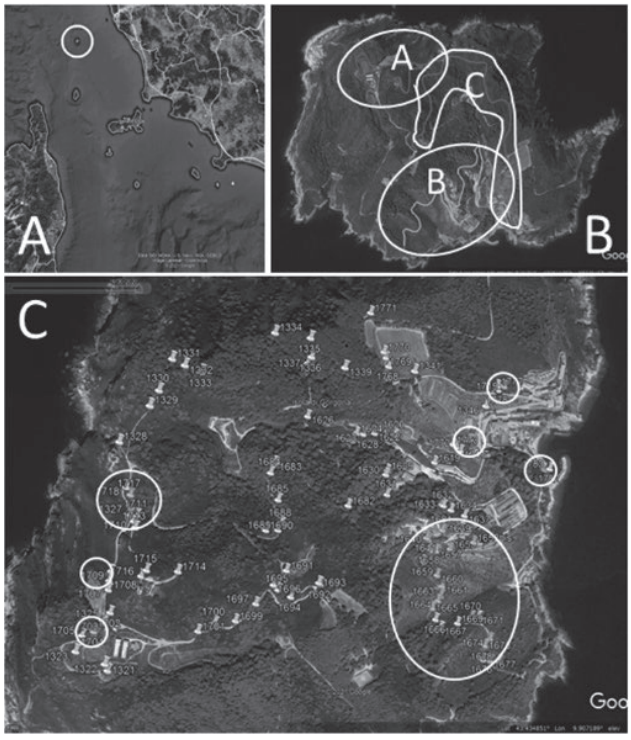

Fig. 1. A. Tuscan Archipelago islands with the Gorgona Island, marked with a white circle. **B**. Distribution of selected transects (A, B, C) on the Gorgona island. **C**. Distribution of allochtonous *Podarcis siculus* (marked with white circles) and the congeneric *P. muralis* (other unmarked waypoints). Figures 1A and B are modified from Google Earth.

At the time when our analyses were performed, there were 532 *P. siculus CYB* sequences in GenBank. Only 394 of these, whose *CYB* sequence covered the 764 bp between np 14,417 and 15,180 (Akopyan et al., 2017; Buglione et al., 2019; Deichsel et al., 2010; Garcia-Porta and Irisarri, 2019; Kolbe et al., 2013; Podnar et al., 2004, 2005, 2007, 2009; Senczuk et al., 2017, 2018; Taverne et al., 2020), were employed for comparisons along with the *P. siculus* reference sequence (PsRS) (NC_011609). Detailed information on the overall 402 samples (7 from this study and 394 as reference) is provided in Table S1.

DNA extraction

Genomic DNA was extracted via the ReliaPrep™ (Promega Madison, WI, USA) gDNA Tissue kit, using the standard protocol for mouse tail. Roughly, 0.5-1 cm of the tail was cut into smaller parts using a scalpel and homogenised in a 2 ml test tube. We added to the samples 100 µl of Tail Lysis Buffer (TLA) and 20 µl of Proteinase K (20 mg/ml), vortexed and incubated at 56 °C

Fig. 2. Adult male of *Podarcis siculus*, showing the typical "*campestris*" pattern (picture taken on Gorgona).

overnight. Then we added 300 µl of Cell Lysis Buffer (CLD) and 20 µl RNase A, vortexed and incubated (56 °C) until clear. DNA was then purified using a standard phenol/chloroform method. Purified genomic DNAs were eluted into Promega elution buffer.

CYB sequencing and data analysis

The seven samples were Sanger sequenced. PCRs were carried out in 50 µl reactions with a standard reaction mix containing 1X Buffer (1.5 mM MgCl2), 0.2 mM of each dNTP, 2 U of GoTaq G2 Polymerase (Promega), 0.3 µM of each primer (CytF and H15425 by Senczuk et al., 2017) and ~100 ng of DNA template, using the following PCR protocol: 95 °C (2 min); 10 cycles at 95 °C (30 s), 52 °C (30 s), 72 °C (2 min); 25 cycles at 95 °C (30 s), 50 °C (30 s), 72 °C (2 min) and a final extension at 72 °C (10 min). PCR products were visualised on a 1% agarose gel and amplicons were sequenced with standard dideoxy sequencing with primers CytF and H15425 using Dye terminator chemistry (Applied Biosystems) and following the manufacturer's protocol. Sequences were output in For and Rev files in .ab1 format, cleaned by hand to remove ambiguous tails, aligned to PsRS (NC_011609) and exported to the standard FASTA format.

Phylogenetic analyses and age estimates of mtDNA haplogroups

A maximum likelihood (ML) tree was built with the software MEGAX using the GTR model (8γ distributed categories) with 1,000 bootstraps (extensive SPR method). It encompassed 402 *CYB* sequences (our

seven sequences, 394 from GenBank plus the reference sequence) and was rooted with the corresponding *CYB* sequence from *P. muralis* (NC_011607) using Geneious 8.1.5 (Biomatters; Kearse et al., 2012). Bayesian estimations were performed using Beast 2.6.0 (Bouckaert et al., 2019) under the HKY substitution model (gamma-distributed rates plus invariant sites) with a relaxed clock (log normal). The clock value of 1×10^{-8} base substitution per nucleotide per year (2% divergence rate Myr^{-1}), was entered as prior. The chain length was established at 50,000,000 iterations, with samples drawn every 1,000 Markov chain Monte Carlo (MCMC) steps after a discarded burn-in of 5,000,000 steps.

RESULTS

Transects

We visually counted 180 *Podarcis siculus* and, among them, we recorded 37 GPS points corresponding to 50 individuals (10 adult males, 30 adult females and 10 juveniles). We counted more than 400 *P. muralis* and, among them, we recorded 92 GPS points corresponding to 74 individuals (20 adult males, 11 adult females and 43 juveniles) (Fig. 1C). We captured seven Italian wall lizards in three different areas of the island (all variables recorded in Table 1), from which a small piece of the tail tip was obtained and preserved in 95% EtOH. Almost all the observed *P. siculus* were distributed along the transects characterized by open and sunny areas (Fig. 1B), while only three individuals were found in the urban context of the island (transect C). Specifically, only some individuals were found along transect A, three only in transect C and almost all the other lizards in transect B. This latter transect is characterized by an abundant olive tree plantation, whose establishment is relatively recent (from 1999 to 2015). On the contrary, *P. muralis* was common and widespread on the island (see Fig. 1C), being relatively scarce only along transect B, especially where the habitat is much open, sunny and cultivated.

Podarcis siculus CYB sequences

We sequenced 924 bp of the *CYB gene* from the seven tails collected from Gorgona Island. We detected three haplotypes (Hd = 0.714 ± 0.127) and a total of seven variable sites (Table 2). On average 3.43 ± 1.08 nucleotide differences were found between any two sequences and the average nucleotide diversity (π) was 0.373% (± 0.066%). When considering all available *CYB* sequences (n = 402), we detected 229 haplotypes (Hd = 0.994 ± 0.001) with 217 variable sites. On average 35.48 ± 1.14 nucleotide differences were found between any two sequences and π was 5.59% (± 0.06%).

Phylogeny of Podarcis siculus CYB sequences

An initial phylogenetic survey by Senczuk et al., (2017) encompassing 277 mtDNA *CYB* sequences revealed three major haplogroups present throughout the species' distribution range. They were named A for Adriatic, T for Tyrrhenian and S for Sicily. The addition of our seven samples from Gorgona together with 118 additional sequences from GenBank (Podnar et al., 2004, 2005, 2007; Mayer et al., 2010; Kolbe et al., 2013; Akopyan et al., 2017; Senczuk et al., 2018; Buglione et al., 2019; Garcia-Porta and Irisarri, 2019; Taverne et al., 2020) provided a more in-depth resolution of the species phylogeny (Fig. 3). All samples fall within haplogroups, A, T and S whose founding nodes were dated, through Bayesian estimates, to 2,602 ± 426, 1,925 ± 3,259 and 4,709 ± 620 thousand years ago (kya), respectively. Haplogroups A and T are sister clades whose ancestral AT node is dated at 3,909 ± 534 kya. The *P. siculus* ancestral mitogenome (PsAM) was estimated at 6,150 ± 735 kya.

Table 1. Biometry of *Podarcis siculus* samples from Gorgona. bmass = body mass (g); svl = snout to vent length; h_l = head length; h_w = head width; h_h = head height (all length in mm).

Sample ID	sex	age	site	Transect	bmass	svl	h_l	h_w	h_h
GORG01	male	adult	Torre Vecchia	A	7.1	65.0	17.5	9.9	7.9
GORG02	male	juvenile	Capanne	B	4.0	58.0	14.5	8.4	6.3
GORG03	male	adult	village	C	8.1	71.0	17.6	10.0	8.2
GORG04	male	adult	Capanne	B	8.2	71.0	17.5	10.1	8.2
GORG05	female	adult	Capanne	B	3.1	55.5	12.7	7.5	5.4
GORG06	female	adult	village	C	4.5	55.5	13.3	7.4	5.8
GORG07	female	adult	Torre Vecchia	A	---	---	---	---	---

Table 2. Nucleotide substitutions identified in the three *P. siculus CYB* haplotypes from Gorgona.

Haplotype	Sample [a]	Mutations relative to the reference sequence (NC011609)							GenBank accession number
		np 14,436	np 14,607	np 14,985	np 15,027	np 15,042	np 15,063	np 15,255 [b]	
Reference	(NC011609)	T	C	A	T	C	A	T	
1	GORG03	C	.	G	.	.	C	.	OM925988
1	GORG05								OM925989
1	GORG06								OM925990
2	GORG02	.	.	G	C	.	T	.	OM925991
3	GORG01	C	T	.	.	T	T	C	OM925992
3	GORG04								OM925993
3	GORG07								OM925994

[a] 924 bp (from np 14,357 to np 15,280) of the *CYB* gene were sequenced for all samples.
[b] This nucleotide position was not included in phylogenetic analyses because outside of the sequence range available for most of the *CYB* sequences from GenBank.

Haplogroup A (n = 112), representative of individuals with Adriatic origins, was indeed mainly sampled around the Adriatic basin (Croatia and Italy), but also in Umbria, Lazio, Campania and Calabria. It harbours the lowest intra-clade nucleotide diversity (1.565 ± 0.125 %) (Table 3) and is composed of three major sub-haplogroups: A1, A2 and A3. Haplogroup A1 (n = 9) encompasses only Croatian individuals and is the youngest (359 ± 133 kya). Haplogroup A2 is the most represented (n = 101) and the oldest (909 ± 164 kya). It encompasses samples from the Italian Adriatic coast, but also from Calabria, Campania, Lazio and Lombardia. Haplogroup A3 (n = 11) (807 ± 219 kya) includes mainly individuals from Calabria, but also one each from Campania and Emilia-Romagna.

Haplogroup T (n = 97) is representative of individuals with Tyrrhenian origins (Toscana, Umbria and Lazio), but also from Emilia-Romagna. It is composed of two major sub-haplogroups, which were renamed from the original study (Senczuk et al., 2017) to T1 and T2 given their major split, which was not considered previously. Haplogroup T1 (n = 45), dated at 990 ± 224 kya, is mainly Toscana-specific with a couple of individuals collected in Umbria. It is further sub-divided into haplogroups T1a and T1b (Ta and Tb, respectively, in Senczuk et al., 2017) though T1a only encompasses one individual. Haplogroup T1b (535 ± 131 kya) was found to include all seven sequences from this study (Fig. 4). Samples GORG 03, 05 and 06 from Gorgona Island share the same haplotype (n. 1 in Table 2) with JX186543 (Kolbe et al., 2013) from Orti Bottagone (WWF Oasis in Piombino). GORG 02 harbours a novel haplotype (n. 2), though similar to those detected in samples from the Giannella peninsula (KY065091-KY065095) (Senczuk et al., 2017). Finally, GORG 01, 04 and 07 harbour the same novel haplotype (n. 3), which appears to be closest related to JX186545 from Pisa (Kolbe et al., 2013) according to the haplotype network (Fig. 5). Thus, in all cases the closest *CYB* sequences were found in mainland Toscana.

Haplogroup T2 (n = 52; 791 ± 184 kya) mainly encompasses individuals from Central Italy. It is composed of two major sub-haplogroups T2c and T2d (Tc and Td, respectively, in Senczuk et al., 2017). Haplogroup T2c (444 kya ± 110 kya) is found in Emilia-Romagna, Toscana, Umbria and Lazio, while haplogroup T2d is younger (246 ± 73 kya) and appears to be Lazio-specific. Haplogroup S (n = 193), the most represented and with the largest intra-haplogroup nucleotide diversity (3.185 ± 0.245 %) (Table 4), is representative of individuals with a Sicilian origin. It is composed of three main sub-haplogroups: S1, S2 and S3. Haplogroup S1 (2,170 ± 403 kya) is mainly found in Calabria, while haplogroup S2 (404 ± 146 kya) appears to be Calabria-specific. Finally, haplogroup S3, which is the most divergent (1,423 ± 230 kya), is almost completely endemic to Sicilia and subdivided into 13 sub-haplogroups (S3a-S3m).

DISCUSSION

Our survey on Gorgona Island reports for the first time the occurrence of the Italian wall lizard, which has probably been accidentally introduced to the island during the last few years. The species is now markedly widespread, despite never being found prior to our survey (Corti, 2006; Corti et al., 2011). The last survey on lizards was carried out at the beginning of 2000 (C. Corti, pers. comm.), and no evidence of *Podarcis siculus* occurrence was reported. Therefore, the time gap between the last and the current survey is well-defined. Nevertheless, we cannot

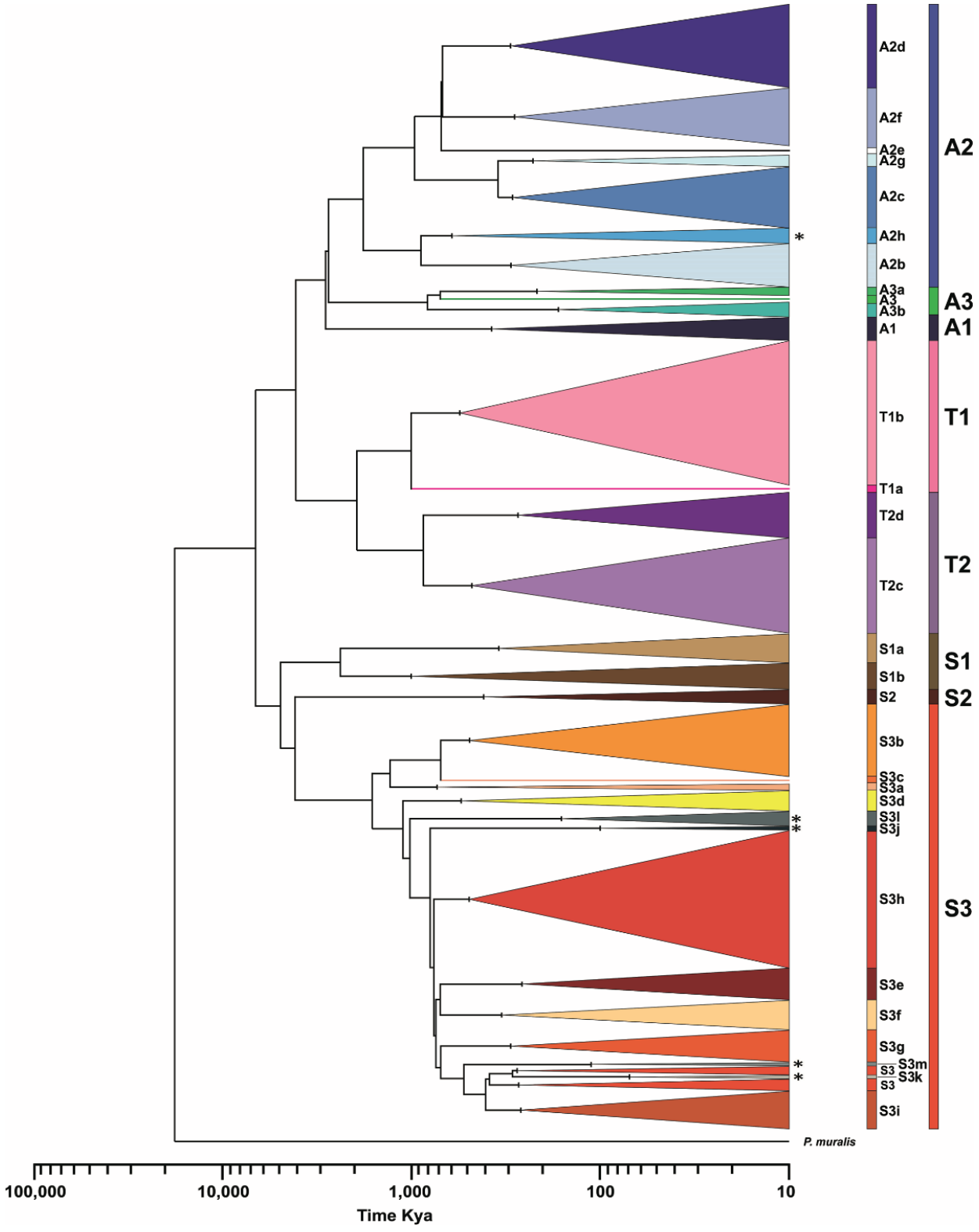

Fig. 3. Bayesian inference phylogeny of *Podarcis siculus CYB* sequences. This tree was obtained via the Bayesian method. It encompasses 402 partial *CYB* sequences (764 bp, nps 14417-15180) and was rooted using *Podarcis muralis* (NC_011607). The time scale is in thousands of years ago (kya). Coloured bars indicate haplogroup/sub-haplogroup affiliation, following colour scheme and nomenclature from Senczuk et al., (2017). New sub-haplogroups are indicated by an asterisk. A2a was removed due missing regions within the sequence.

Table 3. Nucleotide diversity (%) within and between *P. siculus CYB* sequences belonging to different haplogroups and from different geographic areas. Intra-group nucleotide diversities (π) are on the diagonal.

	Haplogroup A n = 112	Haplogroup T n = 97	Haplogroup S n = 193
Haplogroup A	1.565 ± 0.125	5.586 ± 0.148	7.283 ± 0.157
Haplogroup T	---	2.154 ± 0.037	8.093 ± 0.147
Haplogroup S	---	---	3.185 ± 0.245

state precisely when this species reached Gorgona Island. It is worth underscoring that olive trees and grapevines have been transplanted on the island in the last two decades for agricultural purposes, according to Regional and EU projects. Thus, passive transport with plants is a possible scenario, as recently reported in the UK (Clemens and Allain, 2021). Passive transportation of animals, and especially reptiles, has been documented worldwide (Burke et al., 2002; Silva-Rocha et al., 2014; Adamopoulou, 2015; Mizsei et al., 2016; D'Amico et al., 2018; Ribeiro and Sá-Sousa, 2018; Clemens and Allain, 2021). In addition, on Gorgona Island, cattle, horses and sheep have increased in number and much more fodder is imported from the mainland, via boats from Piombino harbour.

Most reptile invaders have a survivorship rate usually at about 10% of the total (Ferreira et al., 2012), supporting the idea that new colonizers frequently survive the introduction and may be more competitive than resident species (Mangiacotti et al., 2013; Kapsalas et al., 2016; Ribeiro and Sá-Sousa, 2018; Damas-Moreira et al., 2020). In particular, the conclusion of Detwiler and Criscione (2014) "invasive metapopulation has rapidly reached the establishment stage as indicated by relatively constant effective sizes and migration rates among introduced subpopulations", appears to fit very well with the high number of adult and juvenile *P. siculus* that we observed on Gorgona Island.

Colonization times and population structure of introduced species are often underestimated and genetic data of insular populations may provide correct information on the original distribution of analysed species (Silva-Rocha et al., 2019). Importantly, some research underlined the different invasion origins (Toscana, Sardegna, Calabria, Sicilia, Silva-Rocha et al., 2012), and possible times of introduction, ranging from the Middle Age for the Balearic Islands to the first half of the XX century for the Almeria and Cantabrian populations, or even more recently (Silva-Rocha et al., 2012). Our data indicate the arrival of the lizards on Gorgona Island from the area occurring between Pisa and Orbetello, particularly because of the overlap (or close relationship) of the three haplotypes observed on the island with those previously reported in a wide area of the coast of Toscana. To explain the detection of three distinct *CYB* haplotypes, at least three unrelated female founders from the mainland have to be postulated, individuals that most likely reached Gorgona Island through distinct introduction events. Different and not related introduction events,

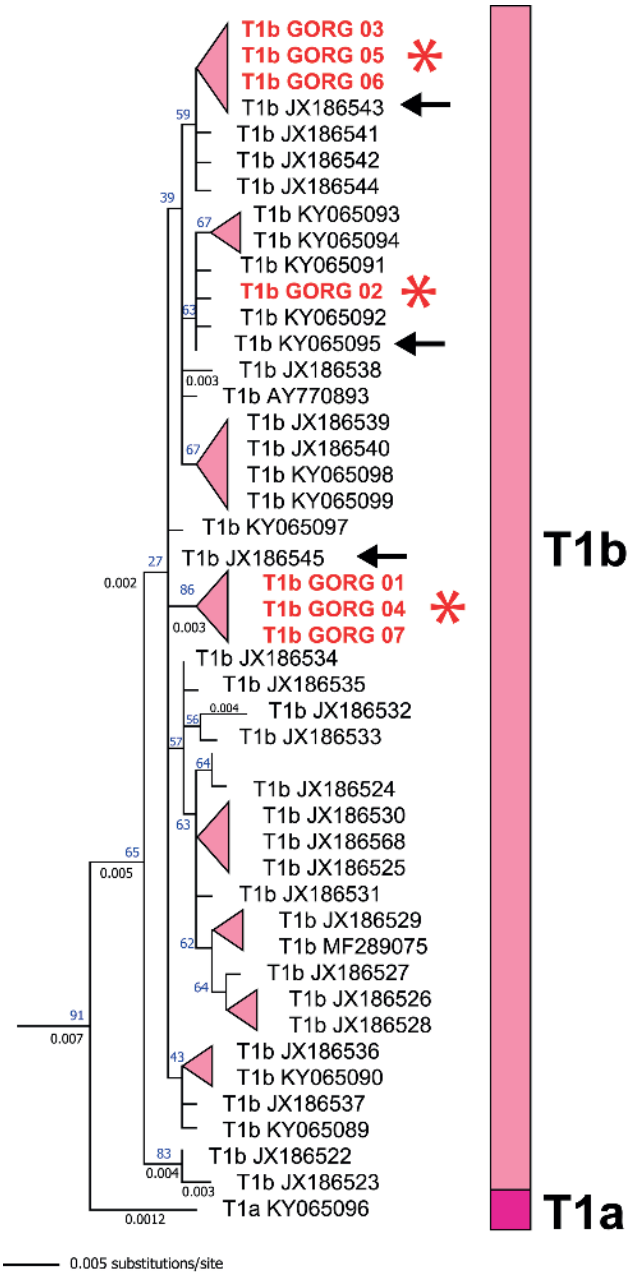

Fig. 4. Maximum likelihood phylogeny of haplogroup T1 sequences. This tree is a subset of the one in Figure S1, encompassing only haplogroup T1 sequences. Numbers at nodes indicate the bootsrap values. Asterisks indicate samples from Gorgona Island and arrows indicate their closest related relative.

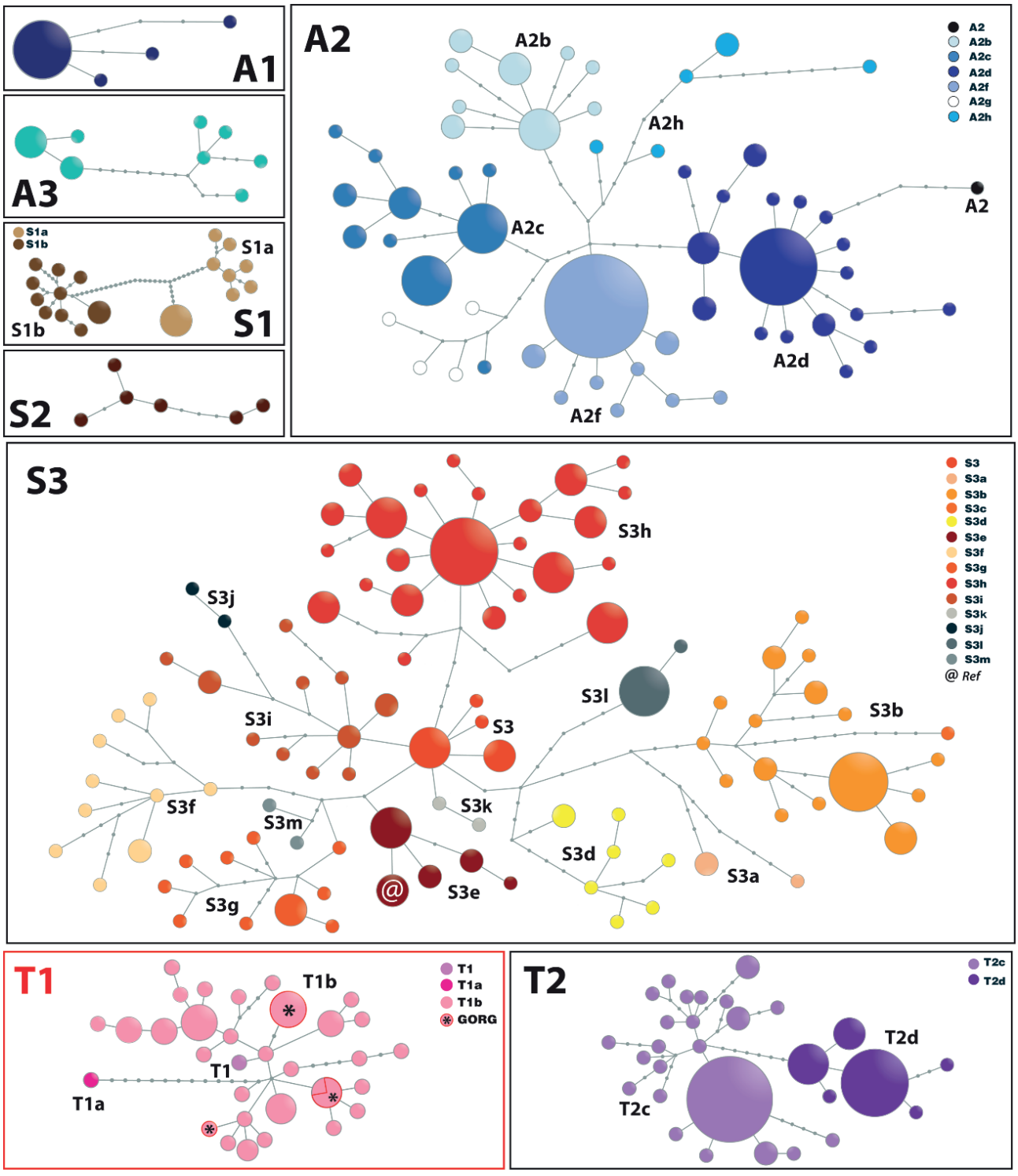

Fig. 5. Phylogeny of the 293 haplotypes found in the 402 *P. siculus CYB* sequences. Partial *CYB* sequences are subdivided into main haplogroups (Senczuk *et al.*, 2017). It was constructed using Fitchi (Matschiner M. (2015), https://evoinformatics.group/fitchi.html). Sizes of circles are proportional to the number of *CYB* sequences, with the smallest circle (for each panel) representing one individual. Dots on branches represent intermediate haplotypes and '@' is the reference sequence. Gorgona Island samples are highlighted with an asterisk.

Table 4. Bayesian age estimates for *P. siculus* haplogroups and subhaplogroups. PsA*CYB* indicates the *Podarcis siculus* Ancestral *CYB* sequence.

Summary Statistic	Age – kya	St Dev – kya
Podarcis muralis	15,670	2550
PsA*CYB*	6,150	735
AT	3,909	534
A	2,602	426
A1	359	133
A2	909	164
A2'3	1,742	317
A2b	268	85
A2c	271	69
A2d	283	70
A2f	252	74
A2g	207	65
A2h	575	145
A3	807	219
A3a	201	83
A3b	148	52
S	4,709	620
S1	2,170	403
S1a	331	112
S1b	992	248
S2	404	146
S2'3	3,902	586
S3	1,423	230
S3a	672	202
S3b	473	123
S3d	528	158
S3e	239	80
S3f	312	88
S3g	297	82
S3h	483	102
S3i	225	65
S3j	100	59
S3k	67	41
S3l	142	49
S3m	113	64
T	1,925	325
T1	990	224
T1b	535	131
T2	791	184
T2c	444	110
T2d	246	73

are the unique logical explanation for having found the three distinct *CYB* haplotypes, similarly to what has been found in the Iberian peninsula (e.g., Silva-Rocha et al., 2012) and in some other countries, where pathways and origins have been determined (Silva-Rocha et al., 2014). These introductions were accidental and the lizards possibly arrived with olive trees and other plants (Clemens and Allain, 2021), rather than together with the fodder for domestic animals or using man-made objects, confirming the high invasive potential of the species (Silva-Rocha et al., 2014; Clemens and Allain, 2021).

Further surveys and molecular analyses are required to understand *i*) the number of colonization events and *ii*) if other founder haplotypes are present. Finally, it could be important to monitor and study ecological and behavioural patterns of Gorgona Island population(s) with respect to those living on the continent, to assess how *P. siculus* interacts with the locally adapted *P. muralis,* and to evaluate if the eradication of this allochthonous species from Gorgona Island should be performed. Experiments on competitive interactions (i.e., chemical avoidance, territorial behaviours, food preference) and biometric analyses of head shape and body size between the two *Podarcis* species may give useful insights into the ecological plasticity of both the residential and the alien lizard.

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SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at <http://www-9.unipv.it/webshi/appendix/index.html> manuscript number 12388

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Molecular analysis of recently introduced populations of the Italian wall lizard (*Podarcis siculus*)

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Abstract. In recent decades, many reptile species have been introduced outside their native ranges, either accidentally through the transportation of goods and materials (e.g., plants, construction materials), but also intentionally through the pet trade. As a paradigmatic example, the Italian wall lizard, *Podarcis siculus*, native to the Italian Peninsula, Sicily and the north Adriatic coast, has been introduced in several nearby islands since historical times (Corsica, Sardinia, Menorca). Besides these regions, scattered populations were later reported from the Iberian Peninsula, France, Switzerland, Turkey, Greece, the United Kingdom and North America. Here, we provide molecular evidence regarding the introduction and origin of *P. siculus* in six new populations outside its native range: Romania (Bucharest and Alba Iulia), inland Croatia (Zagreb and Karlovac), Italy (Lampedusa Island) and Azerbaijan (Baku). Phylogenetic analysis suggests that the Alba Iulia (Romania) population originated from a single clade (Tuscany), while the population from Azerbaijan is admixed including two distinct clades, one similar to those found in Sicily and the other present across the Tuscany clade. Samples from Bucharest also have admixed origins in Tuscany and the Adriatic clades. Less surprisingly, samples from Zagreb and Karlovac are included in the Adriatic clade while those from Lampedusa originated from Sicily. Overall, our results further demonstrate that *P. siculus* is able to establish outside of its native range even under different climatic conditions, not particularly from specific clades or source areas. Also, for the first time in this species, our results indicate that repeated human introductions promote lineage admixture and enhance their invasive potential.

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INTRODUCTION

Long dispersal movements of fauna constitute natural phenomena in the evolution of biological communities (de Queiroz 2005). However, the increasing degree of anthropisation and human-mediated transport are leading to an enormous increase in the rates of animal translocations. This has resulted in a major threat of biological invasions - the process by which an alien species establishes, expands its geographic range and numbers, and exerts ecological or economic impacts in a new area with negative effects on the native biota (Brown et al., 2007). Different steps are required for a species to become invasive. The first step is transportation of the species from its native range, then survive both the transportation and the conditions within the new range, and finally, reproduce and spread, threatening native biota (Williamson and Fitter, 1996). There are currently more than 14,000 alien species recorded in Europe (EASIN, https://easin.jrc.ec.europa.eu/) with more than half originating from outside the territories (Roy et al., 2019), and the number of occurrences is rapidly increasing due to new introductions and due to the growing research and awareness on this topic (Seebens et al., 2017). Evidence of the negative impacts of many alien species (Pimentel, 2011), including reptiles and amphibians (Shine, 2014; Kraus, 2015; Measey et al., 2016) is increasing. This has added urgency for achieving a thorough understanding of factors mediating success at different stages of the introduction-naturalization continuum (Richardson et al., 2000; Blackburn et al., 2011) in order to inform policies and reduce the risk of further invasions. The Millennium Ecosystem Assessment report (2005) showed that invasive alien species are one of the five main drivers of biodiversity loss. There are several mechanisms through which invasive alien species threaten native biodiversity: direct interactions such as predation/herbivory and parasitism, or in direct interactions such as competition for food or other resources, modifications of ecosystems, and introduction of new parasites (Hendrix et al., 2008; Suarez and Tsutsui, 2008; Kenis et al., 2009). However, there are alien species which apparently have little or no detectable effects on their new environment (Strayer, 2012), leading some authors to consider these effects as positive (Sogge et al., 2008; Chiba, 2010; Schlaepfer et al., 2011), although this view is controversial (Simberloff et al., 2012; Richardson and Ricciardi 2013; Cassini, 2020). In most instances the specific source of an introduced population is not known, multiple undocumented introductions are always possible, and putative routes of introduction and transport vectors may not be reliable. Information on the origin and introduction pathways is, however, crucial for determining the degree of invasiveness and for implementing appropriate management policies. In this context, molecular markers can help to reconstruct the history of an introduction, identifying the number of native range source populations, their geographic location and extent, and the distribution of variation from these sources in the non-native range, identifying those where negative effects on native biota are taking place (e.g., Kolbe et al. 2004, 2013; Fitzpatrick et al. 2012).

The Italian wall lizard, *Podarcis siculus* (Rafinesque, 1810), is one such reptile species that has been widely introduced (Kraus, 2009). Previous studies have concluded that the pathways by which the species is being introduced are multiple, ranging from the transportation of materials and goods, especially plant materials like olive trees (Valdeón et al., 2010; Rivera et al., 2011), to the pet trade industry from where individuals escaped or were released (Deichsel et al., 2010), to deliberate introductions as a biocontrol agent against pest insects (Rocha, 2021). It inhabits a wide range of habitats, from natural areas to agricultural and urban environments, and often uses man-made structures for refuge (Capula, 1994; Corti, 2006). From its native distribution in the Italian Peninsula, Sicily and the north Adriatic coast, this species has been introduced in several other places, such as the Tyrrhenian Islands, Corsica and Sardinia, Menorca in the Balearics (Podnar et al., 2005; Senczuk et. al., 2017). Besides these regions, scattered introduced populations are also known from the Iberian Peninsula, Switzerland, Turkey, Greece, United Kingdom and in the United States (Deichsel et al., 2010; Schulte and Gebhart, 2011; Silva-Rocha et. al., 2012; 2014; Kolbe et al., 2013; Garin-Barrio et al., 2020). In some of these locations, the Italian wall lizard has already been demonstrated to be harmful to native species. For example, it outcompetes native *Podarcis* species by being more aggressive (Downes and Bauwens, 2002) and more adaptable to novel situations (Damas-Moreira et al., 2019; Nicolic et al., 2019), feeding earlier (Limnios et al., 2021), eating more and growing faster (Damas-Moreira et al., 2019) and being less parasitized (Tomé et al., 2021), often resulting in spatial exclusion of natives (Nevo et al, 1972; Ribeiro and Sá-Sousa, 2018). It may also hybridize with native, unrelated *Podarcis* species contributing to the dilution of their

genetic identity (Capula, 1993, 2002; Capula et al., 2002), and may undergo fast phenotypic shifts after introduction suggesting great levels of adaptability (Herrel et al., 2008).

All this evidence suggests that the Italian wall lizard is not only an effective colonizer but also a successful invader. In this context, understanding its colonization patterns provides the basis for delineating early and more effective preventive measures (Dorcas et al., 2010). In our study, we provide molecular evidence indicating the origin of *P. siculus* in six populations outside its native range, from: Romania (Bucharest and Alba Iulia; Stănescu et al., 2020; Iftime and Iftime 2021), Azerbaijan (Baku; Iskenderov et al., 2021), Lampedusa island (Lo Valvo and Nicolini, 2001) and inland Croatia (Karlovac and Zagreb; D. Lisičić unpubl.). We investigated the introduction process of *P. siculus* by means of mtDNA sequences in a phylogeographic framework. Clarifying the origin of these alien populations is expected to improve the picture of the colonization pattern revealed by previous studies. We aimed to (i) determine the origin of the introduced populations, (ii) infer the possible colonization routes, and (iii) discuss the management implications from these findings.

MATERIAL AND METHODS

The sampling took place in July, August 2020 and July 2021 in Romania, and June 2019 in Azerbaijan, September 2021 in Croatia and September 2005 in Lampedusa island. In Bucharest, lizards were collected from the Rose Garden of the University of Agriculture and Veterinary Medicine, while in Alba Iulia they were collected on the walls of the recently restored Alba Carolina Fortress. In Azerbaijan, lizards were found on a private landholding located on the shores of the Caspian Sea in the village of Turkan (administratively included in Baku). Specimens from Karlovac and Zagreb in Croatia as those in Lampedusa town were also collected in urban environments.

We collected a total of 16 samples (tail tips) from these six localities (Table 1). The tail tips were removed by applying light pressure and were then stored in 96% ethanol. All lizards were released at the capture location. The geographical coordinates were recorded with a handheld GPS. The geographic references are given in Table 1 and shown in Fig. 1.

DNA extraction was performed using the high-salt method (Sambrook et al., 1989). Partial sequences of 520 base pairs (bp) of the *cytochrome b* (*cytb*) gene were amplified using the primers GluDG-L and CB3H from Palumbi (1991). Amplification of genomic DNA began with an initial denaturation for 15 minutes at 94 °C followed by 94 °C for 30 s, annealing at 52 °C for 60 s with 34 cycles, and extension at 72 °C for 60 s. Products were visualized with 1.5% agarose gel electrophoresis. The suitable amplicons were sent to external service (Beckman Coulter Genomics) for purification and sequencing.

The sequences generated in the present study (GenBank accession numbers: ON365568-ON365583; Table 1) were aligned with sequences downloaded from GenBank. A total of 277 sequences from the Italian Peninsula, Corsica and Sardinia (Senczuk et al., 2017), accession numbers: KY064841-KY065117 were downloaded. Additionally, 41 published sequences from other introduced populations in Eurasia: 33 sequences from the Iberian Peninsula and Menorca (Silva-Rocha et al., 2012; Garin-Barrio et al., 2020), accession numbers: JX072938-JX072960, MW192534-MW192543; seven sequences from Turkey, Greece, and United Kingdom (Silva-Rocha et al., 2014), accession numbers: KP036396-KP036402. The samples from Switzerland (Schulte and Gebhart, 2011) were not included in the analysis because they were not available in GenBank but the locations were added to the map according to Silva-Rocha et al. (2014). One sequence from *Podarcis melisellensis* from GenBank was used as an outgroup (accession number AY185057), following Silva-Rocha et al., (2014). Sequences were edited using Geneious Prime v.2020.1 (https://www.geneious.com). The alignment was performed with MAFFT v.6 (Katoh et al., 2019) and included 330 sequences + one sequence outgroup. The best-fitting model was TIM2+I+G using PartitionFinder2v. 2.1 (Lanfear et al. 2017). A Maximum Likelihood (ML) tree was constructed using RAxML v.7.2 (Stamatakis, 2006) with 1000 pseudoreplicates to assess the confidence of branches. Bayesian Inference (BI) analysis was carried out by MrBayes v.3.2 (Huelsenbeck and Ronquist, 2001) with 5×107 generations and four chains, and subsampling parameters and trees every 100 generations. Finally, 10% of the posterior samples were discarded as burn-in. To inspect the mtDNA *cytb* haplotype diversity, a 95% maximum parsimony haplotype network was constructed using the TCS inference (Clement et al., 2000) in PopART v.1.7 (Leigh & Bryant, 2015). The resulting tree was annotated using FigTree 1.4.3 (Rambaut, 2014). Molecular diversity indices, including the number of haplotypes (H), haplotype diversity (h), and nucleotide diversity (π) were evaluated in R 4.2.0 (R Core Team 2020) using the "pegas" package (Paradis, 2010). Uncorrected genetic distances (p-distances) within clades of were estimated with PAUP v.4.0a10 (Swofford, 2003). Maps were created in QGIS 3.10.8 (QGIS Development Team, 2020).

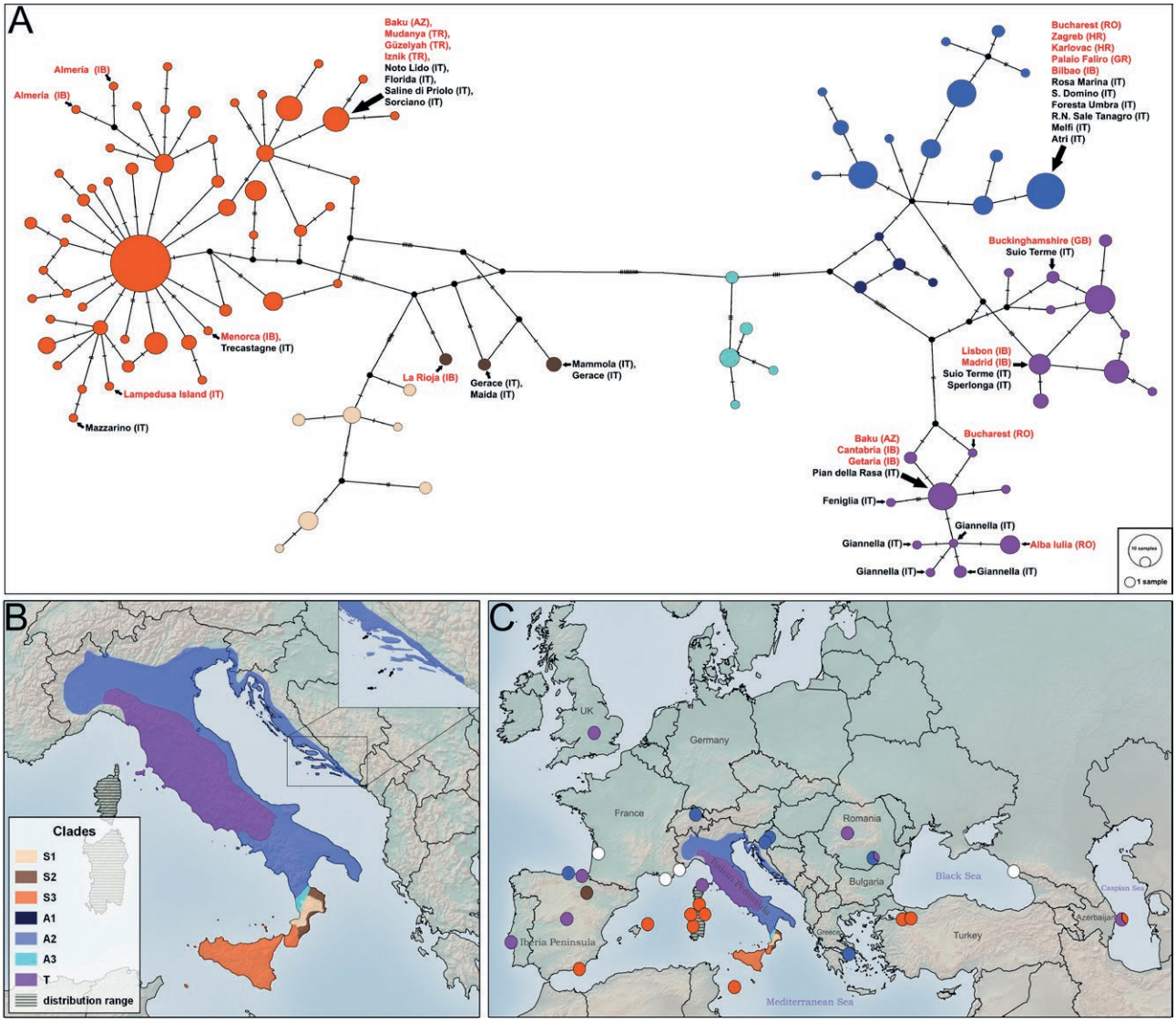

Fig. 1. Network and maps of the natural distribution and introduced populations of *Podarcis siculus* in Eurasia. **A.** Networks of the seven mtDNA clades identified by the phylogeny, colours according to Senczuk et al. (2017). Each circle size is proportional to their frequencies and each filled rectangle represents one substitution. The different colours within each network depict the principal identified clades. The names of the locations of the introduced populations are highlighted in red and the locations from the natural range are highlighted in black; Abbreviation: AZ – Azerbaijan, GB - Great Britain, GR – Greece, HR – Croatia, IB - Iberian Peninsula, IT –Italy, RO – Romania. **B.** Geographic distribution of the mtDNA haplotypes in *P. siculus* and are coloured according to the main haplogroups identified by Senczuk et al. (2017). The natural habitat is represented according to Crnobrnja-Isailović et al. (2009) but modified after Senczuk et al. (2017), namely Corsica and Sardinia are highlighted in gray lines as the introduced area. Clade A1 is indicated by the black arrows on the inlay map. **C.** Map of the natural distribution with mtDNA haplotypes and introduced populations of lizards in Europe and Asia. Populations of unknown origin are highlighted with white dots.

RESULTS

The final alignment included 331 sequences. The BI consensus tree showed a similar topology to the ML tree. The phylogenetic analysis supported the same topology with five well-supported all clades: S1, S2, S3 A1, A2, A3 and T (Appendix 1, Fig. S1) following the terminology in Senczuk et al. (2017). Clades were separated from each other with high support values (1.00-0.98). We identified three clades (S1, S2 and S3) within the Siculo-Calabrian lineage. The central-northern lineage split approximately into two main groups that for simplicity we refer to as "Adriatic" and "Tyrrhenian". The Adriatic group also included two clades with a separation of the clades A1,

Table 1. Novel sequences used in this study and their geographic position.

Genbank number	Location	Country	Coordinates		Clade	Year of the first report
			Latitude	Longitude		
ON365568	Bucharest	Romania	44°470'N	26°065'E	A2	2019
ON365569	Bucharest	Romania	44°470'N	26°065'E	A2	2019
ON365570	Bucharest	Romania	44°470'N	26°065'E	T	2019
ON365571	Alba Iulia	Romania	46°067'N	23°566'E	T	2019-2020
ON365572	Alba Iulia	Romania	46°067'N	23°566'E	T	2019-2020
ON365573	Alba Iulia	Romania	46°066'N	23°566'E	T	2019-2020
ON365574	Alba Iulia	Romania	46°066'N	23°566'E	T	2019-2020
ON365575	Alba Iulia	Romania	46°066'N	23°566'E	T	2019-2020
ON365576	Baku	Azerbaijan	40°363'N	50°212'E	S3	2019
ON365577	Baku	Azerbaijan	40°363'N	50°212'E	T	2019
ON365578	Baku	Azerbaijan	40°363'N	50°212'E	S3	2019
ON365579	Karlovac	Croatia	45°485'N	15°548'E	A2	2021
ON365580	Karlovac	Croatia	45°485'N	15°548'E	A2	2021
ON365581	Zagreb	Croatia	45°796'N	15°976'E	A2	2021
ON365582	Zagreb	Croatia	45°796'N	15°976'E	A2	2021
ON365583	Lampedusa Island	Italy	35°508'N	12°593'E	S3	2001

A2 and A3. The Tyrrhenian clade T was also clearly separated from the others. Tree presented in Appendix 1 (Fig. S1). The molecular diversity indices were demonstrated in Appendix 1 (Table S1). The general sample size (n) is 330 and the introduction sample size (n_{In}) included 57. Also the total number of haplotypes (H) is 106. The haplotype diversity (h) and nucleotide diversity (π) were non-significant for *cytb* but the values were similar to the Senczuk et al. (2017). The uncorrected genetic distances among clades are displayed in Appendix 1 (Table S2). The largest genetic distances are between classes S1 and T (P = 0.0977). Clade A2 had a lower distance to Clade A1 (P = 0.0146). Overall, the six studied populations of the Italian wall lizard were assigned to three clades (S3, A2 and T). The phylogenetic analysis suggests that the Romanian population from Alba Iulia originated from the Tuscany region (clade T) and was included in the haplogroup together with samples from the Giannella and Feniglia (Italy). The population from Bucharest (Romania) revealed admixture: one individual was included in the clade T and close with the samples from Pian della Rasa but the other two belonged to the Adriatic clade (clade A2) and included in a large haplogroup with samples from locations such as Rosa Marina, S. Domino, Forest Umbra, R.N. Sale Tanagro, Melfi and Atrium (Italy). The population from Azerbaijan (Baku) was also admixed including two distinct clades, one similar to the clade found in Sicily (clade S3). This is a large haplogroup that also includes samples from Noto Lido, Florida, Saline di Priolo and Sorciano (Italy). Other Azerbaijani samples came from Pian della Rasa (clade T). The sample from Italy (Lampedusa Island) was included in the clade S3 and close with a sample from Mazzarino. The Croatian samples from Zagreb and Karlovac came from the same haplogroup with Romanian samples from Bucharest in clade A2.

The previously-studied sequences from alien populations of the Italian wall lizard were included in four clades: S3, S2, A2 and T. The Tyrrhenian clade T includes the largest number of samples of introduced populations (n_{In} = 28, n = 66, H = 20), mainly distributed across the north-central Tyrrhenian coast, included the largest number of samples of introduced populations in Eurasia. In addition to Romania and Azerbaijan, this clade was present in the Iberian Peninsula (Madrid, Getaria, Cantabria and Lisbon) and the United Kingdom (Buckinghamshire). Clade A2 (n_{In} = 13, n = 71, H = 16), ranging across the Adriatic coast (excluding clade A1 which is restricted to the Curzolan Islands, Croatia), included two samples from Bucharest (Romania) and Croatia (Zagreb and Karlovac) as well as the published samples from Greece (Palaio Faliro) and Iberian Peninsula (Bilbao). Clade A3 (n_{In} = 0, n = 10, H = 3) includes populations from northern coastal areas of Calabria (Catena Costiera). This clade is more related to the Adriatic clade A2, forming part of the central-northern lineage, than to the other clades found in southern Calabria. We only failed to find any introduced populations originating from the clade A3. Clade S3 is the largest lineage that includes 54 haplogroups (n_{In} = 14, n = 154). Within this clade, we identified five alien populations: two new samples from Azerbaijan and the island of Lampedusa, and also the

Fig. 2. Representative pictures of *Podarcis siculus*. **A.** *P. siculus* from Alba Iulia (Romania); **B.** *P. siculus* from Bucharest (Romania). Photos by T. Sos.

published samples from Southern Iberia (Almería), as well as Menorca and Turkey (Mudanya, Güzelyah, Iznik). Clade S2 (n_{In} = 2, n = 7, H = 7) included only samples from one location (La Rioja, North Iberia). Samples from southern Italy (such as Gerase, Maida and Mammola) are also included in this clade. Clade S1 (n_{In} = 0, n = 17, H = 3) comprising populations from the southern part of the Italian Peninsula was more related to clade S2.

DISCUSSION

This study adds new valuable data to the complex picture of the invasion biology of *P. siculus*, a species with a complex phylogeographic structure, which encompasses multiple lineages across its range (Senczuk et al., 2017; Fig. S1). The results indicated seven highly supported clades within the eastern three-lined lizard (S1, S2, S3 A1, A2, A3 and T). Possibly, the existence of complex topography and multiple refugia across the distribution range of the subspecies have led to the present diversity and distribution pattern of clades (Appendix 1, Table S2). As such, the studied alien populations not only belonged to different lineages as previously reported for other studies, but also more than one lineage was found in two of the introduced populations (e.g., Baku and Bucharest; Fig. 1).

Our results support previous claims that the introduced Italian wall lizard populations have multiple and even admixed origins within their native range (namely the Italian Peninsula). In fact, *P. siculus* may use both vegetation and rocks for foraging, basking and finding shelter (Corti, 2006). This probably allows it to be unknowingly transported with construction materials, plants or other materials associated with construction works, agriculture and gardening (Silva-Rocha et al., 2014). In fact, the habitats of the populations from Bucharest, Zagreb, Karlovac and Baku have all undergone gardening and plant importation (Iftime and Iftime, 2021; Iskenderov et al. 2021; D. Lisičić unpubl.; Fig. 3, B and C). While those in Lampedusa have undergone significant reconstruction works during the past years (M. Carretero pers. obs., respectively; Fig. 3, D). A similar situation occurred in Alba Iulia (Romania), where lizards were found after the reconstruction of the Alba Carolina Fortress (T. Sos pers. obs., respectively; Fig. 3, A). The distribution of this species to the east is associated with an increase in trade, namely the growth of exports of plants from the Mediterranean (Kukushkin et al., 2017). *P. siculus* was also found for the first time in Sochi (southern Russia, see Fig. 1), which is a large port city (Tuniyev et al., 2020). The origin of this population is not known today, but the interesting fact is that this population was found simultaneously with the population in Baku, Azerbaijan (Iskenderov et al. 2021). Populations from France are also of unknown origin, especially the recent discovery of this species in the Gradignan Botanical Garden, Gironde (Berroneau et al. 2021, Fig. 1). Similar pathways of introduction have been reported for the congeneric *P. muralis* (Santos et al., 2019; Jablonski et al., 2019) although the more saxicolous habits of this species makes it less suited than *P. siculus* for using vegetation as an introduction vehicle.

Because these pathways of introduction are human-mediated, the biogeographic signal was expected to be minimal (Helmus et al., 2014). Indeed, we found little or no correspondence between the geographical location of the populations and their phylogenetic lineages. The only exceptions were the populations from inland Croatia and Lampedusa island, which belonged to lineage closest to the native populations (North Adriatic and Sicily, at a distance of 100-200 km, respectively). The population on

Fig. 3. Invaded habitats by *Podarcis siculus* in Europe and Asia: **A.** Alba Carolina Citadel, Alba Iulia (Romania); **B.** University of Agriculture and Veterinary Medicine, Rose Garden, Bucharest (Romania); **C.** private garden in Baku (Azerbaijan); **D.** the island of Lampedusa (Italy). Photos by T. Sos (A, B), T.M. Iskenderov (C), M.A. Carretero (D).

Lampedusa island was discovered in 2001. We collected samples in 2005 and confirmed the existence of this population on the island. Populations from Croatia (Karlovac and Zagreb) were found quite recently (in 2021), which requires further research to confirm the successful introduction of populations. Other finds from Romania and Azerbaijan were reported for the first time in 2019-2020. However, lizards were repeatedly observed in the following years in these localities and juveniles were found, which confirms the successful breeding of lizards in new areas (Stănescu et al., 2020; Iftime and Iftime, 2021; Iskenderov et al., 2021). In its native range, the Italian wall lizard appears to be more thermophilic, but this doesn't seem to be reflected in the climates prevailing in non-native areas. In particular, several introduced populations (Northern Iberian Peninsula, Switzerland, United Kingdom, as well as North America, see Silva-Rocha et al., 2014; here, Romania and Azerbaijan) clearly occur in non-Mediterranean climates, with harsh winters. Moreover, there is no apparent correspondence between the lineage subranges, although this should be further explored with modelling evidence (Carretero and Sillero, 2016).

Another relevant result, reported here for the first time, is the existence of admixed populations, namely in Bucharest and in Baku, which were dense although localized. On one hand, this already reveals repeated introductions from different source regions with contrasting climate regimes (Tuscany and Adriatic in Bucharest; Tuscany and Sicily in Baku). On the other hand, potential hybridization between those contacting haplogroups might produce novel phenotypes adapted to local conditions, hence, increasing the invasive potential of the species (Kolbe et al., 2007), as it has already been reported for *P. muralis* (Santos et al., 2019; Michaelides et al., 2013; While et al., 2015).

These phylogenetic outcomes, added to the partial but repeated evidence of functional negative interactions between *P. siculus* (belonging to multiple lineages and in multiple areas) and native *Podarcis* species, configure an

invasion scenario. The timeframe and spatial scale of such a threat should be uncovered by an assessment of *P. siculus* at a global level. Meanwhile, a principle of caution recommends at least early detection of any new alien population and monitoring of existing ones (Carretero and Silva-Rocha, 2015), particularly in potential invasion hubs such as harbours and railways (Mollov, 2009; Tok et al., 2014). Eradication actions should also be considered in the early stages, when the chances of success are higher (e.g., Buckinghamshire, South East England Hodgkins et al., 2012; Athens, Greece, Adamopoulou and Pafilis, 2019).

Overall, the results obtained here accumulated to the previous evidence strongly suggest that the Italian wall lizard *P. siculus* is an effective invader. Its successful acclimatization to environmental conditions different from those prevailing in its original Mediterranean range increases the probability of becoming invasive.

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SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at < ttp://www.unipv.it/webshi/appendix> Manuscript number 12542.

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Sunny-side up: ontogenetic variation in egg mass temperatures of the wood frog *Rana sylvatica*

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Abstract. The efficacy of most biological processes is temperature dependent and, within physiological limits, on average, warmer is better. This axiom of biology has led to a wide range of adaptations for dealing with temperatures that are outside of an organism's preferred temperature. Many pond-breeding amphibians lay their eggs during early spring, when water temperatures are near freezing. Communal nest-site selection has been proposed as a mechanism to increase developmental temperatures, and temperatures near the center of egg-mass aggregations are elevated relative to egg-masses on the aggregation's periphery. It is unclear whether this spatial variation in temperature is due to concentration of metabolic heat, absorption of solar radiation, or both. Here, we explore finer scale spatial variation within egg masses of the wood frog *Rana sylvatica*, one of the earliest amphibians to breed during the North American spring. We compared peripheral and core temperatures of egg masses that were exposed either to 1) ambient sunlight from above, or 2) sunlight reflected by a mirror from below. We found that differences between core and peripheral temperatures were higher in the control than in the mirror treatment, but core and peripheral temperatures were statistically indistinguishable in both cases. Moreover, the difference in peripheral and internal temperatures increased significantly over the course of development. However, these trends were only significant in ambient sunlight and actually decreased in the mirror group. Our results suggest that the benefits of communal nesting are also experienced by individual egg masses, albeit to a lesser extent. In addition, the lack of effect in shaded egg masses suggests that the thermal advantage is tied to sun exposure and not due to concentration of metabolic heat.

Keywords. Anurans, egg-mass aggregations, developmental temperatures.

INTRODUCTION

A central tenet of biology is that all physiological processes are temperature dependent (Huey, 1991). This is because enzymes that regulate physiology have specific temperature ranges over which they can operate (Knies et al., 2009). The vast majority of these thermal-performance ranges are left-skewed and show improving physiological performance with increasing temperature, until some threshold 'optimal temperature' (T_{opt}) is reached, at which point physiological performance drops off rapidly. The generality of this pattern underlies the adage that, within physiological limits, hotter is better (Frazier et al., 2006; Angilletta et al., 2010).

Organisms in environments characterized by extreme temperatures face unique challenges around life-history and reproduction, since sub-optimal environmental conditions should constrain physiological performance (Skelly, 2004; Tryjanowski et al., 2006; Benard 2015). These challenges are especially prevalent for ectotherms (Huey and Tewksbury, 2009), which depend on environmental sources of warmth to maintain metabolic activity. Temperatures that exceed a critical threshold (e.g., CTmax) may be lethal and during hot periods

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ectotherms may require shaded habitat or underground refugia to behaviorally thermoregulate (Díaz-Ricaurte et al., 2022; Sinervo et al., 2010). High temperatures may lead to dehydration which itself can change thermoregulatory dynamics (Guevara-Molina et al., 2020). Many ectotherms, including insects, reptiles and amphibians, buffer themselves from the negative consequences of sub-optimal temperatures by estivating or entering diapause (Blanckenhorn and Fairbairn, 1995; Ellner et al., 1998; Storey and Storey, 2012) during colder periods of the year. Nevertheless, these same organisms may often face extremely cold conditions that occur at the peak of their reproductive activity in early spring (Costanzo and Lee, 1993).

Many pond breeding amphibians emerge from winter hibernacula as soon as temperatures exceed freezing (Waldman, 1962; Herreid and Kinney, 1967). The first amphibians to emerge at the end of North American winters often breed while ponds are still ice-covered, and females lay eggs in water that is very close to its freezing point (Costanzo and Lee, 1993). Aggregating egg masses in communal nest sites is one potential adaptation to cold. Previous studies have shown that egg masses at the center of these communal aggregations are warmer compared to those on the periphery (Savage, 1961; Hassinger, 1970). Warmer temperatures should be advantageous to developing embryos, since warmer temperatures speed development and tadpoles that reach metamorphosis more quickly will have a higher probability of escaping vernal pools before they dry (Goldstein et al., 2017). Other work (Arrighi et al., 2013) has shown that the influence of diel fluctuations in temperature may be as (or more) important as that of average temperature on developmental rate. In addition, amphibian eggs often have higher concentrations of melanin in their dorsal hemisphere, which may serve as both protection from ultraviolet radiation and a means of absorbing and retaining heat (Licht, 2003).

Although a handful of studies have documented a thermal advantage to aggregated egg masses (Waldman, 1982; Skelly, 2004), the source of thermal variation (e.g., solar vs. metabolic) remains unclear as does the degree to which this thermal advantage occurs at smaller spatial scales (i.e., within individual egg masses). Moreover, all of these studies have been conducted as point estimates in time and so we currently have no information about how developmental progression impacts temperature of the egg mass itself. Developmental stage may be important for impacting both the generation of metabolic heat as well as greater thermal absorption by larger embryos. Finally, it is possible that egg mass aggregations are not at all adaptive but are the result of space constraints within a pond, aggregation due to wind currents, or other neutral exaplanations.

Here we build on previous work to address these shortcomings. The wood frog, *Rana sylvatica*, is among the earliest amphibians to breed in North America, emerging from winter hibernacula as soon as temperatures exceed freezing (Slough and Mennell, 2006). Wood frogs at our study populations near Norwich Vermont, USA, elevation ca. 300m; 43.7153°N, 72.3079°W (WGS 84 web Mercator), typically emerge during the end of March-early April (Brady et al., 2019; Goedert and Calsbeek, 2019). Males and females arrive at breeding ponds and breed explosively (Swierk et al., 2014), most oviposition occurring within a few days of arrival, and eggs are often laid while ponds are still partially covered in ice. These life history traits make wood frogs especially relevant for understanding the effects of temperature on embryo development. First, we test whether individual egg masses also exhibit warmer internal compared to peripheral temperatures. Next, we experimentally test the hypothesis that egg masses warm by absorbing radiant heat from above (i.e., via the pigmented dorsal surface) more efficiently than from below. Lastly, we provide temperature measurements over the time-frame from oviposition to hatching to assess the degree to which development itself may influence temperature variation (e.g., by metabolic warming) within egg masses

METHODS

We collected 10 wood-frog egg masses from a single pond within 36 hours of oviposition in April of 2021. Egg masses were collected by hand and carefully transferred to a plastic holding tank along with ~6L of unfiltered water from the same pond. We assessed Gosner's stage (Gosner, 1960), recording the average the developmental stage of five embryos scored under a microscope for each egg mass. All embryos were at Gosner's stage 10 at the start of the experiment. Developmental stage was thereafter scored by visual inspection to minimize disturbance over the course of the experiment. Egg masses were sectioned into two groups of ca. 75 embryos each (i.e., 150 eggs total from each egg mass) and these two sections of egg mass were then split randomly (by coin toss) into the two groups, such that ~75 individuals from of each of the 10 egg masses were represented in both groups. Each set of 75 embryos in the first group were placed in separate 500 ml cylindrical plastic containers (10 cm diameter, 10 cm deep), covered with an opaque lid and placed outdoors on wire shelving that was suspended approximately 30 cm above a mirror. The mirror group was designed to

reverse the direction of sun exposure from the melanic dorsal to the white ventral side of the embryo. Each group of 75 embryos in the second group were likewise placed in individual 500 ml plastic containers but were covered with a clear plastic lid and placed on wire shelving over a dark green substrate that was covered with leaf litter from the adjacent forest. The two groups were placed in the same outdoor location with no canopy cover. Photoperiod during the experiment was approximately 14:10 (L:D). All sections of egg mass were suspended in approximately 450 ml of pond water, which was changed once, seven days into the experiment.

Every one to two days we chose half of the ten containers in each group at random for temperature measurements. We recorded air temperatures using an outdoor Accu-Rite™ thermometer, and egg-mass/water temperature using a digital thermometer (Thermoworks model RT600C-N) on the periphery of the egg mass and inserted into the center of the egg mass at the same water depth (~2 cm). We recorded air temperatures outside of the containers, and water temperatures at the time of each measurement. We monitored rates of development by recording changes in Gosner stage (Gosner, 1960) during each temperature recording. Temperature measurements continued until hatching or 14 days, at which point the experiment was ended and the hatchlings and a few unfertilized eggs were transferred to larger volume holding tanks. In total we recorded 110 temperatures during 11 of the 14 days.

To minimize the problem of pseudo-replication, we calculated the mean temperature for each group on each day and used these mean values as our unit of observation in all analyses: 11 averages per group, 22 total observations. All data met the criteria for parametric statistics (normality, homoscedasticity, and independence; Sokal and Rohlf, 1995) based on testing in JMP Pro v.16. We tested for relationships between time elapsed since the start of the experiment (days) and air/water temperature using simple linear regression. We tested for a difference in peripheral temperature and core temperature between groups using ANOVA with 1 and 19 degrees of freedom. We tested for a difference in temperature from the center and periphery of the egg mass by subtracting the latter from the former and then using these difference values as the dependent variable using ANOVA with 1 and 19 degrees of freedom. We included Gosner's stage and tested for an interaction between Gosner's stage and experimental group to test whether there was a stage specific difference in temperature between groups in an ANOVA with 1 and 18 degrees of freedom. All tests were two-tailed and were conducted in JMP Pro v.16 (SAS Institute, Cary, North Carolina, USA 1989-2022).

RESULTS

Owing to the vicissitudes of spring temperatures during 2021 (typical of the NE United States), there was no significant correlation between measurement date and either air temperature ($r^2 = 0.03$, $t_{20} = 0.74$, $P = 0.47$) or water temperature ($r^2 = 0.07$, $t_{20} = 1.23$, $P = 0.23$). Mean temperature at the periphery of the egg mass section was not significantly different between the two groups (X ± SE: control = 13.13 ± 1.09 °C, mirror = 12.45 ± 1.05 °C; ANOVA: $F_{1,19} = 0.04$, $P = 0.80$, effect of Gosner's stage $P = 0.23$; Fig. 1A). Mean temperature at the center of the egg mass section was likewise not significantly different (control = 13.21 ± 2.47 °C, mirror = 12.32 ± 2.36 °C; ANOVA: $F_{1,19} = 0.87$, $P = 0.79$, effect of Gosner's stage $P = 0.21$; Fig. 1B). However, the differences between core and peripheral temperatures were significantly larger in the control than in the mirror group (0.39 ± 0.08 vs.

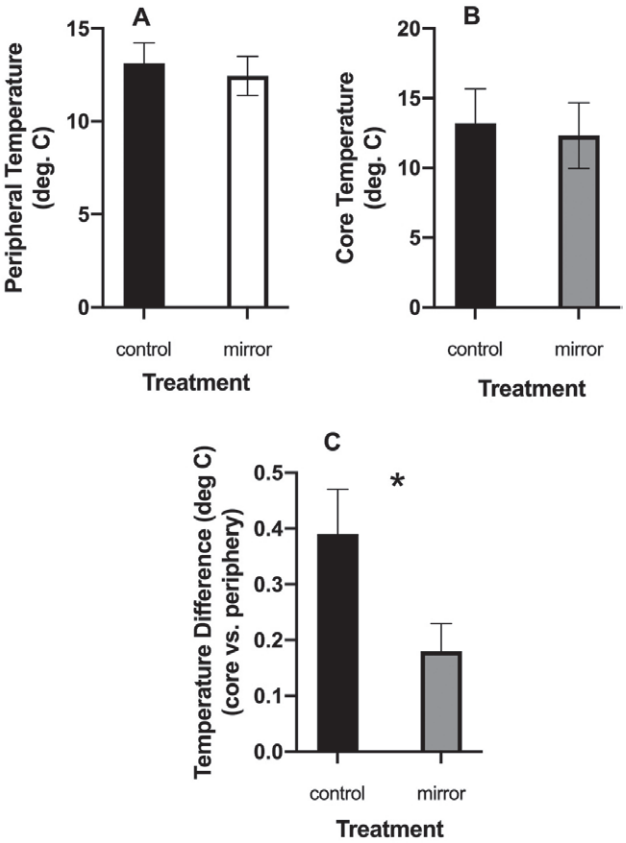

Fig. 1. Temperatures did not differ at the periphery of the wood frog egg mass (A), nor at the core of the egg mass (B) in either group. However, the difference in core and peripheral temperatures (that is, comparing means in panels A and B was significantly higher (* $t_{20} = 2.29$, $P = 0.03$) for the control group compared to the mirror group (C). Histograms bars show mean values ± one standard error.

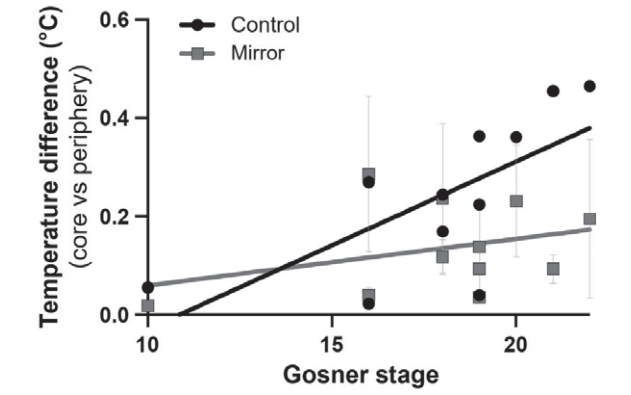

Fig. 2. *Top*. The difference in temperature between core and periphery increased throughout embryonic development of wood frogs (*Rana sylvatica*) for the control group but not in the mirror group. Data points show mean values ± one standard error. *Bottom*. Picture of the study species.

0.18 ± 0.05 °C respectively, ANOVA: $F_{1,19}$ = 6.44, P = 0.02, effect of Gosner's stage P = 0.03; Fig. 1C). Moreover, this difference increased over the course of development resulting in a significant group × Gosner's stage interaction (ANOVA: $F_{1,18}$ = 6.42, P = 0.04; Fig. 2). Hatching success at the conclusion of the experiment did not differ between groups; nearly all containers showed 100% hatching success. Whereas developmental stage and hatching in the mirror group lagged the control group by one day, developmental stage did not vary with position in egg mass section in either group. Nor did hatching dates vary within groups. Given this lack of variation, these results were not analyzed statistically.

DISCUSSION

The challenges associated with life in cold climates select for a variety of adaptations that facilitate heat retention and speed development (Skelly 2004, Sparks et al. 2006, Benard 2015). Ectotherms, which rely on external sources of heat to sustain metabolic processes, exhibit patterns in nature that may represent a limited set of strategies to maximize heat retention. Concentrating melanin and other dark pigments in the dorsal hemisphere of amphibian eggs may enhance thermal absorption within individual egg masses (Licht 2003). Likewise, communal nest sites, in which aggregations of egg masses are formed, may concentrate temperatures on their interior (Hassinger 1970).

We have shown that both of these patterns occur not just in aggregate, but also at the smaller spatial scales of individual portions of egg masses. Eggs in our control group experienced significantly warmer temperatures at the core of the egg mass compared to their peripheral counterparts but the same was not true in the mirror group. This suggests that the darker dorsal side of the developing embryo may briefly enhance thermoregulation during development compared to the white ventral side of the embryo. This effect is likely to be short-lived in nature since the dark pigment quickly subsumes the entire embryo. Wood frog embryos appear to maintain this thermal advantage even following disturbance sufficient to re-orient the embryos in their horizontal plane. We included the mirror group in our study to account for the rotational behavior of flipped embryos. It is worth noting that embryo rotation is likely a result of differences in density and not a response to light, since all embryos retained their normal orientation in both the mirror and control group.

The difference between internal and peripheral temperatures increased significantly over the course of development in our control group but not in the mirror group. This further supports a role for the pigmentation of embryos in enhancing thermoregulation (Clusella-Trullas et. al., 2007, 2009; Stuart-Fox et. al., 2019). The initial difference between dorsal and ventral pigment persists up to about the 13th Gosner's stage in wood frogs (Gosner, 1960), which occurs about one week after oviposition. After stage 13, the embryo enters gastrulation and the distribution of pigment is more evenly distributed throughout the entire embryo (Altig, 1972). As differentiation proceeds, the surface area to volume ratio of the embryo increases dramatically and the uniformly dark body acts as a heat sink. The fact that this ontogenetic shift was absent in the mirror group suggests that despite our attempts to maintain similar degrees of light exposure in the two groups, a mirror may have been insufficient to match the thermal energy absorbed in the control group. An alternative hypothesis is that the difference in peripheral and core temperatures arises due either all, or in part, to metabolic heat production. There are at least three reasons to think that this may not be true: first, ectotherms

produce negligible amounts of metabolic heat (Andrade et al., 2015). Second, we see no reason why differences in metabolism should have arisen between groups. Third, egg masses held in a dark, temperature-controlled room as part of a separate experiment (Calsbeek, unpublished) showed no variation in temperature between the center and periphery of the egg mass.

Given the rapid loss of polarity in the pigmentation of embryos, any thermal advantage to the dorsal orientation of the pigmented embryo should be short-lived (e.g., a few days). Combined with the lack of an effect in the mirror group, we suggest that our results are consistent with a brief thermal advantage to an egg that rests sunny-side up (i.e., darker side dorsal), followed by a rapidly increasing thermal advantage associated with shifts in the surface area to volume ratio of the developing anuran embryo.

Understanding the importance of incubation temperature for amphibians with different oviposition behaviors (e.g., communal versus solitary nesting) could prove important for understanding the potential impacts of climate warming. Future work should include tests for the joint roles of nesting behavior and incubation temperatures in tropical species, since temperatures in the tropics are both warmer and less variable than in the temperate zone (Sinervo et. al., 2010). These differences in thermal regime suggest that tropical ectotherms may be especially vulnerable to climate warming, since even subtle changes in temperature could surpass thermal maxima (Huey and Tewksbury, 2009). As such, small differences in temperature within egg masses could have important implications for rates of development and/or survival for tropical amphibians.

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Ecological niche differentiation in the Anatolian rock lizards (Genus: *Anatololacerta*) (Reptilia: Lacertidae) of the Anatolian Peninsula and Aegean Islands

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Abstract. The genus *Anatololacerta* is distributed in the eastern Mediterranean region including Asia Minor and some east Aegean islands. Recent phylogenetic studies suggested that this genus displayed cryptic diversity and was divided into five species: *A. anatolica, A. pelasgiana, A. ibrahimi, A. finikensis* and *A. danfordi*. The ecological niche differentiations of these species have not been studied so far. Our aims for this study were to predict the potential suitable habitats for the species nested in genus *Anatololacerta*, and to examine the niche overlaps and differentiations via identity and background tests. The occurrence data were obtained from literature and our own field surveys. Occurrence records were rarefied and assessed in a 30 arc-second resolution layer, compatible with several bioclimatic and topographic variables. Species distribution analyses were performed using maximum entropy approach and pairwise niche comparisons were evaluated by identity and background tests. Our results demonstrated that the species delimitation among this genus was not only affected by geographic isolation but also that precipitation and temperature influenced the habitat suitability for these species. Predicted suitability usually well matched the actual species distributions. Moreover, the niche overlap (identity test) analyses verified that allopatric *Anatololacerta* species show clear ecological differentiations. However, a niche overlap between parapatric species *A. pelasgiana* and *A. finikensis*, was confirmed by identity and background tests. It has been suggested that these parapatric species could be more affected by microclimatological parameters than the others. The results of our study are in agreement with the latest phylogenetic study within this genus.

Keywords. Squamata, *Anatololacerta*, niche overlap, precipitation, temperature, MaxEnt, Anatolia.

INTRODUCTION

Ecological factors, e.g., climatic factors, significantly affect the distribution of organisms and may lead to formation of new species (Zhao et al., 2019). Each species often has unique ecological niche characteristics and as a result of it, ecological needs differ even for sympatric or sister species (Soberon and Peterson, 2005). Quantifying

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and visualizing the effects of spatial and temporal ecological patterns on speciation processes have contributed to our knowledge of interactions between species and their environments (Jezkova and Wiens, 2018; Kurnaz et al., 2019; Şahin et al., 2021). In the last two decades, Ecological Niche Modeling (ENM) was frequently used to better understand these processes. ENM is a method used to predict the habitat suitability of species across space by using occurrence records and bioclimatic and topographic variables (Barve et al., 2011; Kass et al., 2018; Hosseinian Yousefkhani et al., 2019). Moreover, ENM is a very beneficial approach to better understand aspects of conservation, ecology, distribution, and evolutionary history of the species (Guisan and Zimmermann, 2000; Araújo et al., 2006; Phillips et al., 2006). Latest tool developments in modeling studies such as *ENMTools* (Warren et al., 2021), *ENMeval* (Muscarella et al., 2014) and *kuenm* (Cobos et al., 2019) provide frameworks able not only to generate maps but also to assess the niche overlap and the possible degree of differentiation among multiple species.

The complex geological history of Western Asia has shaped the Anatolian Peninsula, the Caucasus Mountains, and the Iranian steppes, resulting in high variations in vegetation covers and topographic patterns in these regions (Rajabizadeh et al., 2016). In addition, many environmental dynamics, like atmospheric concentrations of greenhouse gases, precipitation and temperature fluctuations, alterations in land use and cover have influence on ecosystem and biodiversity structure in Mediterranean Basin (Klausmeyer and Shaw, 2009). Despite some parts of the Anatolian Peninsula and its close areas have been studied in terms of ENM species distribution analysis for several herptile species (Gül et al., 2015, 2016, 2018; Hosseinian Yousefkhani et al., 2016, 2019; Heidari, 2019; Candan et al., 2021; Kurnaz and Şahin, 2021a), the western part of the peninsula and/or with Aegean Islands and Cyprus has still been less represented (Kıraç et al., 2022).

The herpetofauna in the Anatolian Peninsula and Aegean Islands is rich (180 species) (Kurnaz, 2020; Baran et al., 2021; Yaşar et al., 2021), almost as the 60 % of whole European continent (301 species) (Speybroeck et al., 2020). Besides, recent discoveries of the new species have been making the herpetofauna richer (Tuniyev et al., 2018; Jablonski et al., 2019; Yılmaz et al., 2021; Kurnaz and Şahin, 2021b; Arribas et al., 2022; Kurnaz et al., 2022). However, even though this region has been investigated in several biogeographic or phylogeographic studies (Kornilios et al., 2012; Skourtanioti et al., 2016; Kotsakiozi et al., 2018; Bozkurt and Olgun, 2020), the effects of environmental conditions on the distribution of reptile species or subpopulations are being studied only in the last decade (Fattahi et al., 2014; Gül et al., 2015; Hosseinian Yousefkhani et al., 2019; Kurnaz and Hosseinian Yousefkhani, 2020, 2021).

Anatololacerta Arnold, Arribas & Carranza, 2007 is an Eastern Mediterranean lacertid genus that is distributed along the western and southern parts of Anatolia and some Aegean islands (Karakasi et al., 2021). However, taxonomic debates on some populations of this genus have been historically controversial. Species of this genus represent an example of cryptic diversity (Bellati et al., 2015; Candan et al., 2016), a common phenomenon among lacertids (Kaliontzopoulou et al., 2012; Barata et al., 2012; Tamar et al., 2015; Freitas et al., 2016; Psonis et al., 2017; Šmíd et al., 2017; Mendes et al., 2018). The recent study on the phylogenetic relationships of *Anatololacerta* clades (Karakasi et al., 2021) classified them into five species: i) *Anatololacerta anatolica* (Werner, 1900) distributed in northwestern Anatolia, Ikaria and Samos islands ii) *Anatololacerta pelasgiana* (Mertens, 1959) in southwestern Anatolia, Symi and Rodos islands iii) *Anatololacerta finikensis* (Eiselt & Schmidtler, 1987) in western part of Mediterranean region and Psomi island iv) *Anatololacerta ibrahimi* (Eiselt & Schmidtler, 1987) central part of Mediterranean region v) *Anatololacerta danfordi* (Günther, 1876) in eastern Mediterranean region. Therefore, the cryptic diversity within this genus inspired us to test if the species delimitations can be affected by bioclimatological and/or topographic factors. That's why the objectives of the present study are i) to predict highly suitable areas for each *Anatololacerta* species distribution and determine which environmental factors are important; ii) to measure and compare the niche divergence within the genus *Anatololacerta*, as a case study for cryptic species.

MATERIALS AND METHODS

Study area and input data

This study was conducted within 25-37° East Longitude and 34.5-41° North Latitude, covering the western and southern parts of Anatolia and Aegean islands (Fig. 1). A total of 159 occurrence data (31 for *A. anatolica*, 46 for *A. pelasgiana*, 22 for *A. finikensis*, 37 for *A. ibrahimi*, and 23 for *A. danfordi*) were obtained from field surveys and literature (Eiselt and Schmidtler, 1986; Mulder, 1995; Baran and Kumlutaş, 1999; Kumlutaş et al., 2015; Yakın and Tok, 2015; Beşer, 2015; Bellati et al., 2015; Candan et al., 2016; Sarıkaya et al., 2017; Beşer et al., 2020; Karakasi et al., 2021). The raw input data for localities are given in Table S1. Data for these species were error-checked and improved to meet appropriate standards for ecological

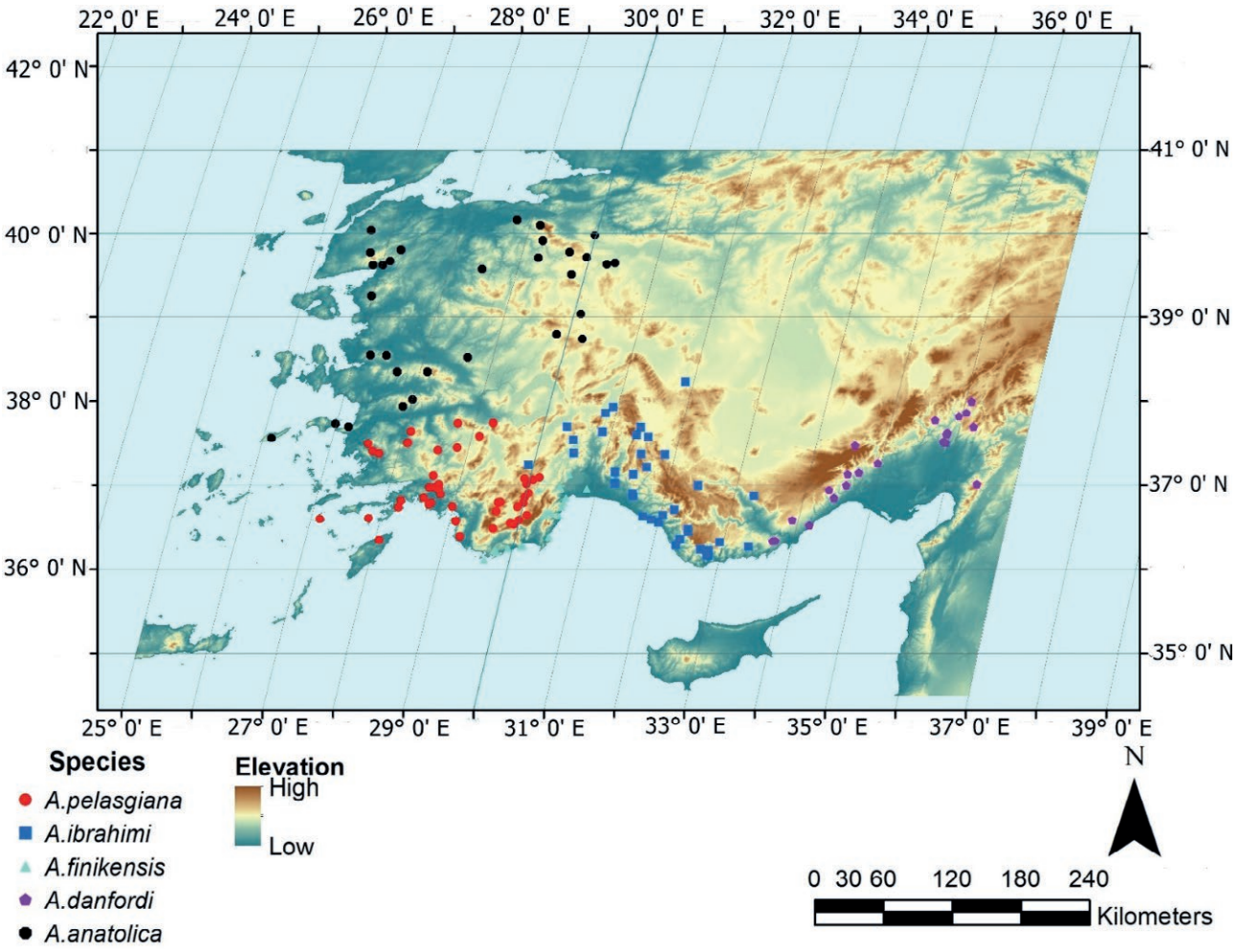

Fig. 1. Species occurrence records for genus *Anatololacerta* in the Anatolian Peninsula and Aegean Islands.

niche modeling in two steps. Firstly, georeferenced data were checked for error and data consistency for geographic coordinates (Chapman, 2005). Secondly, in order to avoid spatial sampling biases and misinterpretation of the habitat suitability analysis and niche overlap tests, the occurrence records for each species were spatially rarefied with keeping one locality in each 2 km by SDM Toolbox 2.0 (Brown, 2014).

Nineteen bioclimatic and one topographic variables were downloaded from WorldClim version 2.1 (Fick and Hijmans, 2017). The bioclimatic data were generated from global ESRI grids for current conditions (~1970-2000). Additionally, three topographic variables were obtained from the studies of Gavashelishvili and Tarkhnishvili (2016), and Gavashelishvili et al. (2018). All these environmental variables were at 30 arc-second resolution (~1 km) (Table S2) and each layer was clipped for the study area in ArcGIS 10.6.1 (ESRI, California, CA, USA) for the whole study area. Pearson Correlations between variables were calculated in R v4.1.3 (R Core Team, 2020) and highly correlated variables were eliminated ($r \geq |0.8|$) (Fig. S1).

Ecological niche modeling

Due to its robustness and dependence on presence and pseudo-absence data, maximum entropy approach was used for niche modelling. The maximum entropy algorithm, which generates the probability of presence of a given species that varies between 0 to 1, provides predictions from presence and pseudo-absence data (Phillips et al., 2009). A total of 2000 background points for each species were randomly sampled across the study area. The potential habitat suitability was modeled by using the *kuenm* package in R for the implementation of Max-

Table 1. Percentage contribution of the environmental layers used in species distribution modeling of *Anatololacerta* species.

Species	Bio 3	Bio 5	Bio 7	Bio 17	River_dist	Lai
A. anatolica	3.1	11.1	35.4	29.5	5.9	15.1
A. danfordi	13.5	1.5	17.8	19.9	3.6	43.7
A. finikensis	19.7	27.3	5.5	35.7	6.5	5.3
A. ibrahimi	0.9	8	7.2	36.6	36.9	10.4
A. pelasgiana	7.3	26	19.6	23	10.7	13.3

Ent 3.4.1 (Phillips et al., 2017; Cobos et al., 2019). To create the models for each *Anatololacerta* species, 80 % of the occurrences were used for the creation of candidate models and the remaining 20 % for independent presence as test data. The bioclimatic and topographic envelopes, derived from environmental variables, were constructed as set for each species (Table 1).

Model selection

To optimize model complexity for all 5 species, 31 combinations of MaxEnt's 5 feature classes [hinge (h), threshold (t), product (p), quadratic (q) and linear (l)] along with 17 regulation multiplier values (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 2, 3, 4, 5, 6, 8, 10) were evaluated. Using these combinations allowed us an optimal approach for generating diverse candidate models in order to select the models that explain our data best (Muscarella et al., 2014; Cobos et al., 2019). After that, candidate models were evaluated and best models were selected using not only AUC values (with the highest values), but also Akaike Information Criterion corrected for small sample sizes (AICc) (with the lowest values) (Hurvich and Tsai, 1989). Significance tests were performed using partial ROC (Peterson et al., 2008), and predictive power with a 5% omission rate (Anderson et al., 2003). Model AUC scores are evaluated as follows: AUC = 0.5: a performance equivalent to random, AUC > 0.7: useful performance, AUC > 0.8: good performance, AUC > 0.9: excellent (Manel et al., 2001). Finally, all model inputs were transformed into binary predictions using minimum training presence as the threshold to distinguish unsuitable from suitable areas (Pearson et al., 2007; Rodríguez-Ruiz et al., 2020).

Niche equivalency

In order to assess the niche overlap among *Anatololacerta* species, *ENMTools* (Warren et al., 2021) was applied to calculate Schoener's *D* (Schoener, 1968) and Hellinger's-based *I* (Warren et al., 2008) niche similarity metrics for niche overlap test due to their simplicity, long usage time and effective method to measure niche similarities (Warren et al., 2008). These indices ranged from 0 (no overlap) to 1 (identical niches).

The significance of the niche difference was assessed by pooling the occurrences from each taxon, and generating 100 pseudo-replicates. Afterwards, one-sided test and an α level of 0.05 was applied to compare the true calculated overlap to the null distribution of niche overlap. This means that the ENM of two species is not equivalent when the overlap value is smaller than 5% of the null distribution.

Background test

Background test was conducted in order to determine the differential availability of habitat for examined species (Warren et al., 2008). The running conditions of the background test were similar to the niche overlap tests (identity test).

RESULTS

Ecological niche models and the contribution of environmental variables

On the basis of minimum training presence threshold, ecological niche modeling predictions for each *Anatololacerta* species were reliable enough to result in realistic maps, and these predictions were separately conducted for each species with lowest AICc values.

A total of 4 bioclimatic and 2 topographic variables contributed to map the predicted distribution of each species (Table 1). The ultimate models were selected based on the lowest AICc from evaluation metric results (Table 2). The MaxEnt models demonstrated a significant ability to generate ecological niche models for *Anatololacerta* species with average test AUC of models as follows: 0.818 ± 0.093 for *A. anatolica*, 0.818 ± 0.083 for *A. pelasgiana*, 0.927 ± 0.045 for *A. finikensis*, 0.830 ± 0.083 for *A. ibrahimi* and 0.895 ± 0.104 for *A. danfordi*.

Based on these results, most of the suitable predicted areas were relatively wider than the present distributions of each species. The potential distribution of all *Anatololacerta* species are shown in Fig. 2 a-e. Although the bioclimatic and topographic variables that contributed to species distribution were the same, their contribution percentiles were different. The distribution of *A. anatolica* is highly associated with the temperature annual range - Bio 7 - (35.4 %), while that of *A. danfordi* with the Mean Leaf Area Index

Table 2. Summary statistics for the best models selected for species distribution maps of *Anatololacerta* species via *kuenm* package. AICc: a corrected AIC score, used for a small sample size by increasing the cost for each parameter; wAICc: the model weight is the relative likelihood for each model, divided by the total relative likelihood for all models that were considered; ΔAICc: the difference between the model with the lowest score (the "best" model) and the AICc score for each model; AUC: area under the curve is a measure of the accuracy of the model; mean AUC ratio ≥1.00, p<0.05 means predictions are significantly better than a random model.

Species	Best MaxEnt features	AICc	wAICc	ΔAICc	AUC	Mean AUC ratio
A. anatolica	hinge	765.536	0.191	0.134	0.818 ± 0.093	1.672 (p = 0.03)
A. danfordi	threshold	583.369	0.584	1.881	0.895 ± 0.104	1.000 (p = 0.02)
A. finikensis	quadratic	394.171	0.182	0.395	0.927 ± 0.045	1.619 (p = 0.03)
A. ibrahimi	threshold	908.851	1.000	0.143	0.830 ± 0.083	1.353 (p = 0.01)
A. pelasgiana	product	993.837	1.000	0.111	0.818 ± 0.083	1.720 (p = 0.02)

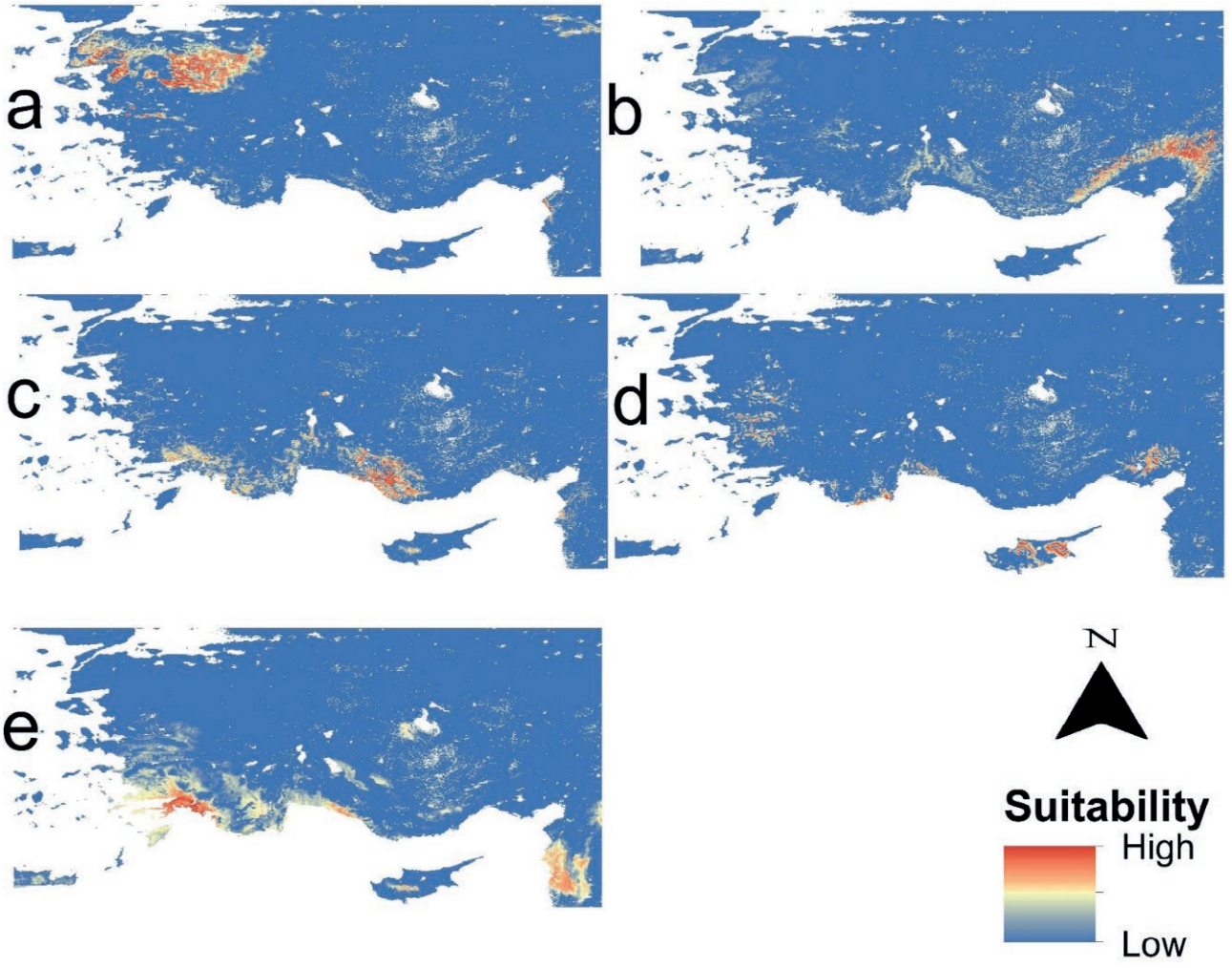

Fig. 2. Habitat suitability predictions of a. *A. anatolica*, b. *A. danfordi*, c. *A. ibrahimi*, d. *A. finikensis*, e. *A. pelasgiana* in the Anatolian Peninsula and Aegean Islands (Warmer colors refer to high suitability).

(43.7 %), and that of *A. finikensis* with the precipitation of driest quarter - Bio 17 - (35.7 %). The distribution of the remaining two species was highly determined by the environmental variables as follows: distance to the river (36.9 %) for *A. ibrahimi* and maximum temperature of the warmest period - Bio 5 - (26 %) for *A. pelasgiana*.

Table 3. Niche overlap analyses among *Anatololacerta* species in the Anatolian Peninsula and Aegean islands.

Comparisons *Anatololacerta* sp.	Measured Niche Overlap		Identity Test		Background Test**	
	Schoener's *D*	Hellinger's based *I*	Schoener's *D*	Hellinger's based *I*	Schoener's *D*	Hellinger's based *I*
pelasgiana vs. *anatolica*	0.418	0.712	0.679*	0.896*	0.362	0.648
pelasgiana vs. *danfordi*	0.529	0.803	0.639*	0.867*	0.307	0.586
pelasgiana vs. *finikensis*	0.666	0.888	0.633	0.862*	0.342	0.610
pelasgiana vs. *ibrahimi*	0.641	0.879	0.693*	0.910*	0.346	0.623
anatolica vs. *danfordi*	0.389	0.669	0.693*	0.909*	0.347	0.625
anatolica vs. *finikensis*	0.341	0.688	0.592*	0.851*	0.328	0.606
anatolica vs. *ibrahimi*	0.453	0.740	0.646*	0.883*	0.305	0.577
danfordi vs. *finikensis*	0.472	0.748	0.582*	0.835*	0.292	0.569
danfordi vs. *ibrahimi*	0.593	0.847	0.649*	0.884*	0.294	0.574
finikensis vs. *ibrahimi*	0.547	0.822	0.655*	0.895*	0.313	0.559

* Identity test showed significant niche differentiation (all p-value < 0.05).
** No background test showed significant overlap (all p-values > 0.05).

On the other hand, even though ENM in geographic space was generally suited to determine geographic isolation between the cryptic species, discussions on species delimitation have been continuing for the last two decades (Raxworthy et al., 2007; Fišer et al., 2018). Therefore, over-predicted areas were discarded from our final discussion.

Niche overlap tests

The measured niche overlaps among all species are presented in Table 3. The null hypothesis of niche overlap between *Anatololacerta* species (except *pelasgiana* vs *finikensis*) were rejected because empirical values for Schoener's *D* and Hellinger's based *I* test statistics were significantly different than the null distribution of overlap test for each species comparisons (Fig. S2 a-j) (*t* test, df = 99, P < 0.05). In other words, the ecological niche models of most of these species were nonequivalent. Background test for the parapatric *A. pelasgiana* and *A. finikensis* confirmed the niche overlap between these species in terms of global bioclimatic and selected topographic variables (Fig. S2 k). On the other hand, background tests for species that represent allopatric diversification patterns demonstrated that empirical values for Schoener's *D* and Hellinger's based *I* test statistics did not significantly differ from the null distribution (Fig. S2 l-t).

DISCUSSION

ENM on environmental layers has revealed not only additional insights into evolutionary lineages (Rissler and Apodaca, 2007) but also niche distinctiveness of species (Nakazato et al., 2010). Climatic niche has a remarkable effect on the area where species occur and each species requires a unique niche according to its ecological needs (Gewin, 2006; Rissler and Apodaca, 2007; Gül, 2019). There has been no study on the ecological niche of all Anatolian rock lizards so far. In this study, we have modeled environmental niches of all *Anatololacerta* species on the Anatolian Peninsula and the Aegean islands. Our results suggested that the niche divergence among the genus was confirmed for allopatric species (Fig. S2 a-j). However, the results for *A. pelasgiana* and *A. finikensis* showed that there is a niche overlap between these species (Fig. S2 k).

The present study showed the differentiation in the requirements of the ecological conditions among the *Anatololacerta* species (Fig. 2 a-e, Table 1). In other words, we assessed ecological niche differentiation to examine the phylogenetic-based taxonomic outputs for this genus. The speciation process within this genus was so far explained only by the geological factors and physical barriers (Schmidtler, 1998; Bellati et al., 2015). Based on the given results, we could assume that the differentiation within the genus and their present allopatric distribution on the Anatolian Peninsula and the Aegean islands is associated with ecological factors as well. The bioclimatological and topographical conditions provided remarkable contributions to the genetic diversity among this genus in terms of allopatric speciation. Even though ENM for each *Anatololacerta* species were generated using the same bioclimatic and topographic layers, the contribution percentiles of these variables were different. For instance, the dominant contributing variable for each *Anatololacerta* species is different: temperature annual

range for *A. anatolica*, mean leaf area index for *A. danfordi*, distance to river for *A. ibrahimi*, precipitation of driest quarter for *A. finikensis*, and maximum temperature of the warmest period for *A. pelasgiana*.

On the other hand, allopatric speciation dynamics were not only supported climatologically but also geographically (Eiselt and Schmidtler, 1986; Bellati et al., 2015). For example, separation between *A. anatolica* and the rest of the genus was highly related to the occurrence of the Great Menderes River. Additionally, *A. danfordi* was isolated from the rest of the genus by Central Taurus Mountains and located in the eastern part of Mediterranean region. Lastly, *A. ibrahimi* was only distributed in the northern and southern slopes of Central Taurus Mountains.

When it comes to parapatric speciation, the niche overlap case, that was demonstrated in the comparison between *A. pelasgiana* and *A. finikensis*, was needed to be discussed in another way, because distribution of both species was limited to only southwestern Anatolia and some Aegean islands. The actual utilization of the niche is significantly influenced by ecological interactions of various sorts. Thus, it could be helpful to use data on different selective regimes to examine the speciation dynamics of these parapatric species (Gavrilets et al., 2000; Mammola et al., 2018).

In order to discuss these speciation dynamics among this genus, it might be also beneficial to have evaluations on climate based historical perspective. Karakasi et al. (2021) revealed that the first split in *Anatololacerta* occurred in Early Pleistocene approximately 1.62 Mya with the separation of *A. anatolica* and the recent one was between *A. pelasgiana* and *A. ibrahimi* (0.56 Mya). The latter split matches the Mindel glacial period. According to the literature, 16 glacial periods have occurred during the last 2.4 million years in the Pleistocene (Webb and Bartlein, 1992; Hewitt 1996, 2000). Moreover, it was highly thought that the last four glacial periods in Pleistocene had a remarkable impact on faunal composition of Anatolia and related areas (Çıplak, 2004). Fluctuations in the temperature during these periods not only affected the movements of old Anatolian populations (Çıplak, 2004) but also shaped the vegetation dynamics with important changes (Jiménez-Moreno et al., 2015). In addition, the precipitation and temperature dynamics for a long time might have an impact on the vegetation patterns along western and southern parts of Anatolian Peninsula (Şahin et al., 2021).

On the other hand, the comparisons among allopatric *Anatololacerta* species revealed that, while their niches are not more similar than expected by chance (Fig. S2 a-j), their niches are not equivalent (Fig. S2 l-u). Studies on allopatric *Neurergus* species in Anatolia (Gül, 2019), speciation dynamics of endemic lizards in Madagascar (Nunes et al., 2022) and diversification of shrews in island dispersal events (Esselstyn et al., 2011) demonstrated that differences between their climatic niches are compatible with the abiotic environmental conditions between the geographical regions where allopatric species have been inhabiting. In fact, although this situation shows that allopatric *Anatololacerta* species living in the same geography have different niche requirements, the isolation areas between the inhabiting zones do not have an effect on the differentiation of environmental characteristics.

If species fit to particular climatic conditions (or various local conditions), it brings to niche differentiation because of the unique adaptations needed to survive and breed (Nakazato et al., 2010). In the present study, ecological niche divergence has been inferred to display ecological speciation of species with allopatric distributions. Our results are compatible with the taxonomic suggestion of the work of Karakasi et al. (2021) that discerns each allopatric clade at the species level.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found at <http://www-9.unipv.it/webshi/appendix/index.html> manuscript number 13089

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Occupancy and probability of detection of the introduced population of *Eleutherodactylus coqui* in Turrialba, Costa Rica

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Abstract. The Puerto Rican Common coqui frog (*Eleutherodactylus coqui*) has a long history as an invasive species in places such as Hawaii. Since its introduction in Costa Rica, scarce information is available to understand why and how the habitat in the Turrialba town is suitable for the species. Our goal was to analyze the habitat selection of *E. coqui* to identify if there are key habitat features that explained its success there. We measured 9 site variables that may affect the habitat selection of *E. coqui* in 92 survey units of 10 m radius distributed over a 500 m radius from its introduction point. We registered the presence/pseudo-absence data of *E. coqui* and environmental variables in each survey unit during eight surveys. We ran occupancy models to determine the influence of the variables on the habitat selection and to estimate its detection probability. We found that sites near the introduction point, containing abundant vegetation, bromeliads, and palms have a higher probability to be occupied by *E. coqui*. The habitat selection in Costa Rica shares characteristics with the populations of Puerto Rico and Hawaii. But, unlike the case in Hawaii, in Costa Rica this species has maintained a limited dispersal because the potentially higher biotic resistance, as well a sedentary behavior. However, the microhabitat conditions used by *E. coqui* in the study site are common throughout the country. Therefore, active management in new populations and environmental education programs to avoid human transportation of the species is critical to reduce its dispersal.

Keywords. Amphibians, conservation, detection probability, invasive species, introduced species, occupancy models.

INTRODUCTION

The study of the factors that determine the establishment and dynamics of an exotic species in a new ecosystem is not only a vital component in the development of biological invasion management strategies, but it also provides important information for understanding the processes that take place in natural ecosystems (Jiménez-Valverde et al., 2011; Wan et al., 2019). In most scenarios the introduced species fail to establish or advance beyond the first stages of invasion (Zenni and Núñez, 2013). However, under the right conditions, these species can colonize and spread over large areas and ecosystems causing severe alterations (Mačić, 2018). Additionally, in some cases rapid evolutionary processes may occur that favor their adaptation to new conditions (Whitney and Gabler, 2008; Carneiro and Lyko, 2020), where characteristics such as behavior, morphological and reproductive traits, and genetic variability of populations of introduced species may differ considerably with respect to the populations in their native range (O'Neill et al., 2018).

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The Common coqui frog (*Eleutherodactylus coqui*, Thomas 1966) is a species native from Puerto Rico with a long history as an invasive species (Lowe et al., 2004). In its native habitat *E. coqui* is one of the most abundant amphibians, and it can be found from the forest floor to the canopy, inhabiting almost all environments (Joglar, 1998). It breeds throughout the year (Townsend and Stewart, 1994). Neonates take 8 to 9 months to become sexually mature (Towsend and Steward, 1994) and lays on average 4-6 clutches of eggs per year, each containing 16-41 eggs per clutch (Towsend and Stewart, 1994). Eggs are generally deposited in covered sites that provide protection from rain and environmental conditions (Townsend, 1989; Beard and Pitt, 2012). Egg development is direct (Towsend and Steward, 1985) and hatch after 14-17 days (Towsend and Steward, 1994).

This anuran was introduced to the Hawaiian archipelago in the late 1980s, where in less than 10 years it had spread throughout an extensive area of the archipelago (Kraus and Campbell, 2002). As in Puerto Rico, *E. coqui* populations in Hawaii are abundant; it has been reported population densities of up to 91000 individuals per hectare at the archipelago, a number three times higher than the estimates reported in Puerto Rico (Beard et al., 2008). These extreme densities have caused not only ecosystem alterations such as changes in the invertebrate community (Choi and Beard, 2012), alteration in the nutrient cycle and herbivory regimes (Sin et al., 2008), but also social and economic effects due to noise pollution produced by their constant vocalizations and the measures required for its control (Beard et al., 2009).

In Costa Rica, the Common coqui frog was introduced around 1998 into the city of Turrialba (García-Rodríguez et al., 2010; Barrantes-Madrigal et al., 2019). Unlike its invasion process in Hawaii, in the Cartago Province it has been kept restricted to a few localities for almost two decades: Turrialba and Juan Viñas (Barrantes-Madrigal et al., 2019). Although Barrantes-Madrigal et al. (2019) provided an update of the invasion status of the species in Costa Rica, since then have been observed few individuals in San Antonio de Escazú (San José Province) (https://www.inaturalist.org/observations/48536340). To continue research on this topic is relevant to understand why this population survived in Turrialba, and what implications could it has with the years across the country.

Although there is much information in the literature about the ecology of *E. coqui*, this information comes mainly from islands (Puerto Rico and Hawaii) where the ecological conditions are different from the continental neotropical context found in Costa Rica. The objective of this work is to determine the habitat selection of the Common coqui frog (*Eleutherodactylus coqui*) population introduced in the town of Turrialba to identify habitat variables that favor its occupation. We predicted that the vegetation structure and the availability of breeding sites would play a relevant role in the selection of the microhabitat of this frog as it has been in its native (Townsend, 1989) and exotic range (Beard et al., 2003). This research is relevant to understand why this population survived in Turrialba, and what implications could it has with the years across the country.

MATERIALS AND METHODS

Study area

The study was carried out in the city of Turrialba, where the initial population of *E. coqui* was found in Costa Rica (9°53'42"-9°54'18"N and 83°40'48"-83°39'54"E; García-Rodríguez et al., 2010; Fig. 1). Turrialba is located on the Caribbean slope and belongs to the Canton of Turrialba, Province of Cartago. It has an elevation range between 600 and 650 m a.s.l, has a warm and humid climate with an average annual temperature of 22 °C, and, due to its location, it is exposed to humid northeast winds and in certain regions can receive up to 7000 mm of rain (Dufour, 1978).

The place where *E. coqui* was first detected is surrounded by an area of heterogeneous composition with residential and commercial areas, including the campus of the University of Costa Rica (Atlantic Branch), but also open areas, pastures, plantations, streams, and small patches of secondary forest such as the Botanical Garden of the Centro Agronómico Tropical de Investigación y Enseñanza (CATIE; Fig. 2).

Sampling design

We delimited a circular area of 500 m radius (78.5 ha) around the point where this species was first reported (García-Rodríguez et al., 2010) as the study site. The extension of the sampling area was defined according to a preliminary sampling where we did not find evidence of the presence of the Common coqui frog outside the 500 m radius area from the introduction site. We assumed that, since its introduction, the species has had the same probability of dispersal in any direction within the selected area. Within this area we delimited three strata: urban, forest and open areas, based on satellite images taken from Google Earth Pro (Google, 2016). In each stratum we randomly distributed 29 circular sampling units (SU) of 10 m radius (314.1 m^2), with a minimum separation of 30 m between each other to capture most of the micro-

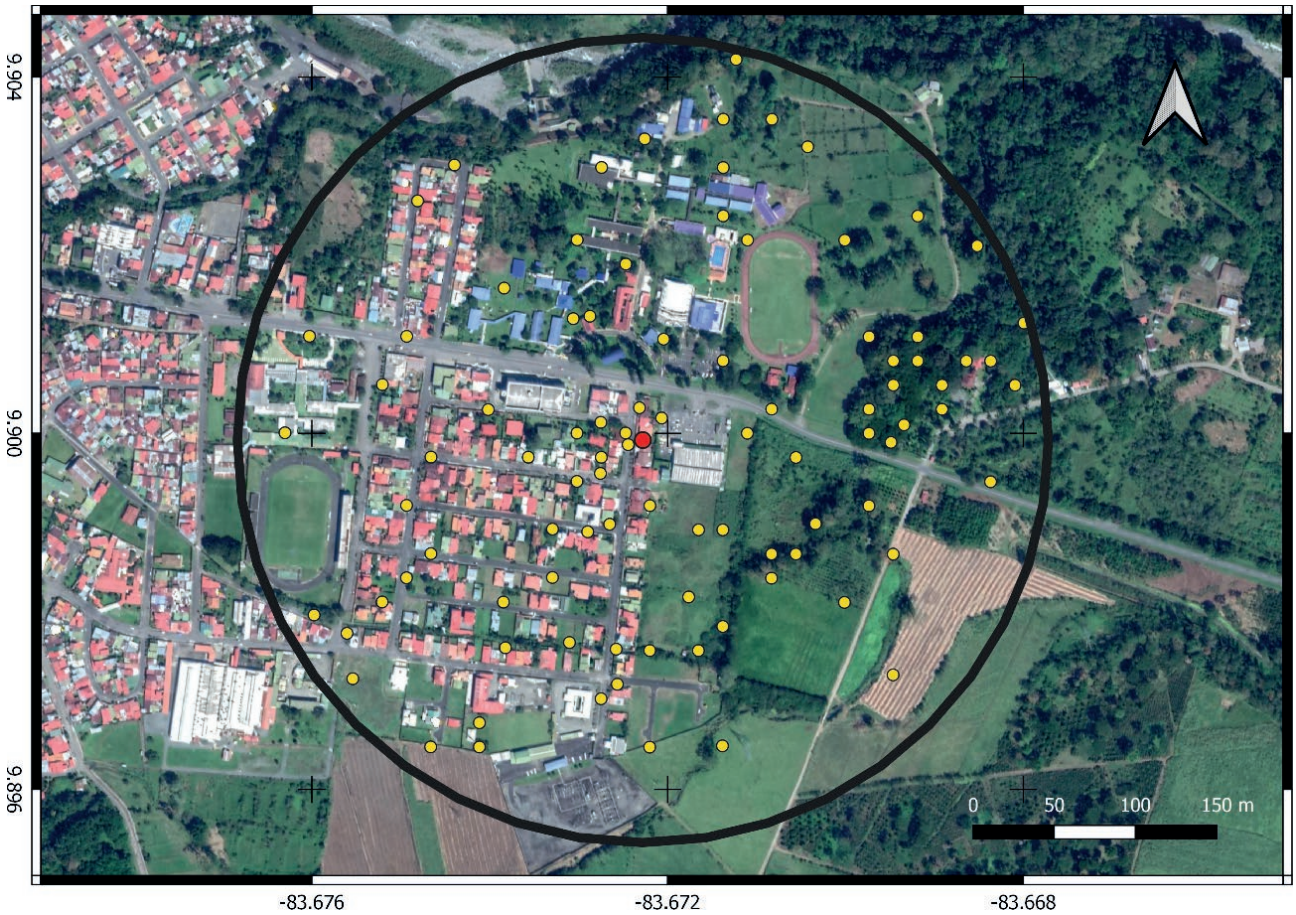

Fig. 1. Point of introduction of *Eleutherodactylus coqui* (red dot) and distribution of sampling units (yellow points) for the analysis of its habitat selection in Turrialba, Costa Rica.

habitat's variability in each stratum (Fig. 1). We considered 30 m as an adequate distance considering that *E. coqui* is a sedentary species, with movements of 3 4.5 m on average around its retreat sites (Woolbright, 1985).

Data collection

SUs were characterized based on nine site covariates distributed in four categories that we considered may influence the habitat selection of *E. coqui* (Table 1). The first category was vegetation, where we estimated volume of vegetation in three vertical strata and tree cover as habitat attributes that could be important in maintaining the environmental requirements of this species (e.g., temperature, humidity), as well as providing foraging sites or perches to vocalize. We registered bromeliads, palms, and leaf litter for their role as possible nesting or refuge sites (Stewart and Pough, 1983; Beard et al., 2003). Bromeliads were registered as presence or absence, where we considered less than five bromeliads as an absence. The percentage of palms and leaf litter in the SU, as well as the percentage of vegetation mentioned above, were calculated dividing the SU into four equal sections by drawing an imaginary line from the central point towards the four cardinal directions, in this way, we visually estimated the percentage of the covariate represented in each section and averaged the result for each SU.

On the category of water bodies, we quantified the distance to rivers as a measure to analyze the association with gallery forest environments. Additionally, to consider the dispersal capability of this species we measured the distance from the site where the first individuals were introduced. Both distance measures were calculated using the *distance* function of the *raster* package (Hijmans, 2016) in the statistical program R v3.3.2 (R Core Team, 2016).

We carried out a minimum of five and a maximum of eight nocturnal surveys (18:00 - 22:00 h) in each SU during October 2016 to February 2017. We implemented a five-minute survey in each SU using the visual and

Fig. 2. Representation of the types of environments contained in the study area for the habitat selection analysis of *Eleutherodactylus coqui* in Costa Rica. **A**. Forest, **B**. Gardens, **C**. Plantation, **D**. Open area-pasture, **E**. Green areas, **F**. Urban areas.

auditory encounter survey technique (Crump and Scott, 1994) to determine the presence of *E. coqui.* During each survey, we recorded if there was presence of the species in the sampling units (SU).

We registered three environmental variables at the beginning of each SU survey: relative humidity (hum), air temperature (temp) and the illuminated percentage of the moon (moon). These variables were chosen because there

Table 1. Detail of covariables used to analyze the habitat selection of the Common coqui frog (*Eleutherodactylus coqui*) in Costa Rica.

Covariable	ID code	Description
Vegetation		
Low height vegetation	veg_low	Percentage of the volume between 0 - 1 m in height within the SU occupied by vegetation
Medium height vegetation	veg_med	Percentage of the volume between 1 - 2 m in height within the SU occupied by vegetation
High height vegetation	veg_high	Percentage of the volume between 2 - 3 m in height within the SU occupied by vegetation
Canopy cover	can_cover	Percentage of canopy cover within the SU (measured with a densiometer)
Retreat sites		
Bromeliads	brom	Number of bromeliads within the SU at a height of less than 3 m.
Leaf litter	leaf_litter	Estimated percentage of leaf litter within the SU
Palms	palm	Percentage of the SU volume occupied by vegetation belonging to plants of the *Arecaceae* family
Water bodies		
Distance to rivers	dist_river	Distance in meters to the closest moving body of water
Dispersal		
Distance to origin	dist_origin	Distance in meters to the point of introduction of *Eleutherodactylus coqui* in Costa Rica.

is evidence in the literature that they influence the calling activity of *E. coqui* and other congeners (Joglar, 1998; Grant et al., 2012). Relative humidity and air temperature were quantified using a digital thermo-hygrometer (SE = ± 5% and ± 0.1 °C respectively). Additionally, the illuminated percentage of the moon was calculated as the percentage corresponding to the lunar phase, where 0% represents new moon and 100% full moon, using a lunar calendar.

Data analysis

We performed a habitat selection analysis using a single-season static occupancy model (Mackenzie et al., 2002). These models are especially useful when detection probability is less than 1, as it is expected for most amphibians. First, we standardized all variables (Mean = 0, SD = 1) due to their different value scales. We built a global model using the relative humidity, air temperature and the illuminated percentage of the moon as observation variables for the detection history, and vegetation, bromeliads, palms, leaf litter, canopy cover, distance to rivers and distance to origin as site covariates. Site and observation covariates were tested to evaluate their correlation, we built a global model with and without each of the correlated variables (Pearson $|r| < 0.6$) and kept those that resulted in the most parsimonious model evaluated by the Akaike Information Criterion (AIC; Burnham and Anderson, 2002). As result, we excluded leaf_litter, veg_low and veg_high from the global model. We assessed the goodness-of-fit and overdispersion of the global model with a parametric bootstrap approach based on the $\chi 2$ statistic with 1000 bootstrap samples (MacKenzie and Bailey, 2004).

We evaluated all possible combination of the global model and ranked the results by their AIC values using the *dredge* function of the MuMIn package (Barton, 2016). For occupancy and detection probability estimation we used a model averaging over the subset of models with a $\Delta AIC < 2.0$ as all of them were considered robust (Weir et al., 2005). Finally, we calculated the relative importance of the estimated parameters for the habitat selection analysis using the importance function of the MuMln R package (Barton, 2016). This function ranks the variable according to the sum of the AIC weights in all models where the variable is included over all possible combinations of the global model. Models were built using the *unmarked* package (Fiske and Chandler, 2011) in the statistical program R v3.3.2 (R Core Team, 2016). All data and the R code used in the analysis is available as supplementary material.

RESULTS

We detected the presence of *E. coqui* in 30 of the 92 SUs on at least one occasion. The maximum distance from the point of introduction at which the species was recorded was 493 m, near the limit of the study area. A subset of 19 models with different combination of variables resulted with a $\Delta AIC < 2$ (Table 2). The estimated c-hat value for site-occupancy model was close to 1 and did not indicate overdispersion or lack of fit (c-hat = 1.08; $\chi^2 = 781.74$; $P = 0.258$). The AIC value was lower when we do not use any of the observation-level variables, however temperature and percentage illuminated of the moon were included in the subset of models with

Table 2. First 10 models of the set of models with the best fit (ΔAIC < 2) used in the habitat selection analysis of *Eleutherodactylus coqui*. p: detection probability; psi: selection probability; nPar: Number of parameters; AIC: Akaike's information criterion; ΔAIC: Difference with respect to the best model; wAIC: Akaike's weight.

Model formula	nPar	AIC	ΔAIC	wAIC
p(.) psi(brom + dist_origin + palm + veg_med)	5	313,04	0,00	0,106
p(.) psi(brom + dist_origin + veg_med)	6	313,29	0,25	0,094
p(.) psi(dist_origin + palm + veg_med)	5	313,47	0,43	0,085
p(.) psi(brom + can_cover + dist_origin + palm + veg_med)	4	313,58	0,54	0,081
p(.) psi(dist_origin + veg_med)	7	314,30	1,26	0,056
p(temp) psi(brom + dist_origin + palm + veg_med)	6	314,36	1,31	0,055
p(temp) psi(brom + dist_origin + veg_med)	6	314,52	1,48	0,051
p(.) psi(can_cover + dist_origin + palm + veg_med)	6	314,64	1,60	0,048
p(.) psi(brom + dist_origin + dist_river + palm + veg_med)	7	314,64	1,60	0,048
p(moon) psi(brom + dist_origin + palm + veg_med)	6	314,68	1,64	0,047

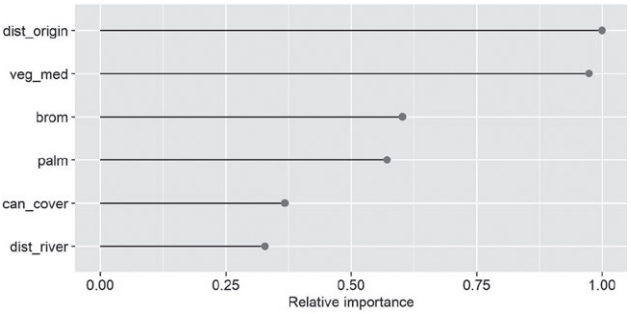

Fig. 3. Relative importance of variables in the habitat selection of *Eleutherodactylus coqui* in Turrialba, Costa Rica. dist_origin = distance to origin, veg_med = medium height vegetation, brom = bromeliads, palm = palms, can_cover = canopy cover, dist_river = distance to rivers.

ΔAIC < 2 (Table 2). The estimated detection probability using the averaged model was 0.666 (95% CI = 0.596 – 0.736). The variables mid vegetation (veg_med) and distance to origin (dist_origin) stand out as the most influential in the habitat selection of the species (Fig. 3). The presence of bromeliads (brom) also obtained a high value (0.60) as did the percentage of palms (palm) (0.57). The other site covariates presented relative importance values lower than 0.36.

DISCUSSION

The distribution of the Common coqui frog (*Eleutherodactylus coqui*) in the study area was explained by site features that favor its occupancy. We determined that the vegetation at a height of 1-2 meters, as well as the proximity to the site of introduction, are the site characteristics that best explain the occupation of the species on a microgeographic scale. In Puerto Rico, individuals of *E. coqui* have been observed from the ground to the top of the trees (Joglar, 1998), however, consistent with our observations, in our study area this species prefers perches with heights of approximately 1 m and has a negative association for higher places (Beard et al., 2003). The Common coqui uses plants to vocalize and forage, to select low vegetation for that purpose fit with previous habitat description and selection in Puerto Rico (Townsend, 1989). Dense and abundant low vegetation cover contributes to maintaining humidity conditions to avoid its desiccation (Beard et al., 2009; Klawinski et al., 2014).

The positive association with the abundance of bromeliads and palms could be explained by the reproductive biology of the frog, because previous research carried out in Puerto Rico and Hawaii highlights the importance of the availability and quality of nesting sites as a limiting factor for the Common coqui population, because the hatching success of the spawn is affected by the structure of the selected sites (Stewart and Pough, 1983; Townsend and Stewart, 1994; Beard et al., 2003). Plant species such as *Cecropia peltata*, epiphytic plants as bromeliads and palms (e.g., *Prestodea montana*) are important for the biology of species in Puerto Rico, especially due to leaf litter produced that could be shelter, nesting site or call perch (Townsend, 1989). In Turrialba this type of vegetation also occurs everywhere, especially in riparian and secondary forest, but not necessarily in gardens or sidewalks in our study site. However, also into gardens and sidewalks where ornamental introduced palms (e.g., *Areca* sp., *Wodyetia* sp.) or *Hybiscus* sp. bushes are common and frequently pruned to 1-2 m heigh. Structurally, our study site provides vegetation requirements that the Common coqui required for breeding and shelter, even

Fig. 4. Common coqui frog (*Eleutherodactylus coqui*) found in a bromeliad, Turrialba, Costa Rica. Photo by J. Barrantes.

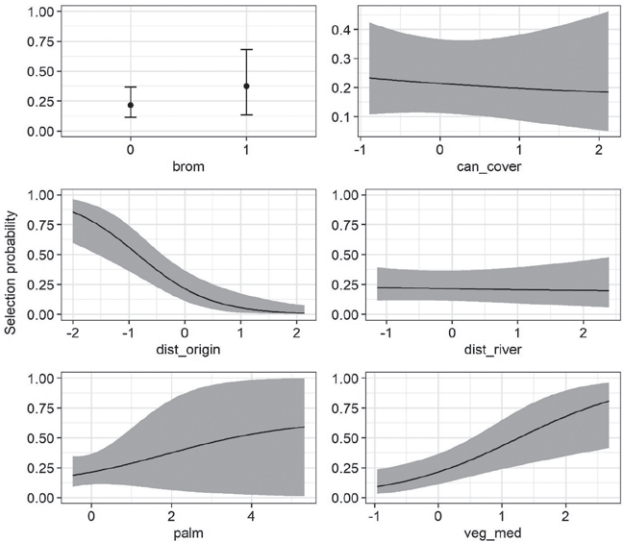

Fig. 5. Selection probability of the variables used to analyze the habitat selection of *Eleutherodactylus coqui* in Costa Rica.

when leaf litter was not abundant in our study site; the species could be using different types of substrates to lay eggs. We hypothesize that *E. coqui* uses bromeliads or other epiphytes (e.g., orchids, ferns) frequently found in trees and gardens for this purpose, because it was common to find individuals retreated inside bromeliads (Fig. 4) or perching in palm leaves. The use of bromeliads and epiphytic plants as shelters during the day is well known for the Common coqui biology (Ovaska, 1992; Fogarty and Vilella, 2003), as they provide a protected substrate where humidity conditions are maintained (Stewart and Pought, 1983), and the same conditions required to deposit their eggs (Townsend, 1989). Although it is common for *E. coqui* to lay its eggs on the ground or surroundings, this species prefers elevated substrates whenever they are available as it allows it to have greater hatching success and makes it easier for males to access high perches, close to the laying, where they can perform their vocalizations to attract females or defend territories (Townsend, 1989).

The detection probability (0.666, 95% CI = 0.596 – 0.736) is similar to values reported in a study from Hawaii (0.58 to 0.73; Olson et al., 2012). These results indicate that, despite being a relatively easy species to detect due to its constant vocalizations, at least three nocturnal surveys (2.73) to each site are required to avoid false negatives in detections of Common coqui individuals with a 95% of confidence. Even when none of the quantified environmental variables had a significant influence on the detection probability, previous studies indicate that the activity of this species is closely associated with humidity conditions (Pough et al., 1983). Humidity in Turrialba is relatively constant and high across the surveyed months (Dufour, 1978). This lack of variation could be the reason why we did not find a significant influence of these variables on the detection probability.

The observed distribution pattern suggests that there is a higher probability of finding Common coqui individuals near the introduction point (Fig. 5). This same pattern has been observed in Hawaii, where their populations are frequently found near points or routes of introduction such as roads or nurseries, and their dispersal throughout the archipelago is mainly due to transport facilitated by humans (Rauschert et al., 2017), with the natural dispersal movements being less important during the invasion process (Everman and Klawinski, 2013). This anuran is a very sedentary species, its movements at night are generally short and maintains an action range of just a few square meters (Woolbright, 1985), limiting its dispersal to more remote areas since its introduction in Costa Rica.

The limited dispersion documented can be related with the highly heterogeneous matrix with cover that contain potential barriers such as high-speed roads, neighborhoods, or even more complex rainforest fragments. Into the Jorge de Bravo neighborhood and surroundings, the Common coqui behaves like strong invader in disturbed areas near the introduction point, but it seems that would be a weak invader outside where natural ecosystems are more dominant because potentially there is more biotic resistance (Meyer et al., 2021). The biodiversity level in the Costa Rican Caribbean is much higher than in islands like Puerto Rico or Hawaii,

especially vertebrate diversity such amphibian, reptiles (Savage, 2002), birds (Stiles and Skutch, 1989) or bats (LaVal and Rodríguez, 2002) that could be potential competitors or predators for a noisy species of *Eleutherodactylus*. For example, other native amphibians with a similar niche than the Common coqui such as Tink frog (*Diasporus diastema*), Pigmy rain frog (*Pristimantis ridens*), Fleischmann's glass frog (*Hyalinobatrachium fleischmanni*) or Green-boned tree frog (*Scinax elaeochrous*) also occur in the study area, including secondary growth, gardens, or perturbed lands (Savage, 2002). It is likely that competition, prey abundance, predation and other factors can influence the habitat selection and dispersal of this species. Previous work has highlighted that the way in which introduced species interact with native biota at different perturbation levels is an important determinant of their invasion success (Shea and Chesson, 2002; Meyer et al., 2021). Further studies are needed in this field to understand the influence of these interactions, both for the target species and for the native species with which it coexists.

Our study suggests that the habitat selection of the introduced population of *Eleutherodactylus coqui* in Costa Rica shares characteristics with the populations of Puerto Rico and Hawaii, where low vegetation and refuge sites during the day are decisive. However, unlike the case in Hawaii, in Costa Rica this species has maintained a limited dispersal because the biotic resistance and sedentary behavior discussed previously. Therefore, the scenario of a natural dispersion sounds like a less probable one based on what has been recorded in our study site into the Turrialba town thought the last 20 years (Barrantes-Madrigal et al. 2019). Moreover, all the populations in Turrialba, Juan Viñas, and potentially Escazú, where introduced on purpose or accidentally by humans (Barrantes-Madrigal et al., 2019). According with our results, the species could potentially colonize areas with open vegetation or crops with small bushes such as parks or sun coffee plantations from lowlands or middle elevations. Other species of *Eleutherodactylus* that also succeed in open vegetation are abundant in Puerto Rican sun coffee plantations (Monroe et al. 2017), for example. However, in the other hand, other similar species to the Common coqui such as *Eleutherodactylus planirostris* or *E. johnstonei* has been restricted to a single record or locality, without an important expansion or succeed to stablish new populations (e.g., *E. johnstonei*; Savage, 2002; Barquero and Araya, 2016). Thus, even when an extreme aggressive invasion scenario like the observed in Hawaii is unlikely to occur at country scale in Costa Rica at least soon, because the microhabitat conditions used by *E. coqui* in the study site are common in other neighboring towns in the lowlands from Caribbean or Pacific slopes, we consider that rural and peri urban areas with a mixed matrix of agropastoral-urban systems could be more likely to be invade by the Common coqui in further years only if transportation by humans continue.

Anecdotically, during surveys made by Barrantes-Madrigal et al. (2019), we identified that an important number of people from our study area sympathized with the sound produced by the Common coqui, even feeling proud of having the species living in their homes. This can increase the transportation risk of Common coqui frogs between people, both intentional and accidental, something that did not happened with other species like *E. johnstonei*. On the contrary, it was identified that other neighbors from our study area had noise problems due to the extreme local abundance of the frog in their gardens trying to manage the population with invasive and non-friendly environmental methods but with few succees. We encourage the environmental authorities from Minister of Environment (MINAE) to develop an early warning system and apply immediate management measures in new locations where this species is detected to prevent its establishment and spread. Additionally, we recommend increasing research and monitoring efforts on the possible negative effects on the ecosystem of the study area and to identify other pathways that could facilitate their dispersal to new regions, mainly those related to movement by humans. Our observations could serve as the basis for making microhabitat management decisions in parks or gardens in Turrialba where the species represents a nuisance to its inhabitants or a threat to other native species. It would be critical to develop an environmental education program to local people from Turrialba or Juan Viñas to avoid moving the species to new places where biotic resistance could be lesser or environmental conditions could be even more beneficial for the Common coqui establishment.

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SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at < http://www.unipv.it/webshi/appendix> Manuscript number 13209.

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One site, three species, three stories: syntopy of geckoes *Euleptes europaea* (Gené, 1839), *Hemidactylus turcicus* (Linnaeus, 1758), *Tarentola mauritanica* (Linnaeus, 1758) in a coastal area of southern Tuscany (central Italy)

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Abstract. Ecological aspects of syntopic geckoes were rarely addressed in the Mediterranean basin. We reported basic information on habitat use, and activity patterns of three species found in syntopy in Cala Violina site (divided in three subareas), a highly touristic beach located in southern Tuscany, central Italy, during 2009-2010. The most abundant species at first capture is *Hemidactylus turcicus* (94 individuals), while *Tarentola mauritanica* and *Euleptes europaea* are less represented (28 animals in both cases). Total captures and recaptures were 175. Sex ratio did not differ from 1:1 in all the species, nor sexes of adults did differ in size. Ambient temperatures did not differ in *T. mauritanica* and *E. europaea*, while were different in *H. turcicus*. Despite the humidity of capture sites did not vary among species, we recorded the highest number of *E. europaea* at 95% and *H. turcicus* at 62% humidity. Wind influenced negatively *T. mauritanica* and *H. turcicus* presence, not on *E. europaea*. Higher observation rate took place between 21:00 and 22:00. After 23:00, only *Euleptes* was active. Height from the ground was different only in *H. turcicus*. General Linear Models showed that interaction substrate-height at capture was important for *Euleptes*, not for the other two species. Along the area, *E. europaea* was more concentrated in the northern patch, while *T. mauritanica* and *H. turcicus* distributed more homogeneously. We suggest limitation of human presence for conservation purposes.

Keywords. Syntopy, geckoes, *Tarentola*, *Hemidactylus*, *Euleptes*, central Italy.

INTRODUCTION

The study of the ecology of the Italian reptile species is particularly advanced for some groups, especially for tortoises (e.g., Chelazzi and Carlà, 1986; Rugiero and Luiselli, 2006), pond turtles (e.g., Rovero and Chelazzi, 1996; Lebboroni and Chelazzi, 1998; Zuffi et al., 2004, 2007), as well as for snakes (e.g., Luiselli et al., 1996; Zuffi, 2008; Zuffi et al. 2009; Scali et al., 2011) and lizards (e.g., Perez-Mellado and Corti, 1993; Sacchi et al., 2007; Salvidio and Oneto, 2008; Bombi et al., 2009; Zuffi et al., 2011, 2012).

Most information about Italian lacertilia *sensu lato* has been provided in Atlases and Distributive Maps (Vanni and Nistri 2006; Corti et al., 2011), despite quite anecdotal and descriptive. On the contrary, complete and scrutinized data concern phylogeographic and taxonomic features (Harris et al., 1998; Oliverio et al., 1998; Gamble et al., 2008), and, partially, ecological-behavioural features (Vervust et al., 2007; Biaggini et al., 2009; Marsili et al., 2009; Sacchi et al., 2015; Scali et al., 2016). However, research considering comparative aspects in different reptile species are relatively limited (i.e., Capula, Luiselli and

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Rugiero, 1993; Capula and Luiselli, 1994; Carvalho Jr et al., 2008; Gordon et al., 2010; Maura et al., 2011; Simbula et al., 2019) and further studies are strongly needed.

There are four gecko species in Italy (Corti et al., 2011): *Tarentola mauritanica* and *Hemidactylus turcicus,* distributed in most of the Mediterranean coastal environments, and *Euleptes europaea* and *Mediodactylus kotschyi* more localised, in western Mediterranean Italy and in south-eastern Italy (Apulia), respectively. Although the distribution of *Tarentola*, *Hemidactylus* and *Euleptes* is to some extent overlapped in north-western Italy, namely in coastal Tuscany (Vanni and Nistri, 2006), the only site where the three species actually occur in sympatry and in syntopy is in Southern Tuscany, in the municipality of Scarlino, province of Grosseto (Radi, 2013). On average, available data on *Euleptes europaea* refer to a few sites only in Liguria (Tinetto, Tino, Genoa), Sardinia (Sassari, Gallura) and Tuscany (Castiglione della Pescaia and the Tuscan Archipelago) and regard morphology and population dynamics (Salvidio and Delaugerre, 2003; Salvidio and Oneto, 2008; Salvidio et al., 2011, for a review). Ecological data on *Hemidactylus turcicus* and *Tarentola mauritanica* of Italy are quite descriptive (Capula and Luiselli, 1994; Luiselli and Capizzi, 1999; Aprea et al., 2011; Zuffi et al., 2011) with the exception of a few studies on sympatric geckoes in Italy and Croatia (Lisicic et al., 2012; Simbula et al., 2019). Our research, as far as we are aware, is likely the first one aimed at comparing three Gekkota species in sympatry and syntopy, and analysing and comparing biometrical features, population structure and ecology patterns in a quite unique zoogeographic context.

MATERIAL AND METHODS

Study area

Study area is in Cala Violina, municipality of Scarlino (province of Grosseto), which is a highly frequented touristic place during summer. This area extends for 1000 × 300 m and falls within the "Monte d'Alma" 108 SIR (Sito di Interesse Regionale, Regional Interest Site; IT51A0008), and pSIC (Sito di Interesse Comunitario, EU Interest Site; Natura 2000 IT51A0008) and the A.N.P.I.L. "Costiere di Scarlino" (Area Naturale Protetta di Interesse Locale, Protected Natural Area of Local Interest) (42.856850°N, 10.774386°E) (Fig. 1). We have focused the field activity on the maximum area extension, which is about 670 m long sector; the area is characterized by a central sandy part. Proceeding towards the far ends of the promontories, that are quite high and boulder-like, the sandy part gradually changes into coarse

Fig. 1. Satellite picture (source: Google Earth) of Follonica Gulf delimited by Piombino promontory (LI) to the North, and by Punta Ala promontory (GR) to the South. Cala Violina is pointed by a white pin.

soiled sandstone cliffs. Going down from the shore to the Mediterranean scrub it is possible to find dissolved bedrock and sandstone slopes, transitional environments in which geckoes live. Climate is Mediterranean, with average rainfall of 600-800 mm during winter, and average temperatures of 14 °C (Selvi and Stefanini, 2005). Specifically, in Follonica, the closest meteorological station to the study area, average rainfall and temperatures are 655.2 mm and 15.7 °C respectively (Barazzuoli et al., 1993).

Sampling and measurements

Sampling was carried out with censuses during two annual sessions, in 2009, from 23rd July to 22nd November and in 2010, from 07th April to 30th August. We did 20 sampling days in 2009 (18 out of 20 during the night) and 22 in 2010 (20 out of 22 during the night) for 42 sampling days. Each sampling lasted five hours on average for a total of 210 hours of field night sampling. Sampling occurred from 20:00 to 02:00 solar hour to avoid touristic disturbance and to match species' activity. The area is naturally divided in three sectors T1, North, ca 220 m long T2, central, ca 70 m long, and T3 south, ca 350 m long, by two forest tracks (the first 24 m wide, the second 4 m wide) loading to the beach from the forest, for a total of 670 m transect length (Fig. 2). We have considered the three sectors as a unique survey area.

We captured geckoes by hand, or with a noose on a long stick, and placed in cotton sacks before data recording. At each capture, we registered solar time, substrate type (sandstone, loose ground, boulders, sand, vegetation), ambient temperature, humidity (thermo-

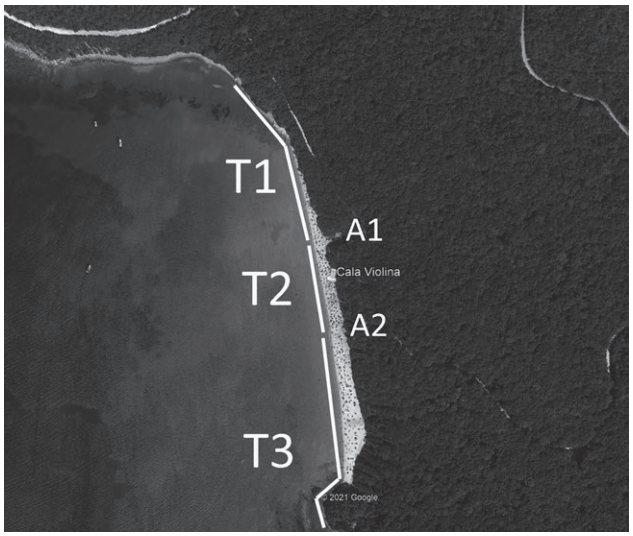

Fig. 2. Satellite image (Google Earth) of Cala Violina. White lines show three transects (T1, T2, T3), A1 and A2 indicate access to the beach.

hygrometer HANNA, HI9565, precision 0.1 °C, 0.1% humidity), wind (with the empirical Beaufort scale), animal position (height from the ground, distance from transect starting point). Morphological data were snout to vent length, tail length, head length, width and height, eye diameter, distance between eye and nostril, internarial distance, interorbital distance. We also determined size and sex class, as described in full by Atzori et al. (2007). We therefore considered males and females, juveniles (medium size, unsexable) and new-borns (very small size, unsexable). Geckoes were marked with acrylic water pens for short term recognition and with a cut of coded sub-digital scales (see Atzori et al., 2007) for a long-term recognition. We did not apply sub-digital marking to the new-borns of all the three species due to their markedly small size, and to the adults of the European leaf-toad gecko, due to the extremely reduced size of lamellae and very thin fingers. We assessed female reproductive status by manual palpation and, in some cases, using a direct light placed on the female vent, to detect eggs for transparency. In the whole period, we captured 175 geckoes: 110 *Hemidactylus*, 34 *Euleptes* and 31 *Tarentola*. We excluded recaptures and visual encounters from this research. We therefore analyzed 159 unique gecko records, 100 of which were *Hemidactylus* (59 in 2009, 41 in 2010), 31 were *Euleptes* (21 in 2009, 10 in 2010), and 28 *Tarentola* (14 in 2009, 14 in 2010). We defined three age classes: males, females, and juveniles. We considered juveniles and new-borns together as juvenile category. We tested sex-ratio differences within and among species using a log-linear model (with binomial distribution). We tested differences in size and biometry (all variables were normally distributed, Kolmogorov-Smirnov test, $P > 0.05$) with a General Linear Model (multivariate GLM, with species and sex as fixed factors and their interaction). We applied this analysis only to the adults.

Given all environmental variables, we used a multivariate GLM to test if species do differ in some way between years and among them. In addition, to describe ecological relationships among gecko species, we applied a Principal Component Analysis (Varimax Procedure, eigenvalue ≥ 1, rotated matrix), to all environmental variables and two biometrical features (SVL, body mass), extracting the most correlated variables within each main component. Therefore, we were able to describe the components driving the average ecology of the three species of geckos. To analyse the spatial distribution of captured and observed geckoes, we normalized the three subsectors, creating a unique transect. We also considered the width of the two tracks used to reach the beach: 26 and 4 metres, respectively.

Temperature, humidity, wind presence and hourly distribution of captures were not normally distributed, and we therefore considered them in GLM and PCA analyses. We carried out univariate and multivariate analyses with SPSS 20.0 release.

RESULTS

We sampled 31 *Euleptes* (17 males, eight females and six juveniles), 100 *Hemidactylus* (35 males, 36 females and 29 juveniles, and 28 *Tarentola* (nine males, seven females and 12 juveniles). Sex ratio of adults (juveniles were excluded) did not differ from 1:1 in each of the three species (Wald test = 0.785, df = 1, P = 0.376).

The three species are markedly different for all considered biometric features (Table 1). *Euleptes* is the smallest, *Hemidactylus* is intermediate and *Tarentola* the largest (all with P values < 0.0001, but inter-nasal P = 0.015). They do not show sexual difference (P values from 0.093 for head width to 0.663 for SVL) nor sex × species interaction (P values from 0.130 for head width to 0.695 for inter-orbital). All statistics are reported in Supplementary Table 1.

Ecological variables recorded at each gecko capture (Table 2) showed differences in most cases: multivariate GLM showed a marked difference between 2009 and 2010 for wind ($F_{2,131} = 5.807$, P = 0.017) and humidity ($F_{2,131} = 36.593$, P < 0.0001). Species differed in site position ($F_{2,131} = 4.011$, P = 0.020), height from the ground ($F_{2,131} = 11.670$, P < 0.0001) and hour of capture ($F_{2,131}$ =

Table 1. Average in mm and grams ± 1 SD of selected variables for each gecko species.

Variable	*Taxon*	Average	SD
Head length	*Euleptes*	10.230	0.312
	Hemidactylus	13.996	0.173
	Tarentola	16.419	0.367
Head width	*Euleptes*	6.809	0.191
	Hemidactylus	9.251	0.106
	Tarentola	11.767	0.224
Head height	*Euleptes*	3.471	0.126
	Hemidactylus	5.561	0.070
	Tarentola	6.965	0.149
Eye diameter	*Euleptes*	2.036	0.059
	Hemidactylus	2.701	0.033
	Tarentola	3.012	0.069
Nostril eye	*Euleptes*	2.795	0.087
	Hemidactylus	3.695	0.048
	Tarentola	5.010	0.102
Inter-nasal	*Euleptes*	1.735	0.052
	Hemidactylus	1.863	0.029
	Tarentola	1.964	0.061
Inter-orbital	*Euleptes*	4.567	0.132
	Hemidactylus	4.702	0.073
	Tarentola	6.630	0.155
bmass	*Euleptes*	1.292	0.235
	Hemidactylus	3.152	0.130
	Tarentola	5.208	0.276
SVL	*Euleptes*	38.939	1.086
	Hemidactylus	50.534	0.601
	Tarentola	55.377	1.276

8.587, $P < 0.0001$). Year × species interaction was significant only for humidity ($F_{2,131} = 3.781$, $P < 0.025$).

PCA (sampling adequacy = 0.551; sphericity Bartlett test = 424.286, $P < 0.0001$) extracted four main components, explaining about 68% of total variance (Table 3). The rotated matrix showed the first component describing species and body size, the second describing period, site position, ground type and height from the ground, the third describing wind and humidity, the fourth describing temperature and ground type (Table 4). The distribution of the three species of geckos according to four components are shown in figures 3-5. Figure 3 shows that smaller geckoes, as *Euleptes* and smaller *Hemidactylus* and *Tarentola*, tend to be distributed to the northern part of the study area. Figure 4 shows that *Euleptes* is associated to low or no wind but with high humidity, while the other two species are more associated to a relative absence of humidity. Figure 5 shows that

Table 2. Variability of ecological variables recorded at gecko capture. Wind in m/sec, umidity in %, site position and Hmslm in m, Hour as in hours (solar time).

Variable	Species	Average ± SD	Sample
Wind	*Euleptes*	0.28 ± 0.45	29
	Hemidactylus	0.22 ± 0.45	77
	Tarentola	0.16 ± 0.37	25
Umidity	*Euleptes*	77.38 ± 17.70	29
	Hemidactylus	69.66 ± 18.17	77
	Tarentola	72.20 ± 20.60	25
Site position	*Euleptes*	157.21 ± 45.14	29
	Hemidactylus	119.65 ± 69.67	77
	Tarentola	119.24 ± 60.10	25
Hmslm	*Euleptes*	2.74 ± 1.15	29
	Hemidactylus	1.61 ± 1.04	77
	Tarentola	1.72 ± 1.05	25
Hour	*Euleptes*	17:55 ± 08:13	29
	Hemidactylus	21:22 ± 00:32	77
	Tarentola	21:14 ± 00:17	25

Table 3. First four principal components explaining about 68% of total variance.

Component	Eigenvalues			Weights of rotated factors		
	total	% variance	% cumulated	total	% variance	% cumulated
1	2.653	26.533	26.533	2.405	24.048	24.048
2	1.687	16.873	43.407	1.705	17.053	41.101
3	1.359	13.592	56.999	1.377	13.767	54.868
4	1.105	11.052	68.051	1.318	13.183	68.051

Table 4. Rotated component matrix and main contribution of each variable (in **bold**) to each component.

Variable	Component			
	1	2	3	4
species	**0.679**	0.157	0.074	-0.270
month	-0.202	**0.622**	0.206	-0.008
wind	-0.179	0.365	**-0.771**	-0.087
T°	0.124	-0.041	-0.032	**0.853**
umidity	-0.225	0.264	**0.817**	-0.187
siteposition	-0.228	**-0.717**	0.208	-0.099
ground	-0.026	**0.518**	-0.073	**0.586**
hmslm	-,484	**-0.548**	0.077	0.078
bmass	**0.898**	-0.022	-0.049	0.199
SVL	**0.844**	-0.076	-0.089	0.276

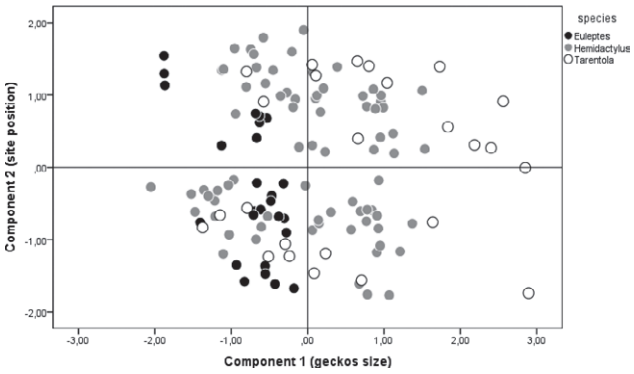

Fig. 3. Geckos distribution along the Component 1-Component 2 relationship.

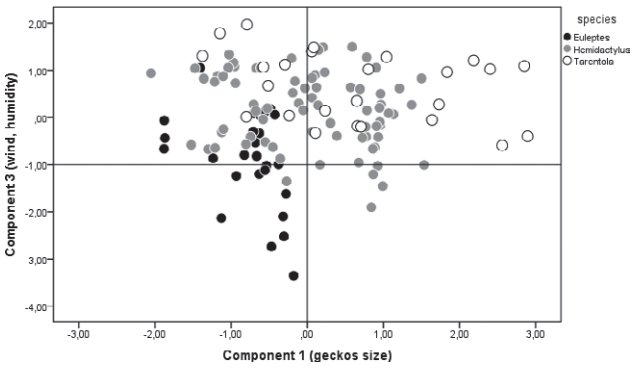

Fig. 4. Geckos distribution along the Component 1-Component 3 relationship.

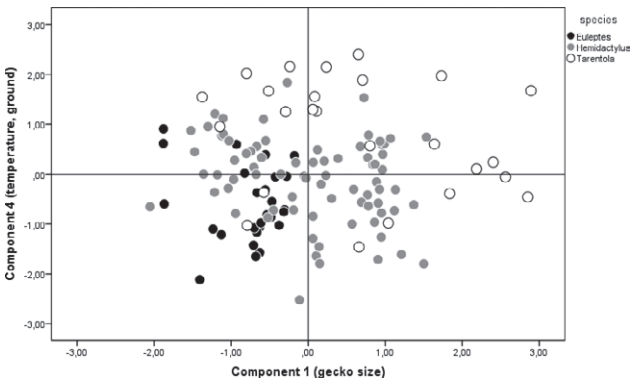

Fig. 5. Geckos distribution along the Component 1-Component 4 relationship.

Euleptes and *Hemidactylus* are active at lower temperatures and on similar substrate ground than *Tarentola*.

Multiple histograms show the occurrence of captures of the three species along the normalized transect (670 m long). Red bars indicate and delimitate the accesses to Cala Violina (Fig. 6).

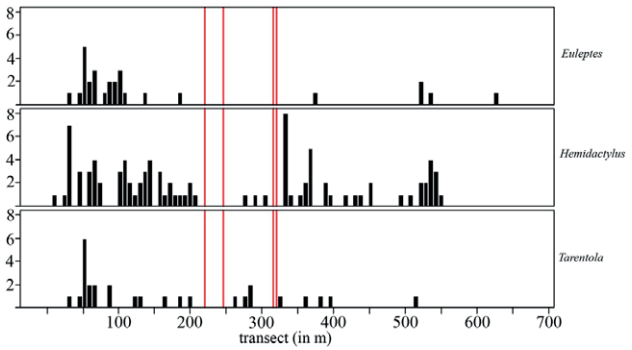

Fig. 6. Capture frequency distribution of target species in the study area. Red lines represent the borders of forest tracks to the beach.

All the species showed a different distribution, *Tarentola* and *Hemidactylus* were quite homogeneous, while *Euleptes* was more concentrated in the first patch of the area (transect 1). Spatial distribution was not normal (Kolmogorov Smirnov Z = 2.149, P < 0.001), and the observed differences in spatial distribution were significant (P = 0.007).

DISCUSSION

The gecko community in this area shows a sex-ratio not differing from the expected 1:1, for all the species considered. Average body size of the leaf-toad gecko and of the Turkish gecko falls within the range of the species in Italy (Corti et al., 2011), while the Moorish gecko results smaller in size with respect to available data (Tuscany: Atzori et al., 2007). In our studied sample, we did not find any significant sexual difference within each species. This matter may occur in some other populations of *Euleptes* (Delaugerre, 1985), likely due to the biotic capacity of the site (Salvidio et al., 2011). *Hemidactylus* males generally present much larger heads than the females, while size is similar for the two sexes (Corti et al., 2011). In *Tarentola*, sexual dimorphism is significantly marked for some features: larger eye diameter and bigger head in males (more voluminous head than equal sized females) and, same SVL, lighter body mass in females (Zuffi et al., 2011). In our study area, the difference between males and females may be underestimated because the number of the two sexes of juvenile age, or as young adults, probably does not present yet the strongly different sexual dimorphism when considering adults. Interestingly, as pointed out by Simbula et al. (2019) where diurnal vs nocturnal *Tarentola* were considered, sexual dimorphism was not significant, a similar matter as occurred in our population. Furthermore, noctur-

nal individuals attained a smaller body size than diurnal ones (Simbula et al., 2019), suggesting an analogous pattern also in our sample. Body size did differ for almost all parameters among the species.

We have recorded, on average, a different association of species as regards site position, height from the ground and hour of observation. Between the two years humidity and wind were different, with a significant interaction among species and year for humidity only.

To date, ecology studies on these three species of geckoes are relatively scarce, covering trophic ecology (Capula and Luiselli, 1994; Luiselli and Capizzi, 1999; Hòdar et al., 2006), or underlining competition for spatial niches in sympatric populations of *Hemidactylus* and *Tarentola* in Croatia (Lisicic et al., 2012), and on lizard and geckoes' community in central Italy (Simbula et al., 2019). Overall, our research is among the very few field works on species assemblages and, actually, it is the first work on comparative ecology of these three species of geckoes in condition of syntopy.

Tarentola seems to prevail in the northern portion of the area, while *Hemidactylus* is more common in the northern and southern portion, as supported by GLM and Chi square analyses. According to our data, wind and humidity had more effects on *Tarentola* and on *Hemidactylus* respectively (Table 2), the species are differently distributed in the area (e.g., site position) and placed at different heights from the ground, *Euleptes* being relatively higher than the other two species, with a different hour distribution of observation. However, in accordance with data and analyses, the three species seem to occupy and frequent the area not in a markedly different way. PCA results showed a different distribution of age classes (as size component) relatively to the site position especially for *Hemidactylus* and *Tarentola* (Fig. 3) and a relatively importance of wind, humidity, temperature and ground type for geckoes observation (Figures 4-5). The whole scenario is in accordance with previous ecological observations (see for instance Delaugerre, 1984; Simbula et al., 2019).

The similar occurrence of species along the transect despite the slight, significant, differences among them in many ecological parameters, resembles the pattern found and underlined by Simbula et al. (2019). Specifically, the "The observed overlap in spatial resource use was higher than expected by chance, thus showing a shared resource use instead of a partitioning pattern" (Simbula et al., 2019).

For what we know so far, Cala Violina is the only syntopy area for *Euleptes europaea*, *Hemidactylus turcicus* and *Tarentola mauritanica,* and it arises a pivotal importance for the conservation of the three species. On average, we must stress that human presence in this area, as a marked touristic presence for most of the geckoes active season, is on one side undoubtedly a risk for the three species. On the other side, according to recorded data, it is not possible yet to assert if studied populations of the three species have suffered numerical losses because of direct anthropic actions (killings, capture, and removal) or indirect (alteration or damage to habitat, increment of tourism), because there are not yet studies underlining the risk factors. Locally, arsons, inappropriate woodcutting and touristic impacts are the strongest risk factors in the study area. The touristic activity on the seaside probably plays a negative role on daily activity of *Tarentola mauritanica*. In fact, the study area is characterised by intensive daily seaside activity in the spring-summer seasons (from June to September). Cala Violina is featured in S.I.R. (Site of Regional Interest) 108 "Monte d'Alma" (IT51A0008), in homonym pS.I.C. (cod. nature 2000 IT51A0008), and in A.N.P.I.L. (Area Naturale Protetta di Interesse Locale, "Protected Natural Area of Local Interest") "Costiere di Scarlino", but the actual presence of *Euleptes europaea* justifies the opportunity to propose the realization of a Biotope. The "Rete Ecologica Regionale" (Ecological Regional Network), together with the "Piano Territoriale di Coordinamento" (PTC, "Territorial Coordinational Plan) applicable in the Grosseto Province, represent two essential instruments enforcing the importance of environmental connectivity and natural resources protection. Biotopes, in fact, are an innovative aspect in the field of planning management, to define further restrictions congruent to the latest acts of territory planning. In a conservation and management planning for this area, priority should be given to intervention finalized to protect slopes and cliffs in which these geckoes live and spent most of their life history traits. Protective intervention for slopes and cliffs is desirable to avoid further human disturbance, and particularly to avoid damages to natural fissures and crevices (abandon of garbage and other objects in fissures, destruction of portions of loose soil by sun beds, chairs and other), likely used for egg deposition, and surely for daily hideaway. These protective measures could be made up by wood barriers adequately distant from sides (0.5 – 1 m), which do not consist of a landscape obstacle, and with explicative posters making visitors aware of the site peculiarities, fauna richness and of all the protection measures. Besides, it would be appropriate to evaluate through a dedicated study how much touristic impact influences these geckoes and other herpetofauna activity in the area, and to limit, if necessary, the daily access to Cala Violina with a maximum number of people per day (a maximum of 700/day since the last year; P. Biagini pers. comm). Measure of human disturbance on a lizard community has been recently carried out in Spain on a population of

wall lizard (*Podarcis muralis*), in a strongly touristic area (Amo, Lopez and Martin, 2006), where the authors pointed out that tourism had harmful effects on physical condition and relations host-parasite in this reptile. Therefore, it would be desirable to verify the actual situation in Cala Violina, because of this and other studies (e.g., Attum et al., 2006; French et al., 2008).

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SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at <http://www-9.unipv.it/webshi/appendix/index.html> manuscript number 11547

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Comparative cytogenetics on *Zamenis lineatus* and *Elaphe quatuorlineata* (Serpentes: Colubridae)

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Abstract. Because of their peculiar genomic and chromosomal characteristics, reptiles are extraordinary model organisms to study karyotype and sex chromosome evolution, but despite the growing interest in their evolutionary cytogenetics, only a small fraction of species have a known karyotype. We performed a comparative cytogenetic analysis on *Elaphe quatuorlineata* and *Zamenis lineatus*, using classic and molecular techniques. We provide the karyotype of these two species and an assessment of their chromosomal features. Chromosome analysis was performed with standard karyotyping, C-banding, sequential C-banding + CMA_3 + DAPI and Ag-NOR staining. On *E. quatuorlineata*, we also performed CMA_3-methyl green staining and Fluorescence in situ Hybridization mapping NOR loci (NOR-FISH). *Elaphe quatuorlineata* and *Z. lineatus* show a very similar karyotype of 2n = 36, with 8 macro- and 10 microchromosome pairs, but differ in the morphology of the pair 8, which resulted submetacentric in the former and metacentric in the latter species. By comparing our data to those available from the literature on congeneric species, we analysed the occurrence of primitive and derivate chromosomal characters and provide cytotaxonomic insights, which further support the species status of *Z. lineatus.* In both species, the 4th pair was identified as the sex chromosome pair (ZZ/ZW) and NORs were localized on a microchromosome pair. We finally highlight in both genera *Elaphe* and *Zamenis* different stages of heterochromatinization of the W chromosome, in agreement with the progressive diversification model of sex chromosome as already shown in different reptile taxa.

Keywords. Chromosome, evolution, karyotype, NORs, squamates, snakes.

INTRODUCTION

Classic cytogenetic through differential staining and banding of chromosomes permits to describe and compare karyotypes, whereas the molecular cytogenetic approach, employing Fluorescence *in situ* Hybridization (FISH) with specific probes, allows the detection of particular sequences present in genomes (Matsuda et al., 2005; Dumas and Sineo, 2014), including repetitive DNA sequences (Scardino et al., 2020a). Among those repetitive DNA elements are the ribosomal DNA (rDNA), encoding rRNA. These elements have been successfully used as markers for comparative cytogenetic studies and phylogenetic analyses. The rDNA is organized into 2 families: 5S (minor) and 45S (major) rDNA. The latter comprises the genes for 18S, 5.8S, and 28S rRNA and is located in the so-called nucleolus organizer regions (NORs). The NORs can be identified either by silver staining, which detects only transcriptionally active loci, or more accurately, by FISH, which permits the identification of both active and inactive NORs. The location of the rDNA loci in the karyotype may show a species-specific pattern, so rDNA loci are often used for complex karyotype characterizations (Scardino et al., 2020a). Indeed, compara-

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tive chromosome analyses can be useful to identify plesiomorphic and apomorphic states and the occurrence of different evolutionary lineages (Deakin and Ezaz, 2014; Damas et al., 2018; Scardino et al., 2020b). Chromosome rearrangements may precede or follow molecular evolution, directly promoting cladogenesis or resulting from phylogenetic diversification (Noor et al., 2001; Rieseberg, 2001). In either case, they represent discrete evolutionary markers able to detect different evolutionary trends or apomorphisms in the taxa studied (Dobigny et al., 2004; Olmo, 2008; Dumas et al., 2015).

Squamate reptiles, due to their peculiar genomic and chromosomal characteristics, are exceptional model organisms in the study of karyotype evolution and sex chromosome diversification of vertebrates (Olmo, 2008; Alam et al., 2018). Squamates display a remarkable variability in chromosome number and morphology, number and location of different chromosome markers and the occurrence of environmental genetic sex determination, with the independent evolution of simple and multiple sex chromosome systems with either male or female heterogamety (Olmo, 2008; Pallotta et al., 2017; Deakin and Ezaz, 2019; Sidhom et al., 2020; Mezzasalma et al., 2021 a). The cytogenetic approach used for the study of chromosome rearrangements and different morphologies and/or levels of heterochromatinization of the heteromorphic sex chromosomes have been previously used in different phylogenetically closely-related European squamate taxa such as the snakes of the genus *Hierophis* (Fitzinger, 1843), *Anguis fragilis* Linnaeus, 1758, and *A. veronensis* Pollini, 1818, and geographically distinct populations of *Coronella austriaca* Laurenti, 1768 (Mezzasalma et al., 2013, 2015, 2018b; Mezzasalma and Odierna, 2021). However, despite the growing interest in the evolutionary cytogenetics of squamate reptiles, only a small fraction of the described squamate species have a known karyotype (Olmo and Signorino, 2006; Mezzasalma et al., 2021), leaving most of their chromosomal diversity still unexplored. This is also true for some peculiar Mediterranean reptile species such as the European four-lined snake *Elaphe quatuorlineata* (Bonnaterre, 1790) and the Italian Aesculapian snake *Zamenis lineatus* (Camerano, 1891).

In this work, we performed a comparative cytogenetic analysis on *E. quatuorlineata* and *Z. lineatus*, using a combination of standard staining and banding techniques. We provide the first karyotype description of these two species and an assessment of their chromosomal features. By comparing our data to those available from the literature on phylogenetically closely-related species, we evidence and discuss the occurrence and distribution of primitive and derivate chromosomal characters in the species studied and provide cytotaxonomic insights, which support the species status of *Z. lineatus*. We also highlight that both genera *Elaphe* and *Zamenis* show progressive evolutionary stages of the W chromosome, supporting the heterochromatinization model of sex chromosome diversification (see e.g., Mezzasalma et al., 2021).

MATERIAL AND METHODS

We analysed two samples (one male and one female) of *Z. lineatus* and one female of *E quatuorlineata* from Piedimonte Matese, Campania, Italy. Specimens were anesthetized on ice and, after taking a 0.5 ml of blood aliquot from the caudal vein, they were released in the capture site. Chromosomes were obtained from blood cultures following Odierna et al. (2004). Namely, blood aliquots were incubated for four days at 30 °C in 5 ml of lymphocyte medium culture (3.8 ml of DMEM, 0.5 ml sterile distilled water, 0.5 newborn calf serum, 0.1 ml antibiotics, 0.1 ml PHA). Chromosome harvesting was performed by adding 0.1 ml of Colcemid (10 μg/ml) and two hours later the cells were collected by centrifugation (1000 rpm/min), incubated for 30 min in 5 ml of hypotonic solution (KCl 0.075 M) and fixed in methanolacetic liquid (methyl alcohol + acetic acid, 3:1). Slides were prepared using the air-drying method, as described in Mezzasalma et al. (2019). The cytogenetic analysis was performed with traditional karyotyping (5% Giemsa solution at pH 7 for 10 min) and additional chromosome staining and banding techniques; in particular, C-banding was performed following Sumner (1972) and sequential C-banding + CMA_3 + DAPI according to Sidhom et al. (2020), which highligh CG and AT-rich regions, respectively. Nucleolus organizing regions (NORs) were identified following the Ag-NOR staining method described by Howell and Black (1980). Given quantity and quality of metaphase plates, on *E. quatuorlineata* we also performed Chromomycin A_3-methyl green staining (CMA/MG) (a staining method usefulf to hightlight CG-rich chromosome regions) as described by Sahar and Latt (1980) and Fluorescence *in situ* Hybridization (NOR-FISH) following Mezzasalma et al. (2018a), using as probe the PCR-amplified and biotinylated 18S rRNA gene of the gekkonid *Tarentola mauritanica* (Linnaeus, 1758). In brief, after denaturation in 70% formamide and 2x SSC for 2 min at 80 °C, slides were incubated overnight at 40 °C with the hybridization mixture (10 ng/ml biotinylated 16 dUTP probe 0.1 μm/ml *Escherichia coli* DNA in 50% formamide and 2x SSC). After washing in 2x SSC, cytochemical detection was performed using 5 μm/ml FITC-conjugated ExtrAvidin (Sigma) in 4x SSC + 1% BSA + 0.1%

Tween 20, pH 7. After washing three times in 4x SSC and 0.1% Tween 20 for 10 min at 42 °C, the detection of FISH signals was performed with ExtrAvidin FITC (Sigma Aldrich) counterstained with propidium iodide (PI) (200 ng/ml) in 2x SSC, pH 7, for 2 min at room temperature. Metaphase plates were scored and recorded with an optical and an epifluorescent microscope (Axioscope Zeiss) equipped with an image analysis system. Karyotype reconstruction was performed after scoring at least five metaphase plates from each sample studied and chromosomes were classified according to Levan et al. (1964).

RESULTS

The karyotypes of *E. quatuorlineata* and *Z. lineatus* are both composed of 2n = 36 chromosomes, with 8 macrochromosome pairs and 10 microchromosome pairs (Fig. 1A, 2A). The two species also show the same chromosome morphology with the exception of the chromosome pair 8, which resulted submetacentric in *E quatorlineata* and metacentric in *Z. lineatus* (Table 1, Fig. 2A). Arm number (AN) resulted = 50 in both colubrids. Morphometric parameters of each macrochromosome pair of both species studied are reported in Table 1.

C-banding and Ag-NOR revealed in both species the occurrence of NOR loci on a microchromosome pair, as confirmed by NOR-FISH in *E. quatuorlineata* (Fig. 1B-D). C-banding showed heterochromatin content on autosomes, mostly concerning telomeric and centromeric regions in both *E. quatuorlineata* (Fig. 1E-G) and *Z. lineatus* (Fig. 2B-D). Furthermore, in the female samples of both species, one element of the 4th macrochromosome pair resulted to be largely heterochromatic, allowing us to identify this pair as a homomorphic ZW sex chromosome pair (Fig. 1E-G, 2B-D). This W chromosome resulted highly positive with both CMA_3 and DAPI in *E. quatuorlineata* (Fig. 1E-G), whereas it was clearly evident with DAPI and less evident with CMA_3 in *Z. lineatus* (Fig. 2B-D).

Table 1. Chromosome morphometric parameters. Chr. = Chromosome number; RL = Relative length (Chromosome length/total karyotype length*100); CI = Centromeric index (short arm length/chromosome length*100); m = metacentric; sm = submetacentric; t = telocentric.

Chr.	*Elaphe quatuorlineata*		*Zamenis lineatus*	
	RL	CI	RL	CI
1	19.4 ± 0.8	44.9 ± 3.4 (m)	19.3 ± 1.0	48.4 ± 3.4 (m)
2	16.5 ± 0.5	34.8 ± 4.0 (sm)	16.1 ± 0.7	35.2 ± 4.0 (sm)
3	11.1 ± 0.5	48.4± 3.8 (m)	10.8 ± 0.4	43.2 ± 3,8 (m)
4(Z)	7.7 ± 0.6	45.9 ± 2.8 (m)	8.3 ± 0.6	45.4 ± 2.8 (m)
4(W)	7.6 ± 0.4	45.1 ± 3.4 (m)	8.4 ± 0,3	44.9 ± 3.4 (m)
5	7.5 ± 0.7	48.3 ± 4.3 (m)	7.9 ± 0.5	42.8 ± 4.3 (m)
6	6.0 ± 0.4	0.0 ± 3.0 (t)	7.1 ± 0.6	0.0 ± 3.0 (t)
7	5.6 ± 0.7	36.0 ± 3.1 (sm)	6.3 ± 0.5	36.3 ± 3.1(sm)
8	5.3 ± 0.4	36.9 ± 3.3 (sm)	4.4 ± 0.6	40.1 ± 3.3 (m)
9-18	20.6 ± 1.1		19.8 ± 1.3	

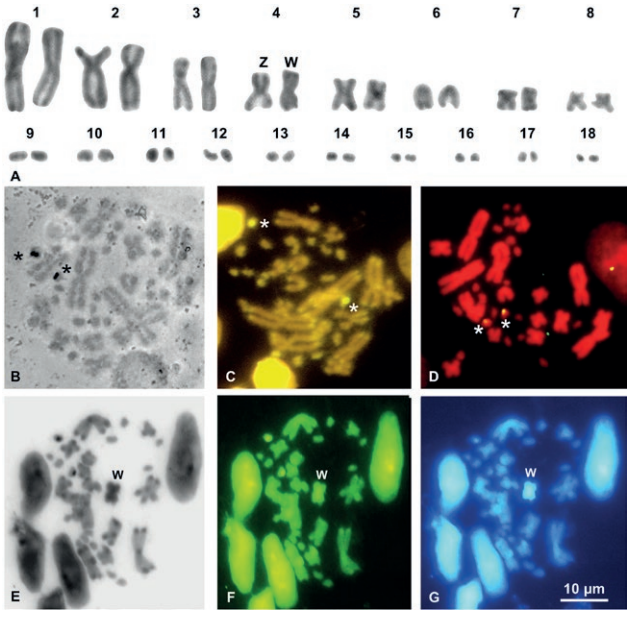

Fig. 1. Karyotype and metaphase plates of *E. quatuorlineata* stained with Giemsa (A), Ag-NOR (B), CMA_3/MG (C), NOR-FISH (D), C-banding + Giemsa (E), + CMA3 (F), + DAPI (G). * = loci of NORs.

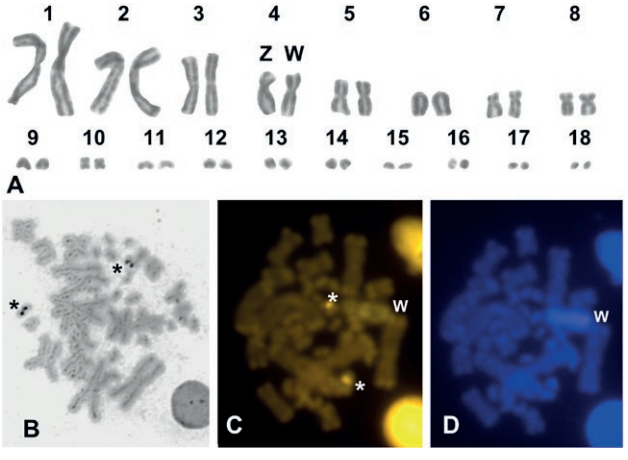

Fig. 2. Karyotype and metaphase plates of *Z. lineatus* stained with Giemsa (A), Ag-NOR (B), C-banding + CMA3 (C), + DAPI (D). * = loci of NORs.

DISCUSSION

Our results show that the karyotypes of *E. quatuorlineata* and *Z. lineatus* have the same diploid number (2n = 36) and a similar general structure, but a different morphology of chromosome pair 8.

In order to highlight the occurrence of simplesiomorphic, sinapomorphic and apomorphic states and add data for the reconstruction of the chromosomal evolution in the genera *Elaphe* and *Zamenis*, we compared the newly generated karyotypes to those available from the literature on congeneric species as well as with that of the hypothesized Ancestral Snake Karyotype (ASK) (see Kobel, 1967; Bianchi et al., 1969; Singh, 1972; Itoh et al., 1970; De Smet, 1978; Augstenová et al., 2017; Rovatsos et al., 2018; Cole and Hardy, 2019) (Fig. 3).

This comparison permits to show that *E. quatuorlineata* and *Z. lineatus*, as well as most congeneric species with a known karyotype, have different chromosomal characters which are considered simplesiomorphisms and found in the hypothesized ASK (see Kobel, 1967; Bianchi et al., 1969; Singh, 1972; Itoh et al., 1970; De Smet, 1978; Augstenová et al., 2017; Rovatsos et al., 2018; Cole and Hardy, 2019; this paper). These shared ancestral characters include: diploid number, number of macro- and microchromosome pairs, the general morphology of several macrochromosome pairs and the localization of NOR loci on a microchromosome pair (see also Cole and Hardy, 2019). All this evidence permits to confirm that *Elaphe* and *Zamenis* are karyologically very conservative, but for the morphology and sequence content of the W chromosome, which are variable among different taxa (see also Augstenová et al., 2017; Cole and Hardy, 2019; Mezzasalma and Odierna, 2021).

Nevertheless, the karyotypes of *E. quatuorlineata* and *Z. lineatus* also possess some peculiar derivate features, which characterize their respective karyotype from those of phylogenetically related species. In *Elaphe*, autosomal

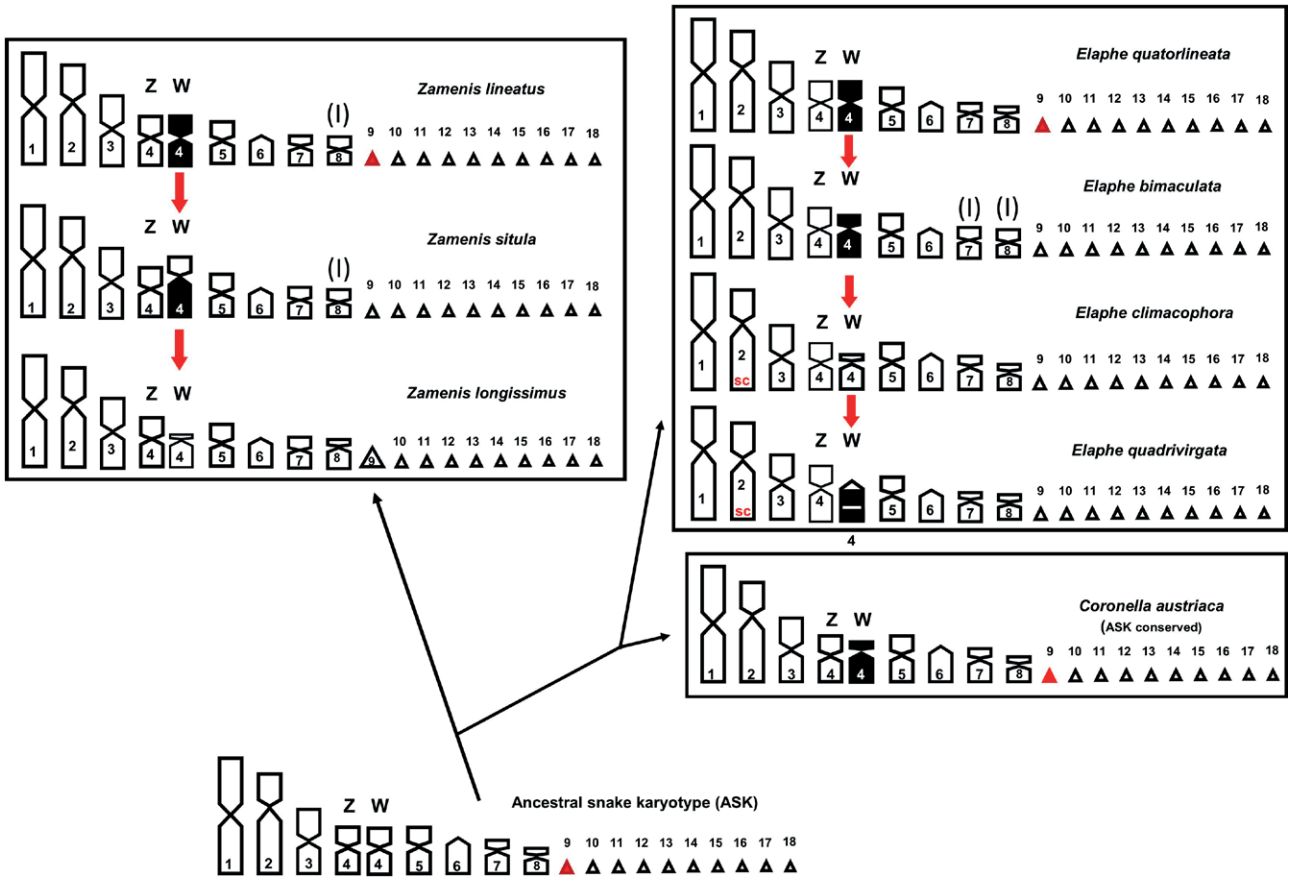

Fig. 3. Original karyograms of *Z. lineatus* and *E. quatuorlineata* compared with the Ancestral Snake Karyotype (ASK) and available literature data on congeneric species (Kobel, 1967; De Smet, 1978; Itoh et al., 1970; Augstenová et al., 2017; Rovatsos et al., 2018; Cole and Hardy, 2019; Mezzasalma and Odierna, 2021). sc = secondary constriction, (I) = chromosome inversion. Red chromosomes = NOR-bearing pair. Black regions/chromosomes = heterochromatin. Red arrows indicate progressive steps of sex chromosome diversification.

rearrangements from the hypothesized ancestral snake karyotype involve a putative inversion of the 8th pair in *E. quatuorlineata* and in *E. bimaculata* that can be considered a sinapomorphism, and in the second species, also an inversion of the 7th pair, as previously showed (see Fig. 3) (Itoh et al., 1970; Rovatsos et al., 2018), which can be considered an apomorphism.

Furthermore a translocation of loci of NORs on the 2nd chromosome pair in *E. climacophora* and *E. quadrivirgata*, evidenced by a secondary constriction (see Itoh et al., 1970), represent a further apomorphism, which is not present in the species here analyzed. *Zamenis lineatus* shows a metacentric 8th chromosome pair, which probably originated by means of a pericentromeric inversion as previously showed also in *Z. situla* (Augstenová et al. 2017), thus representing a sinapomorphism linking the two species.

It should also be noted that in the previously described karyotypes of *Z. longissimus* (Kobel 1967; De Smet, 1978), a different macrochromosome number (8 and 9, respectively) is reported, without any changes in the total chromosome count (2n = 36). The additional macrochromosome pair reported by De Smet (1978) is probably due to the amplification of NOR-linked heterocromatin of the NOR microchromosomes bearing pair, but more focused analyses are needed to confirm the occurrence of intraspecific chromosomal variability in *Z. longissimus*.

Progressive steps of the configuration of the heterogametic W chromosome are important events in reptiles and are clearly visible in many species, supporting the general heterochromatinization hypothesis of sex chromosome diversification (Augstenová et al., 2017; Alam et al., 2018; Cole and Hardy 2019; Mezzasalma et al., 2020). In fact, it is widely accepted that heteromorphic sex chromosome pairs begin their morphological and molecular diversification starting from a homomorphic state (Gamble et al., 2014; Mezzasalma et al., 2021). From this condition, two alternative pathways are known to potentially lead to a fully differentiated sex chromosome pair: a progressive heterochromatinization of the heterogametic chromosome or the insurgence of an inversion in the homomorphic proto-W chromosome (Wright et al., 2016; Natri et al., 2019; Mezzasalma et al. 2021). In either cases, the progressive diversification of the W element eventually leads to its evolutionary isolation (loss of recombination) and degeneration, finally reaching the size of a microchromosome (Marshall Graves, 2016; Mezzasalma et al., 2016; Wright et al., 2016).

Progressive steps of the configuration of the heterogametic W chromosome are visible in the species here analysed and in the phylogenetically closely-related taxa *Elaphe* and *Zamenis* (Fig. 3) (see also Kobel, 1967; De Smet, 1978; Itoh et al. 1970; Augstenová et al. 2017; Rovatsos et al., 2018; Mezzasalma and Odierna 2021). In particular, the W chromosome appears at a relatively earlier stage of diversification in *E. quatuorlineata*, in which it resulted largely heterochromatic, but homomorphic to the Z (this paper). More advanced diversification stages are represented by the W elements of *E. bimaculata* and *E. climacophora*, in which the morphology of the W chromosomes progressively diverged from the Z, reaching a telocentric configuration in *E. quadrivirgata* (Fig. 3) (see also Itoh et al., 1970; Rovatsos et al., 2018).

The W chromosome is homomorphic but largely heterochomatic in *Z. lineatus*, representing an initial diversification step from the Z element (this paper). A progressive addition of heterochromatin may produce a heterogametic chromosome, which appears sensibily larger than the Z: a condition similar to that reported in *Z. situla* (Augstenová et al., 2017) (Fig. 3).

Furthermore, it is possible to highlight that the differences in the morphology of the W chromosome and of the 8th and 9th chromosome pairs found between *Z. lineatus* and *Z. longissimus* (Kobel 1967; De Smet, 1978; this paper) are in agreement with their species status, originally proposed using a combination of morphological and molecular data (Lenk and Wüster, 1999; Utiger et al., 2002).

This evidence underlines that in squamate reptiles the cytotaxonomic approach is a useful tool for characterizing closely-related lineages as already shown in other squamate taxa (Mezzasalma et al., 2013, 2015, 2018b; Mezzasalma and Odierna, 2021).

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