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On the origin of the easternmost known population of *Podarcis erhardii* (Bedriaga, 1876) (Reptilia: Sauria)

Borislav NAUMOV [1], Emiliya VACHEVA [1,*], Simeon BORISSOV [1], Simeon LUKANOV [1], Angel DYUGMEDZHIEV [1], and Irina LAZARKEVICH [2]

1. Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, 2 Gagarin Street, 1113 Sofia, Bulgaria
2. Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria
* Corresponding author: E. Vacheva, E-mail: emilia.vacheva@gmail.com

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Abstract. The presence of *Podarcis erhardii* was recently recorded on the Black Sea coast near the village of Varvara, Bulgaria, more than 100 km to the East of its established range, and in syntopy with another species, *P. muralis*. The origin of this easternmost known population of *P. erhardii* was investigated by using both mtDNA and external morphological characteristics from individuals of *P. erhardii/muralis* from three sites - north and south of Varvara and near Sinemorets. Our results confirmed the presence of *Podarcis erhardii* only south of Varvara, while *P. muralis* was confirmed in each of the three investigated sites. We believe that this isolated population of *P. erhardii* presents an accidental introduction due to human activity, rather than a result of the natural expansion of the species' range. In addition, we highlight the very high genetic similarity between *Podarcis muralis* individuals from this study with individuals from Ropotamo and NW Turkey, which are clearly separated from the other *P. muralis* haplotypes included in our analysis.

Keywords: Lacertidae, distribution, mitochondrial DNA, Balkan Peninsula.

Introduction

The genus *Podarcis* Wagler 1830, with its currently recognized 24 species (Speybroeck et al. 2020), is among the most numerous genera of the Lacertidae family in Europe. The Erhard's wall lizard (*Podarcis erhardii* (Bedriaga, 1876)) is a Balkan endemic that occurs in Albania, North Macedonia, the southernmost part of Serbia, SW and S Bulgaria, and Greece, incl. many of the Aegean islands (Sillero et al. 2014). In mainland Greece, the easternmost locality of the species is located at the 26th meridian (Valakos et al. 2008), i.e. about 180 km west of the Black Sea; the species has not been reported for Turkey (Kurnaz 2020). In Bulgaria, *P. erhardii* is found only in the southwestern part of the country and in the Eastern Rhodope Mountains, up to about 1000-1250 m a.s.l., in rare cases up to 1600 m (Stojanov et al. 2011). The easternmost localities are Ivaylovgrad, Mezek (Buresch & Zonkow 1933), Svirachi and Mandritsa (Petrov et al. 2001), and the distance between these sites and the Black Sea is 150-160 km. Until recently, it was accepted that the species did not reach the Black Sea coast, but two publications from 2022 are likely to change the knowledge about the eastern limit of its range.

Koynova et al. (2022) reported the observation of 3 adult *Podarcis erhardii* near the village of Varvara on the Bulgarian Black Sea coast (ca. 50 km SE from the city of Burgas): one individual of unknown sex from 29.05.2021 and a pair (male and female during courtship) from 30.07.2021. The photograph of the observed pair provided in the paper shows that both individuals closely resemble *P. erhardii* in dorsal coloration. Mollov et al. (2022) supplement these data with exact coordinates of the site at Varvara and mention the finding of four more specimens on 16.04.2022 in the immediate vicinity (two locations with exact coordinates are given, but without descriptions and photographs of the specimens). Therefore, apart from the expertise of the cited authors, the only documented evidence of the presence of *P. erhardii* near the village of Varvara is the photograph mentioned above. It should be mentioned that this locality falls within the natural range of *P. muralis* (Laurenti, 1768) – a species that is widespread in Bulgaria, incl. the Black Sea coast, and is very similar to *P. erhardii* both in external morphology and habitat preferences (see Stojanov et al., 2011). It should also be noted that the external morphology of *P. erhardii* varies widely, and as a result, numerous subspecies have been described (e.g., Gruber 1986 mentions 28 subspecies, 25 of which are from the islands alone). More recent studies (Poulakakis et al. 2003, Lymberakis et al. 2008, etc.) show that the populations of *P. erhardii* are paraphyletic, and that subspecies defined based on external morphological features do not correspond to different molecular phylogenetic clades. Koynova et al. (2022) explicitly say that to clarify the origin of the population of *P. erhardii* from Varvara, additional research, including molecular evidence, is needed.

Given the latter, the main objective of the present study was to clarify the taxonomic status and origin of *Podarcis* sp. from Varvara and surrounding territories by analysis of mtDNA sequences and characterization of external morphology.

Materials and methods

Study area and sampling

The lizards studied originate from three sites along the Bulgarian Black Sea coast (located on the very shore of the sea): two in the area of the village of Varvara (north and south) and one in the area of the village of Sinemorets (Figure 1). The sites were visited on May 31st and June 1st, 2023, and attempts were made to capture any *P. muralis/erhardii* observed by hand. Each caught individual had exact geographical coordinates assigned (handheld GPS receiver, accuracy ± 5 m), we measured basic morphological traits (digital caliper, precision of 0.01 mm), took photographs (digital camera with a

resolution of 180 dpi), and collected a tissue sample (tail tip) for DNA analysis. After the manipulations were completed, the lizards were released at the location of their capture.

Molecular procedures and phylogeny

Total genomic DNA was isolated from the tissue samples of captured lizards using the DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's instructions. To provide comparison with previously studied populations, we amplified through PCR a fragment of the mitochondrial cytochrome b gene using the primers LGlulk (5′-AACCGCCTGTTGTCTTCAACTA-3′) and Hpod (5′-GGTGGAATGGGATTTTGTCTG-3′) (Podnar et al. 2007, Schulte et al. 2012, Michaelides et al. 2013, Oskyrko et al. 2022a). Then, PCR products were purified and sequenced by Macrogen Europe B.V. Chromatograms were visualized, trimmed, and assembled with the software CodonCode Aligner v.8.0.2 (CodonCode, Dedham, MA, USA). The obtained sequences were used to query the NCBI database with the BLAST tool. Previously published DNA sequences (Jablonski et al. 2019, Oskyrko et al. 2022a, Poulakakis et al. 2005, Psonis et al. 2017, Salvi et al. 2013) from *P. muralis* and *P. erhardii* from nearby territories, as well as outgroups, were selected and downloaded from GenBank (Appendix 1). Alignments were prepared in Mega X (Kumar et al. 2018). All fragments were checked for stop codons with DAMBE (Xia 2018). The best substitution model was estimated for each codon position using the PartitionFinder version. 2.1.1 (Lanfear et al. 2016). A phylogenetic tree was inferred through Bayesian inference (BI) using MrBayes v. 3.2.7 (Ronquist et al. 2012). Four simulations of Markov chains with 4 x 10^6 generations were run, sampling one of every 100 trees. The chain parameters were monitored using the Tracer version. 1.7.1 (Rambaut et al. 2018). The first 25% of trees were discarded as burn-in.

External morphology

The morphological characterization of the caught individuals was made based on the following quantitative (metric and meristic) traits: body length (Lcorp), tail length (Lcd), head length (Lcap), head width (Latcap), length of the fore limb (PA), length of the hind limb (PP), number of dorsal scales at the midbody (Sq), number of transverse rows of ventral scales (V), and number of the femoral pores (Pf). The pattern of the dorsal coloration and the size of the massetericum (based on the photographs taken) were used as qualitative traits. Individuals were divided into two groups: PodA – individuals with *P. erhardii* mtDNA, and PodB – individuals with *P. muralis* mtDNA (see Results). Based on the morphometric marks, we calculated six indices (simple ratios) that describe some body proportions (Lcap/Lcorp, Latcap/Lcorp, PA/Lcorp, PP/Lcorp, Latcap/Lcap, and PA/PP). These indices and meristic traits were used to statistically test for differences between the two groups using the Mann-Whitney U test (via PAST 4.07 (Hammer et al. 2001)). The following qualitative traits were also analyzed: occipital line type, dorsal stripes pattern, supraciliary lines type, ventral coloration pattern, and massetericum size. These traits are widely used to distinguish *P. erhardii* from *P. muralis*, but it should be noted that there are extensive variations; therefore, none of them, used alone, is completely reliable (see Gruber 1986, Gruschwitz & Bohme 1986, Stojanov et al. 2011, and Speybroeck et al. 2016).

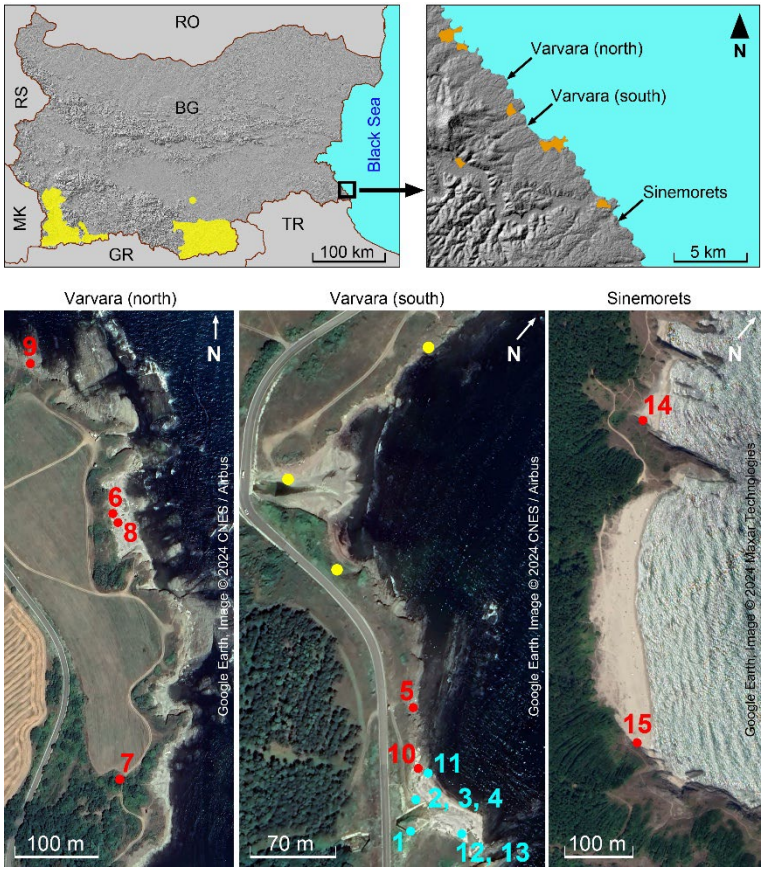

Figure 1. Above left: natural distribution range of *P. erhardii* in Bulgaria (in yellow) according to Stojanov et al. (2011) and location of the study area (black rectangle). Above right: location of the study sites. Below: exact locations of the lizards captured in the three sites [blue and red dots indicate individuals with haplotype PodA and PodB, respectively (numbers indicate the sequence numbers of captured individuals), and yellow dots indicate the locations of *P. erhardii* according to Mollov et al. (2022)].

Results

A total of 15 individuals (11 females and 4 males) were captured: 4 from Varvara (north), 9 from Varvara (south), and 2 from Sinemorets (Figure 1). The reported values of the metric and meristic traits of the lizards and the calculated morphometric indices are given in Appendix 2.

Molecular characteristics

A total of 15 new DNA sequences from the cytochrome b gene (of 294 bp length) were obtained. There were only 2 unique haplotypes from these, hereinafter referred to as PodA (PQ416642) and PodB (PQ416643). The PodA haplotype was observed in individuals 1, 2, 3, 4, 11, 12, and 13 (see Appendix 1) and matched *P. erhardii* samples in the NCBI database. The PodB haplotype, observed in individuals 5, 6, 7, 8, 9, 10, 14, and 15, matched *P. muralis*. The DNA alignment used for phylogeny included the two unique sequences from the southern Black Sea Coast of Bulgaria and 13 unique sequences from *P. erhardii* and *P. muralis,* as well as 3 outgroups (Appendix 1). The Bayesian phylogenetic tree strongly supported most nodes (Figure 2). The two species of interest formed two well-supported clades. The PodA haplotype was arranged within *P. erhardii* closest to the samples from the western Balkans (Northern Greece, North Macedonia, and Serbia, labeled as 'GR: Grevena' on Figure 2). PodB was identical to previously published sequences from Bulgaria ('Ropotamo' and 'Bjala voda', Jablonski et al. 2019) and from European Turkey ('Kapakli', Salvi et al. 2013), and clustered with *P. muralis* from Pinarozu, Turkey, both forming a clade, sister to all other *P. muralis* samples.

Morphological characteristics

The Mann-Whitney U test demonstrated no significant differences between females with PodA and PodB haplotypes regarding body proportions and the number of dorsal and ventral scales (in all cases $U > 5.00$, $p > 0.05$). There was a marginally significant difference in the number of femoral pores, which was greater in the PodA group than in the PodB group ($U = 2.50$, $p = 0.047$). It should be noted that the possible morphological differences between the groups were likely not detected because of the limited test power (due to the small sample size). A better idea of the external differences between the two groups was obtained by analyzing qualitative features.

In regards to the dorsal coloration, the following was observed (Figure 3): in all individuals of the PodA-group the occipital line is short and can be traced up to the first 1/3 of the length of the back, while in individuals of the PodB-group the occipital line is absent or broken in two rows of dots (except for #10, grouped with Pod-A by this trait). In females of the PodA-group the dorsal stripes are strongly and evenly speckled with dark spots and the supraciliary lines are contrasting and visible, while in females of PodB-group the dorsal stripes are weakly and unevenly spotted and the supraciliary lines are less pronounced (except for #10, grouped with Pod-A by these traits as well). The ventral coloration (Figure 4) is uniform and almost free of spots in all individuals of the PodA-group, while most individuals of the PodB-group show visible spots, especially on the throat (except for #10 and #5).

Regarding the lateral pholidosis of the head (Figure 5), in individuals from the PodA group, the massetericum is relatively small and hardly noticeable (except for #12), while in those from the PodB group, the massetericum is significantly larger, except for #10.

Discussion

The results of the DNA analysis confirmed the presence of *Podarcis erhardii* only in the site south of Varvara (referred to by Koynova et al. 2022 and Mollov et al. 2022), while *P. muralis* was confirmed in each of the three investigated sites. In view of this, we believe that the local population of *P. erhardii* does not represent the easternmost point of the species' natural range, but is the result of an accidental introduction due to human activity. Corroborating this is the fact that the area between the Eastern Rhodopes and the Black Sea coast in SE Bulgaria (i.e., the "gap" between the main range of *P. erhardii* and the Varvara site) has been the subject of at least two faunal studies focused on amphibians and reptiles [Stoev (2000) on the Sakar Mts. and Derwent Uplands, and Mollov et al. (2022) on the Strandzha Mts.] and in addition, this area has also been subject of our field research [multiple irregular visits during the period 2012-2022].

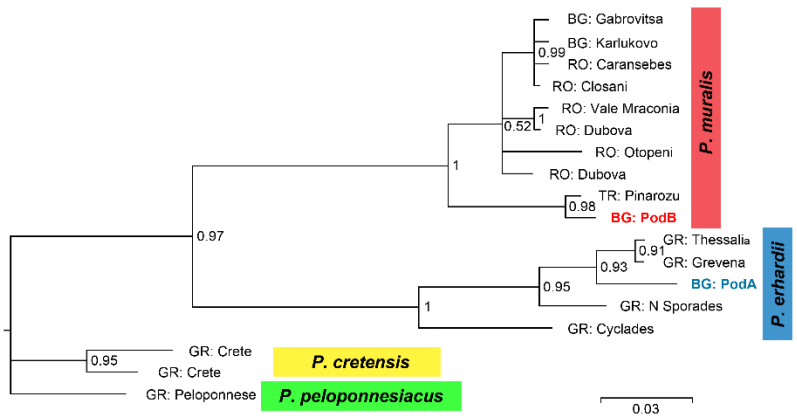

Figure 2. Mitochondrial phylogeny based on the cytochrome b gene (294 bp fragment) of related *Podarcis* species from the Balkans.

Figure 3. Dorsal view of the captured lizards. Above: the specimens from PodA-group; below: the specimens from PodB-group; sex is given in brackets.

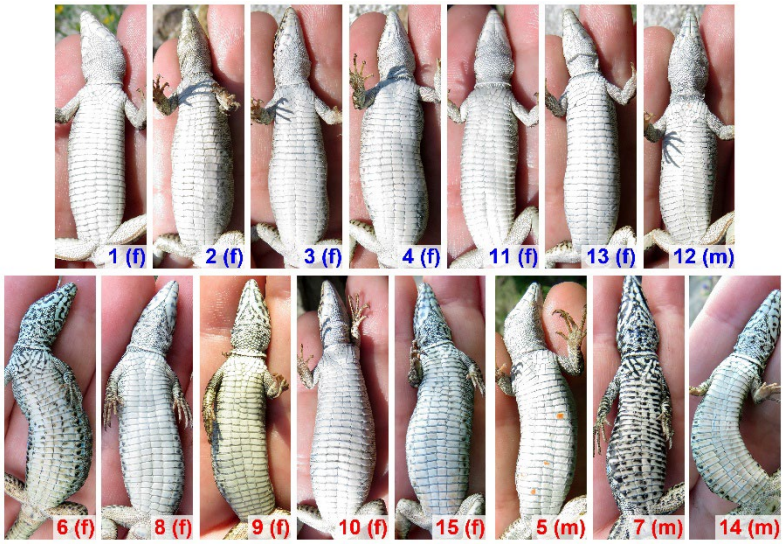

Figure 4. Ventral view of the captured lizards. Above: the specimens from PodA-group; below: the specimens from PodB-group; sex is given in brackets

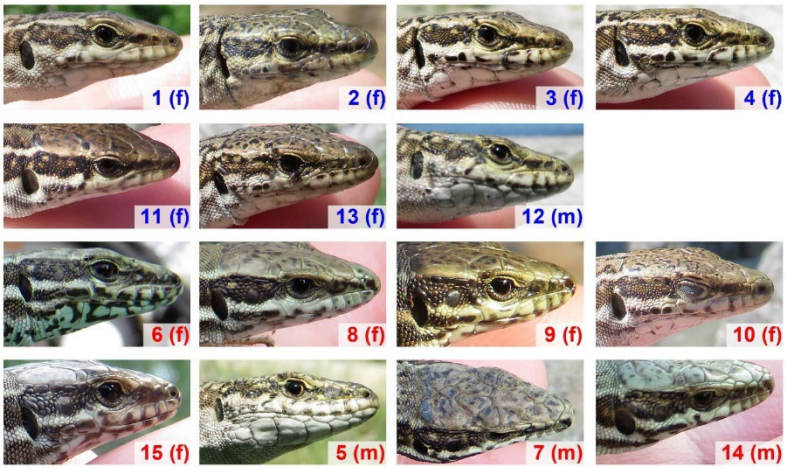

Figure 5. Lateral view of the head (right side) of the captured lizards. The above two rows: the specimens from PodA-group; the below two rows: the specimens from PodB-group; sex is given in brackets.

Comparison with the available Cytb sequences demonstrated that the Varvara population is genetically most similar to the *P. erhardii* populations from Thessaly and Grevena (Greece), Tetovo (North Macedonia), and Makrovi (Serbia). Still, it should be noted that the analysis did not include sequences from the geographically closest parts of the known range of the species (Eastern Rhodopes, South Bulgaria). The existence of populations resulting from accidental introductions is not rare in the species of the genus *Podarcis*, even when islands are excluded. The most typical example in this respect is *P. siculus* (Rafinesque-Schmaltz 1810), found in many sites far outside its natural range (Oskyrko et al. 2022b). A number of non-native populations are also known for *P. muralis* (Oskyrko et al. 2020, 2022a), and the case of *P. erhardii* discussed here suggests that introduced populations could be expected for this species as well. In this sense, it can be assumed that the population at Varvara will continue to exist and perhaps even expand its spatial range, since the seashore in this area provides suitable habitats for the species (steep, rocky, and stony slopes).

According to the analyzed color and pholidosis traits of the studied individuals, two morphotypes can be defined, which largely correspond to the diagnostic descriptions of *P. erhardii* and *P. muralis* provided by other authors, and especially by Stojanov et al. (2011): 1) occipital line short, supraciliary lines contrasting and clearly visible (in females), dorsal stripes strongly and evenly mottled with dark spots (in females), ventral side without or with very few spots, massetericum small or absent; 2) occipital line divided into two rows of dots or is absent, supraciliary lines weakly expressed (in females), th dorsal stripes weakly spotted (in females), ventral side with few or many spots, especially on the throat, massetericum clearly noticeable. The first morphotype includes all individuals with *P. erhardii* mtDNA except #12 (it has a clearly visible massetericum), and the second includes all individuals with *P. muralis* mtDNA except #10 (it has a short occipital line, contrasting supraciliary lines, evenly striated with darkly spotted dorsal stripes, no spots on the ventral side, and no massetericum, i.e. completely falls into the first morphotype) and #5 (it has no dark spots on the ventral side). These three exceptions could be explained in one of two ways. First, the defined morphotypes are not strictly specific for the two species, meaning that virtually all diagnostic traits for distinguishing the two taxa in external morphology are uncertain due to the extensive variation. Second (and more likely), as all three individuals are from the site of Varvara (south)[the only one where both haplotypes were found], it can be assumed that the morphotype-genotype discrepancy is the result of hybridization, but this cannot be proven by the methods used here.

In addition, we should highlight the very high genetic similarity found between our individuals with mtDNA of *Podarcis muralis* (haplotype PodB) and *P. muralis* from the following sites: Ropotamo (Bulgarian Black Sea coast), Byala Voda (Strandzha Mts., SE Bulgaria) (Jablonski et al. 2019), Kapakli and Pinarozu (NW Turkey) (Salvi et al. 2013). These populations form a clade clearly separated from the other *P. muralis* haplotypes included in our analysis (the sequences from other parts of Bulgaria and those from Romania). This is in agreement with the proposed hypotheses of multiple independent Pleistocene refugia in the southern Balkans (Salvi et al. 2013). However, more comprehensive research is needed to clarify if the populations of *P. muralis* from the southwestern coast of the Black Sea represent a separate taxon.

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+ Appendix 1 and Appendix 2

Appendix 1.

List of species localities and Genbank accession numbers of the previously published cytochrome b sequences used in this study.

Species	Locality	Isolate	Accession number Cytb	Source
Podarcis erhardii	Greece: Grevena	Pe4	AY896059	Poulakakis et al. 2005
Podarcis erhardii	Greece: Thessalia	Pe22	AY896061	Poulakakis et al. 2005
Podarcis erhardii	Serbia: Makrovi[1]	Pe40	AY896073	Poulakakis et al. 2005
Podarcis erhardii	North Macedonia: Tetovo[1]	Pe33	AY896075	Poulakakis et al. 2005
Podarcis erhardii	Greece: N Sporades	Pe30	AY896091	Poulakakis et al. 2005
Podarcis erhardii	Cyclades	Pe138	AY896099	Poulakakis et al. 2005
Podarcis muralis	Romania: Closani	PM6	ON666679	Oskyrko et al. 2022a
Podarcis muralis	Romania: Dubova	R017	ON666669	Oskyrko et al. 2022a
Podarcis muralis	Romania: Dubova	R016	ON666668	Oskyrko et al. 2022a
Podarcis muralis	Romania: Otopeni	PBuc5	ON666651	Oskyrko et al. 2022a
Podarcis muralis	Romania: Vale Mraconia	ADN0166a	ON666645	Oskyrko et al. 2022a
Podarcis muralis	Romania: Caransebeş	ADN0165	ON666644	Oskyrko et al. 2022a
Podarcis muralis	Bulgaria: Karlukovo	BGPm018	ON666634	Oskyrko et al. 2022a
Podarcis muralis	Bulgaria: Gabrovitsa	BGPm003	ON666630	Oskyrko et al. 2022a
Podarcis muralis	Turkey: Pinarozu	DB16341	KF372271	Salvi et al. 2013
Podarcis muralis	Bulgaria: Ropotamo[2]	612BG	MG851963	Jablonski et al. 2019
Podarcis muralis	Bulgaria: Byala voda[2]	838_BG	MG851964	Jablonski et al. 2019
Podarcis muralis	Turkey: Kapakli[2]	DB16307	KF372265	Salvi et al. 2013
Podarcis muralis	Turkey: Kapakli[2]	DB16310	KF372264	Salvi et al. 2013
Podarcis cretensis	Greece: Crete	Pe61	AY896054	Poulakakis et al. 2005
Podarcis cretensis	Greece: Crete	Pe65	AY896057	Poulakakis et al. 2005
Podarcis peloponnesiacus	Greece: Erymanthos Mt.	Ppel396	KX657922	Psonis et al. 2017

[1] Sequence identical to Greece: Grevena

[2] Sequence identical to PodB from Bulgaria

Appendix 2.

Morphological characterisation of the studied individuals [ID = individual number (corresponds to the ones in Fig. 1, 3 and 4); Haplotype: PodA = *P. erhardii* mtDNA; PodB = *P. muralis* mtDNA]: (A) values of the measured metric and meristic traits [Lcorp = body length; Lcd = tail length; Lcap = head length; Latcap = head width; PA = length of the fore limb; PP = length of the hind limb; Sq = number of dorsal scales at the midbody; V = number of transverse rows of ventral scales; Pf = number of the femoral pores] and (B) values of the calculated morphometric indices.

A

ID	Haplotype	Sex	Lcorp	Lcd	Lcap	Latcap	PA	PP	Sq	V	Pf
1	PodA	F	59	106	12.68	7.94	23	37	60	26.5	22
2	PodA	F	75	104	14.48	8.83	23	36	53	28	20
3	PodA	F	72	n/a	13.89	8.87	22	36	70	27	23
4	PodA	F	69	124	13.22	8.72	32	36	57	27.5	21
5	PodB	M	73	30x	15.55	10	25	38	66	25	23.5
6	PodB	F	66	119	12.35	7.79	20	31	66	26	21
7	PodB	M	65	130	14.62	8.84	24	37	63	24	18.5
8	PodB	F	58	102	10.89	6.9	19	28	69	26	18.5
9	PodB	F	56.5	102	10.4	6.86	18.5	29	60	26.5	17.5
10	PodB	F	71	119	13.69	8.72	23	36	68	28	19.5
11	PodA	F	55	n/a	10.73	6.99	19	32	64	27.5	22
12	PodA	M	70	113	15.31	8.74	26	42	68	26	21
13	PodA	F	72	120	13.26	7.94	23	37	60	28.5	n/a
14	PodB	M	58.5	96	11.39	6.84	19	30	61	26	19.5
15	PodB	F	67	n/a	12.74	7.75	22	33	60	28	20.5

B

ID	Lcap/Lcorp	Latcap/Lcorp	PA/Lcorp	PP/Lcorp	Latcap/Lcap	PA/PP
1	0.215	0.135	0.390	0.627	0.626	0.622
2	0.193	0.118	0.307	0.480	0.610	0.639
3	0.193	0.123	0.306	0.500	0.639	0.611
4	0.192	0.126	0.464	0.522	0.660	0.889
5	0.213	0.137	0.342	0.521	0.643	0.658
6	0.187	0.118	0.303	0.470	0.631	0.645
7	0.225	0.136	0.369	0.569	0.605	0.649
8	0.188	0.119	0.328	0.483	0.634	0.679
9	0.184	0.121	0.327	0.513	0.660	0.638
10	0.193	0.123	0.324	0.507	0.637	0.639
11	0.195	0.127	0.345	0.582	0.651	0.594
12	0.219	0.125	0.371	0.600	0.571	0.619
13	0.184	0.110	0.319	0.514	0.599	0.622
14	0.195	0.117	0.325	0.513	0.601	0.633
15	0.190	0.116	0.328	0.493	0.608	0.667