SALAMANDRA **61**(3): 299–306
15 August 2025 │ ISSN 0036–3375

SALAMANDRA
German Journal of Herpetology

An unexpected discovery behind an old mistake: a relict evolutionary lineage of Wall Lizards (Sauria: Lacertidae) on a remote Aegean islet

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Received: 27 November 2024
Accepted: 21 May 2025 by Oliver Hawlitschek

Abstract. The Balkan Wall Lizards are divided in two phylogroups, the "*Podarcis tauricus*" and "*P. erhardii*" species complexes. Both include cryptic species that were recently discovered in maternal molecular phylogenies and have undergone taxonomical rearrangements. The central Aegean islands host two of these species, one from each phylogroup, that present completely allopatric geographic distributions; the endemic *P. milensis* is found on the Milos island-group, while almost all other Cycladic islands are home to *P. erhardii*. Velopoula, a small isolated islet, lies at the westernmost edge of the Milos island-group and for the past many decades has been considered part of the *P. milensis* distribution. In this study, we collected DNA sequence data from the lizards of Velopoula, to discover that they, in fact, belong to *P. erhardii*. We built an updated, time-calibrated mitochondrial phylogeny of *P. erhardii*, including specimens from the islet and unrepresented regions of the neighboring mainland, in order to evaluate the phylogenetic position of the Velopoula population and infer its origin and diversification history. According to our results, this population represents a highly divergent lineage that split at the Miocene/Pliocene boundary. This rejects the hypothesis of a recent colonization of Velopoula from another insular or mainland *P. erhardii* population and provides evidence for a new, relict taxon endemic to this islet. Through a thorough bibliographic search, we retrace the mistaken taxonomic assignment of the Velopoula Wall Lizards and discuss their assignment as a new potential species within the "*P. erhardii*" species complex.

Key words. Squamata, Aegean islands, Balkan Peninsula, molecular systematics, *Podarcis* lizards, molecular clock, species delimitation.

Introduction

Wall lizards of the genus *Podarcis* are the predominant reptile group of Southern Europe (Arnold & Ovenden 2002) and have served as an ideal model for phylogeographic studies in this region (Camargo et al. 2010). The genus includes 28 currently accepted species (Uetz et al. 2024), many of which are endemics of the broader Balkan Peninsula and neighboring islands. The Balkan assemblage of *Podarcis* species forms two large phylogenetic groups: "*P. tauricus*" and "*P. erhardii*" (Poulakakis et al. 2005). During the past two decades, molecular phylogenies, largely based on mitochondrial DNA markers (mtDNA), have revealed paraphylies that contradicted the established taxonomies, but also unveiled hidden diversity in the form of cryptic species for each of these Balkan

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groups (Poulakakis et al. 2003, 2005, Lymberakis et al. 2008, Psonis et al. 2017, Kiourtsoglou et al. 2021). As a result of the still-ongoing taxonomical rearrangements, the "*P. tauricus*" group currently includes *P. tauricus*, *P. melisellensis*, *P. gaigeae*, *P. milensis*, and *P. ionicus*. On the other hand, the "*P. erhardii*" group includes *P. erhardii*, *P. peloponnesiacus*, *P. cretensis*, *P. levendis*, and *P. thais*.

The central Aegean islands, mostly belonging to the Cyclades archipelago, host two of the *Podarcis* species, one from each group, that present completely allopatric geographic distributions (Fig. 1). The Milos Wall Lizard *P. milensis* is found on the Milos group of islands at the southwest part of the Cyclades, specifically the larger islands of Milos, Kimolos, Polyaigos, Antimilos and the islets of Ananes, Gerakunia (or Falkonera) and Velopoula (or Parapola) (Supplementary Fig. S1). Almost all other Cycladic islands and some east Aegean ones are home to *P. erhardii*, with very few exceptions that do not host Wall Lizards at all (Fig. 1). Velopoula is a small (1.85 km^2), uninhabited, rocky islet found between the Cyclades island-group in the east and the Peloponnese in the west (Fig. 1, Supplementary Fig. S1). From a biogeographic point of view, this islet presents several interesting features. It is isolated from the mainland and other islands with a minimum distance of about 35 km and surrounded by very deep sea barriers, as sea depth reaches 250 m in the west and 350 m in the east. Sea level never dropped more than 120 m during the glacial maxima (Perissoratis & Conispoliatis 2003), meaning that the islet (at times a relatively bigger version of it) has been continuously isolated for the past 2.6 My throughout the glacial and interglacial cycles of the Quaternary.

As part of a comprehensive study on the endemic Milos Wall Lizard, we visited all islands and islets of occurrence to collect field observations, ecophysiological data and tissue samples for genetic analyses. Upon our inspection on the site, the lizards of Velopoula stood out with respect to their coloration patterns (Fig. 2); they did not resemble the typical "*milensis*" phenotype that, especially in males, is characterized by black flanks, throat and sides of the head with large light spots (Fig. 1). In fact, they better matched the "*erhardii*" phenotype with light and dark dorsolateral stripes (Fig. 1), despite their overall dusky colored appearance (Fig. 2). Preliminary genetic results showed that the population of Velopoula indeed belongs to the *erhardii* group.

The scope of this study was to evaluate the phylogenetic position of the Velopoula Wall Lizard population, in order to infer its origin and diversification history. For this purpose, we generated DNA sequences of the focal population but also other important unrepresented regions, such as the Peloponnese. We have combined our data with published sequences to build an updated mitochondrial phylogeny of *P. erhardii*. We were bound to utilize only mitochondrial markers, since no nuclear markers have been included so far in this species' published phylogenies. However, mitochondrial markers have proven very useful for phylogeographic reconstructions of Wall Lizards and the delineation of species, especially in the Aegean region.

Materials and methods

A total of 57 *Podarcis* samples (53 ingroup) were included in our analyses, sequenced for two mitochondrial markers, i.e. segments of approximately 500 bps of cytochrome b (cytb) and of 16S rRNA (16S). More specifically, 42 sequences were generated here and 72 sequences were retrieved from GenBank. Sampling localities are shown in Fig. 1, specimen data (working codes, sampling localities, GenBank Accession Numbers and references) and PCR amplification details are given in the Supplementary Tables S1 and S2, respectively. To root the phylogenies, we used *P. peloponnesiacus* and *P. cretensis* (from the *P. erhardii* group), *P. milensis* (from the *P. tauricus* group), and *P. muralis* as the most distant outgroup.

We aligned sequences in ClustalX v.2.0.12 (Larkin et al. 2007) and estimated the genetic divergence for each marker, as uncorrected p-distances among sequences and groups in MEGA7 (Kumar et al. 2016), using default parameters.

The construction of phylogenetic trees was based on three methods: Neighbour Joining (NJ), Maximum Likelihood (ML) and Bayesian Inference (BI). The most suitable models and partitions were selected for each of the different datasets that were used in downstream analyses (Supplementary Table S3). The NJ tree was calculated in MEGA7, based on p-distances, with the statistical support of the nodes tested via 1,000 bootstrap replicates (Felsenstein 1985). Partitioned ML was carried out in IQ-TREE 1.6.12 (Nguyen et al. 2015). Nodal support was tested via SH-aLRT tests with 10,000 replicates (Guindon et al. 2010), 10,000 ultrafast bootstrap alignments (Hoang et al. 2018) and 100 standard bootstraps. Finally, BI was performed in BEAST v1.10.4 (Suchard et al. 2018). Four runs were conducted with a chain length of 5×10^7 iterations, sampling every 5,000 iterations. The independent runs were combined, after a 10% burn-in, to produce a maximum clade credibility tree. The posterior probabilities (pp) were used for nodal support.

To estimate divergence times, we also performed four runs in BEAST with the parameters and approach described above, and under an uncorrelated log-normal relaxed molecular clock with a birth–death speciation prior on cladogenesis. In order to conform to the speciation model, we assembled a reduced dataset that included one sample for each main mtDNA "species" (see results). To delimit "species" we applied the multi-rate PTP (mPTP; Kapli et al. 2017), using the ML mtDNA tree as input. For the clock calibration, we applied the age of the split between *P. cretensis* and *P. peloponnesiacus* (normal distribution; mean value 5.3 Mya, standard deviation 0.1 Mya), which has been used numerous times (e.g. Psonis et al. 2018, Spilani et al. 2019, Kiourtsoglou et al. 2021) and its validity has been confirmed in a recent study that estimated a fossil-calibrated phylogeny of the genus *Podarcis* (Salvi et al. 2021). The same study estimated the age of the most recent common ancestor of the "*P. erhardii*" species complex; hence we used that age and intervals as a secondary calibration point (normal distribution; mean value 8.7 Mya, standard deviation 1.3 Mya).

Results and discussion

Phylogenetic position of Velopoula's Wall Lizards

All our phylogenetic analyses returned trees of similar topology and nodal support, with the Wall Lizards of Velopoula nested within the nominal species *P. erhardii*. Figure 2 presents the ML tree with the statistical support values from all analyses and statistical support methods. *Podarcis erhardii* splits into nine clades, identified as nine "species" by mPTP, each with a distinct geographic distribution. One of them corresponds to the Velopoula population, which is delimited as a distinct mitochondrial "species" under the phylogenetic species concept (see Kapli et al. 2017). This rejects the assumption that Velopoula's population is the result of a recent, even human-mediated dispersal.

Based on the tree topology, the relative branch lengths, the nodal support values, the levels of genetic diversity and the estimated divergence times, the nine clades found within the nominal species *P. erhardii* form three major phylogroups, one of which corresponds to Velopoula. The other two are distributed in the north and central Balkan Peninsula including the Sporades island-group (named here "North", i.e., the northern part of *P. erhardii*'s distribution), and the south Balkan Peninsula and the Aegean islands (named "South", i.e. the southern part of *P. erhardii*'s distribution), respectively (Fig. 2). Published studies have also identified these two major phylogroups before (Poulakakis et al. 2005, Lymberakis et al. 2008, Psonis et al. 2021), albeit with the difference that our geographic sampling shows that the southernmost continental populations (Peloponnese, Attica and south Evvoia; Fig. 1) are

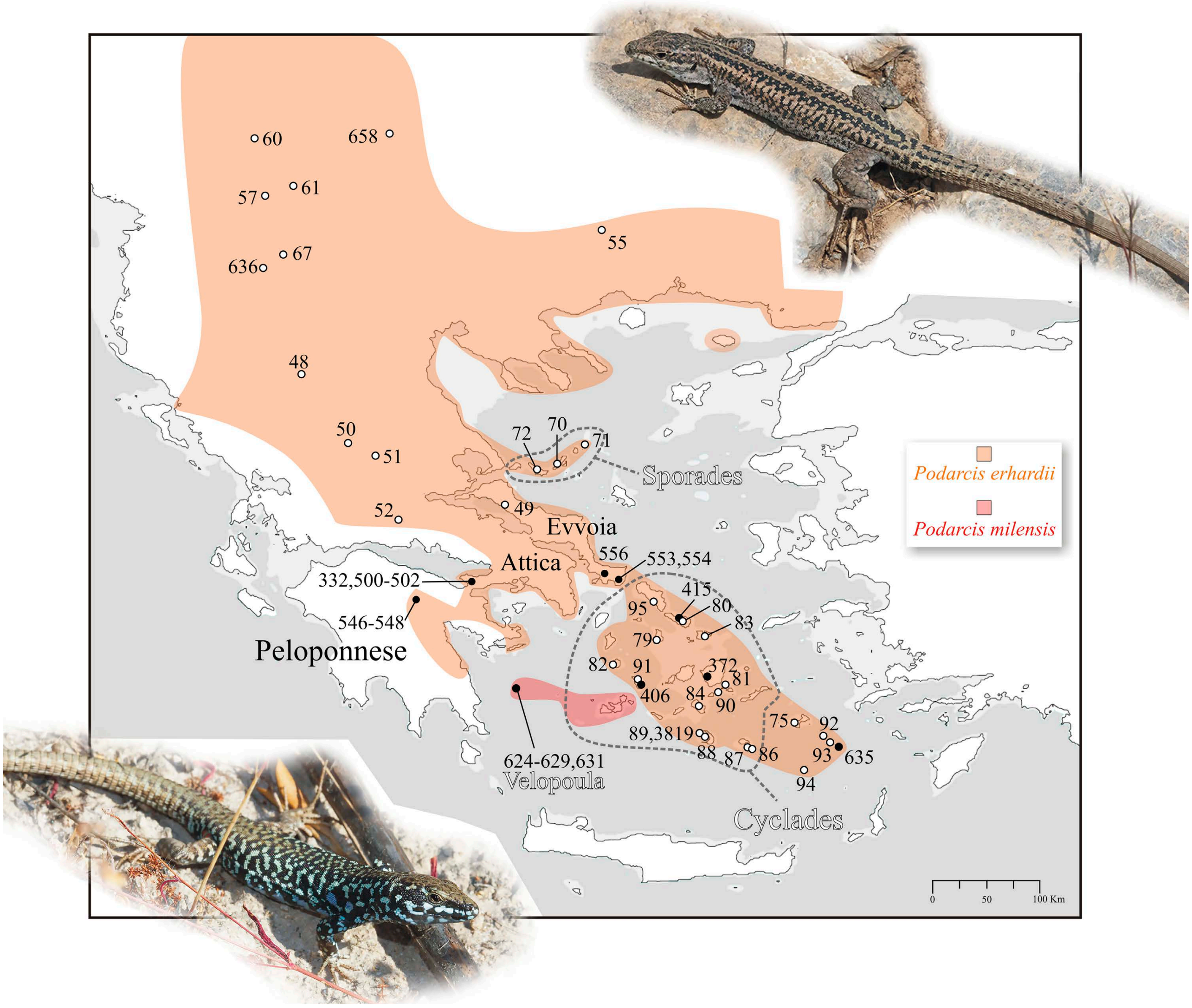

Figure 1. Map showing the geographic distributions of *Podarcis erhardii* and *P. milensis*. Photographs of male representatives of the two species are shown at the upper right and lower left corners, respectively. Circles indicate the sampling localities of the current study (black) or of sequences retrieved from GenBank (white). Numbers refer to specimen codes given in the Supplementary Table S1.

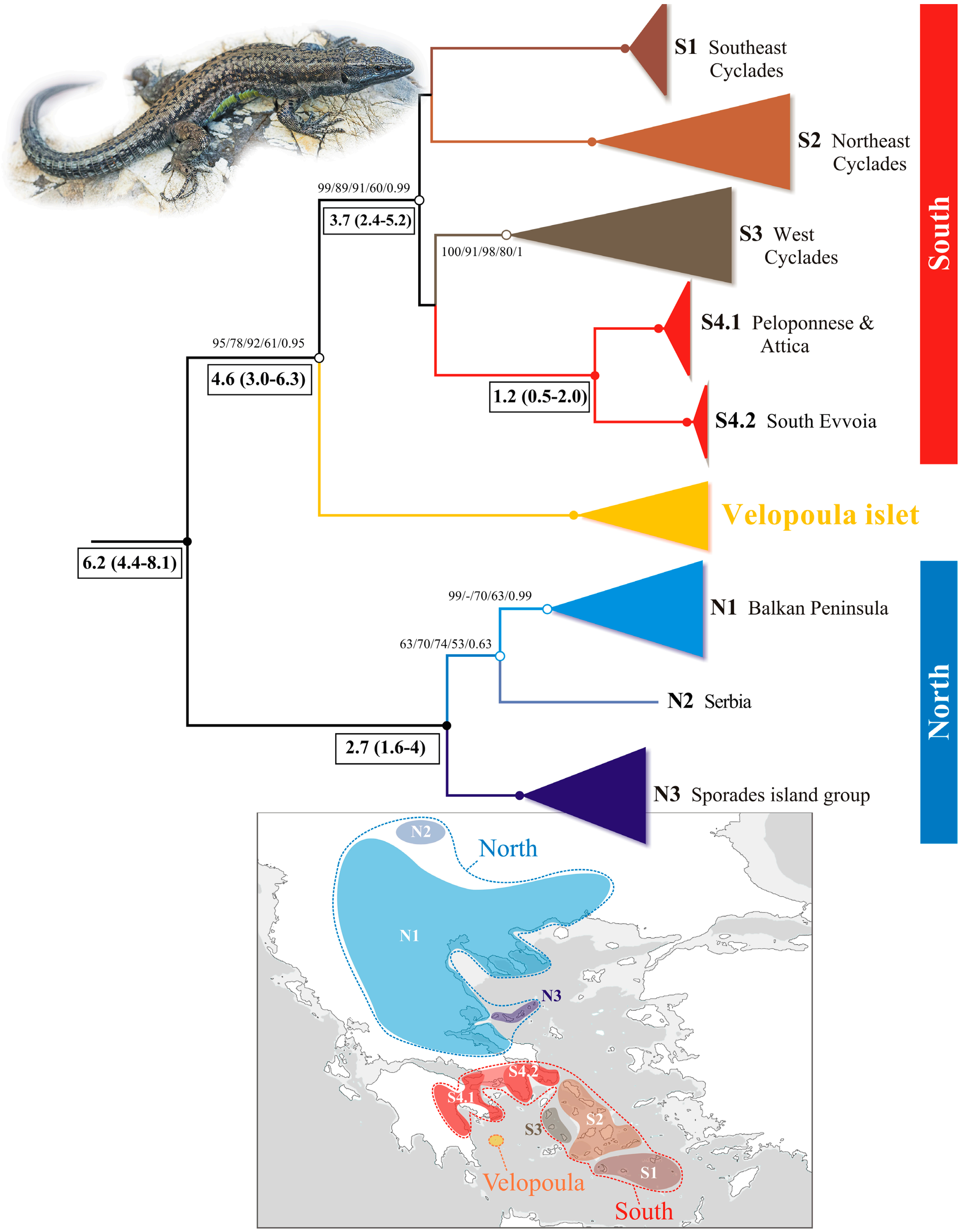

Figure 2. Above: Maximum Likelihood tree, reconstructed with IQ-TREE, based on the mtDNA dataset. The outgroup is not shown and the major phylogenetic clades are collapsed, according to the nine phylogenetic "species" identified by mPTP. Numbers next to the internal nodes are statistical support values: Neighbor-Joining bootstraps/SH-aLRT tests/ultrafast bootstraps/standard bootstraps/posterior probabilities. Nodes with fully colored circles indicate absolute support. Numbers in boxes show the ages of the respective nodes in million years (median and intervals). Clades are color-coded according to their general geographic identity (South: S1–S4; North: N1–N3; Velopoula Isl.). A representative male from the population of Velopoula is shown. Below: Map of the approximate geographic distribution of each clade, with the same color-coding.

not related to the northern ones, but to the Cyclades populations forming the "South" clade. This suggests that the continental populations do not constitute a monophyletic clade. The Velopoula phylogroup is recovered here for the first time and it seems that it is more related to the South group, with medium to high statistical support, depending on the method. The South group includes four distinct clades, three of which are corresponding to the southeast, northeast and west Cyclades, respectively. The fourth one includes populations from the southernmost continental part of *P. erhardii*'s distribution (Peloponnese, Attica and south Evvoia; Fig. 2) and is further split into two subclades, one corresponding to Evvoia and the other one grouping Peloponnese and Attica. The four clades within the South group have unresolved relationships, forming a polytomy. The North group also shows some internal structure with the populations of the Sporades island-group placed in a differentiated clade.

Podarcis erhardii biogeographic history and the origin of Velopoula's lineage

According to our divergence-times estimations (Supplementary Fig. S2), which agree with some published phylogenies (Psonis et al. 2021), the diversification history of *P. erhardii* dates back to the late Miocene or the Miocene/Pliocene boundary (Fig. 2). At that time, we observe a phylogeographic split between the north Balkan and south Balkan distribution of the *P. erhardii* ancestor. At the same period, or shortly after that (early Pliocene), the Velopoula clade also splits from the other south Balkan populations. The statistical support for Velopoula+South is not as robust as other relationships on the tree, while the intervals of estimated times for both events overlap.

During the time from the late Miocene to the early Pliocene, palaeogeographic and palaeoclimatic changes in the area led to a speciation wave within *P. erhardii*. This could be attributed to the general geoclimatic history of the broader Aegean region, but it could more specifically point to the Messinian Salinity Crisis (MSC; 5.96–5.33 Ma), i.e. the dramatic sea level lowering of the Mediterranean basin and its subsequent reflooding (Hsü et al. 1977, Krijgsman et al. 2010). The local events of the MSC had a significant climatic impact coupled with vegetation and habitat turnover (Cerling et al. 1997, Fortelius et al. 2002). These geoclimatic changes have been implicated for major diversification events in the region, especially for reptiles (e.g., Kornilios et al. 2012, Kyriazi et al. 2013, Thanou et al. 2023). Additionally, both the radiation within the "*P. tauricus*" group and among the Peloponnesian *Podarcis*, *P. levendis* and *P. cretensis* have been dated at the Miocene/Pliocene boundary and ascribed to the same events (Psonis et al. 2018, Salvi et al. 2021).

Palaeogeographic reconstructions show that during the Messinian (late Miocene) the southernmost parts of the Balkan region, including the Cyclades, formed a continuous land (Dermitzakis 1990). However, in the early Pliocene, a sea-barrier had formed isolating a landmass in the east connecting present day Cyclades, Attica and south Evvoia from a landmass in the west corresponding to east Peloponnese (Dermitzakis 1990). This west landmass expanded to an area that today includes the Velopoula islet (Anastasakis et al. 2006; see Supplementary Fig. S3). In this context, we may propose that from the late Miocene to the early Pliocene the geographic distribution of the *P. erhardii* ancestor was fragmented into three major parts: one in the north (the ancestor of the North phylogroup), one in the southeast (the ancestor of the South phylogroup) and one in the southwest. The latter was distributed in an area that is now almost totally submerged and the corresponding phylogroup is now almost totally extinct, with the exception of the one relict population of Velopoula.

Podarcis erhardii on Velopoula and the story behind an old mistake

The discovery of *P. erhardii,* instead of *P. milensis*, on the islet of Velopoula was unexpected and puzzling and raised two questions. Is it possible that *P. milensis* does occur on the islet but was not encountered? This is very unlikely due to the number of individuals that we recorded (>100), the total time spent on the islet during two separate visits (≈ 9h) and the number of experienced researchers (8) participating in field work. Additionally, there is not a single island where *P. milensis* and *P. erhardii* co-exist: their distribution in the Cyclades shows that their occurrence is mutually exclusive (Fig. 1). Is it, then, possible that *P. milensis* was indeed the Wall Lizard recorded on Velopoula by researchers in past decades, but was later completely displaced by *P. erhardii* that colonized the islet recently? The resulted phylogeny and the estimated divergence times showed that the unique mito-type found in Velopoula diverged > 3 Mya, therefore this hypothesis is rejected. A thorough search in the literature offers a third possibility.

Müller (1938) mentioned that the Milos Wall Lizard is found on the four larger islands (Milos, Kimolos, Polyaigos, and Antimilos) and examined additional samples from the islet of Gerakunia, which he identified as a separate subspecies of Milos Wall Lizards (*Lacerta erhardii gerakuniae* at the time, *Podarcis milensis gerakuniae* now). The first to ever mention Velopoula with respect to the lizards living there was Wettstein (1953). He proposed that "the Milos archipelago was apparently previously connected to the Peloponnese by a land bridge, on which *L. muralis* [*P. milensis*] reached Milos. It is to be expected that lizards similar to muralis or milensis live on the islands that remain as remnants of this bridge. This has already been confirmed for Gerakunia (…) The islets of Velopoula and Karavi still await research". There is no cited reference to the described geological formations and a palaeogeographic reconstruction of such a land bridge or its remnants is not found in the literature. The sea depths and isobaths cannot support this either. Later on, Buchholz (1962) repeated the same assumption on the existence of a land bridge and its remnant islets and proceeded to declare that "I am of the opinion that the Wall Lizard (…) once populated these islands from the Peloponnese, over the land bridge marked above. The correctness of this assumption is supported by the fact that the lizards of Velopoula and Falkonera (Gerakunia) are unmistakably closely related to those of Milos". Although this is presented as "unmistakable", none of the above mentioned publications or any other publication until the early 1970s had reported a visit to Velopoula or examined specimens from the islet.

This bibliographic entanglement continued when Clark (1972) published a short list of islands and islets and the recorded reptiles, where we read "Island of Velopoula (Parapola), 30 miles S.E. of Spetsai towards Milos in the outlying Cyclades: *Lacerta milensis* [*Podarcis milensis*]". Clark (1972) was the first to actually visit the islet and confirm the presence of a Wall Lizard population, but the entry in his catalogue is not accompanied by any remark, comment, or systematic examination of specimens. To this day, the Wall Lizards of Velopoula were assumed to belong to *P. milensis gerakuniae*, since Velopoula is the next islet east of Gerakunia, regardless of the great distance and deep sea-barriers between them. We strongly believe that Clark and other researchers after him, including ourselves, unreservedly relied on the published knowledge of previous decades. However, we should also note that the Wall Lizard population of Velopoula and especially male individuals are characterized by a darker, sometimes almost monochromatic black coloration, which may be mistaken for the dark morphotype of *P. milensis.*

Taxonomic implications

In agreement with other authors, our results corroborate the status of the nominal species *P. erhardii* as a species-complex, with the two major mitochondrial phylogroups of North and South identified as potential species (Psonis et al. 2021). Here we report a new, third phylogroup restricted on the small islet of Velopoula. Our species delimitation analysis recognized nine clusters within the maternal phylogeny. Clearly, these do not constitute nine different species, but rather a working hypothesis of the maximum number of true species to be tested for validity within *P. erhardii*. At the other end of this spectrum, the minimum number of species within *P. erhardii* is three, corresponding to the three major phylogroups, or two if South+Velopoula is considered a single species. Furthermore, the representation of Velopoula Wall Lizards with nuclear and genomic data is essential prior to any update in the current taxonomy of *P. erhardii,* in order to test for incongruence between maternal and biparental phylogenies and detect possible introgression among the three lineages. For example, contradicting mitochondrial and genomic phylogenies, as well as extensive introgression, were revealed among deeply divergent lineages within the common lizard *P. muralis* (Yang et al. 2021).

This study did not aim and was not designed to provide a thorough taxonomic re-evaluation. However, conclusions can be drawn, when the levels of genetic diversity and the estimated divergence-times are compared to the other Balkan *Podarcis*. The radiation within the *P. erhardii* nominal species is similarly old, or even older than the radiation within the "*P. tauricus*" species-complex (five species) and the radiation among the remaining species of the "*P. erhardii*" species-complex (four species: *P. peloponnesiacus*, *P. cretensis*, *P. levendis*, and *P. thais*), all dated at the Miocene/Pliocene transition or later. The genetic diversity among all clades for both markers is shown in the Supplementary Table S4. The mean p-distance values between Velopoula and all others range between 6.7–9.8% for cytb and 2.2–3.5% for 16S. These values are lower between subclades within the other two phylogroups (North and South). For a comparison, mean cytb p-distance values for pairs of currently recognized species in the same Balkan group are 5.8% between the two Peloponnesian species (*P. peloponnesiacus* & *P. thais*), 7.2% (*P. levendis* & each of the Peloponnesian species), 7.3% (*P. cretensis* & each of the Peloponnesian species), and 8.8% (*P. cretensis* & *P. levendis*). For 16S, the respective values are 3.4%, 3.2%, 4.4%, and 4.7% (Spilani et al. 2019). In this context, the Velopoula clade exhibits values similar or higher than those reported between distinct species of the "*P. erhardii*" species-complex or among species of the other Balkan group, "*P. tauricus*" (Psonis et al. 2017).

The levels of genetic diversity, the divergence times and the isolation of the Velopoula population indicate that this may represent a new species for the genus and for the Greek herpetofauna. This would be the second case of such a narrow endemic species found on a small islet, after the description of *P. levendis* from the much smaller islets of Pori and Lagouvardos (Lymberakis et al. 2008). Besides a more targeted molecular-genetic approach of multiple independent genomic markers and coalescence methods, a thorough morphological analysis will clarify the taxonomic situation within *P. erhardii* and the status of Velopoula's Wall Lizards.

Acknowledgements

The project was supported by the National Environment & Climate Change Agency (N.E.C.C.A.) under the Call "Actions to protect, conserve and promote biodiversity. Field studies of endemic, endangered and nationally important species of Greece" (Project Number: 82538 "An in-depth investigation into the diversity, ecology and conservation status of the Milos wall LiZard – MiLiZ" and 14848 "NISOS – Navigating Aegean Islets: in Search of the MilOs wall lizard Secrets"). Sample collection was conducted with permission from the Hellenic Ministry for the Environment and Energy (permit code 12539/339/08-03-2023).

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Supplementary data

The following data are available online:

Supplementary document 1. Information about analysed specimens.

Supplementary Figure S1. Map of the islands constituting the Milos island-group.

Supplementary Figure S2. Chronogram resulting from the molecular clock analysis.

Supplementary Figure S3. Palaeogeographic reconstructions of the region.

Supplementary Table S1. List of specimens analyzed in this study.

Supplementary Table S2. Primers and PCR conditions.

Supplementary Table S3. Partitions and models in phylogenetic analyses.

Supplementary Table S4. Genetic diversity among phylogenetic clades.