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

Oleksandra Oskyrko[1,2,*], Lekshmi B. Sreelatha[1,12,13], Iolanda Silva-Rocha[1], Tibor Sos[3,4], Sabina E. Vlad[5,6,7], Dan Cogălniceanu[5,6], Florina Stănescu[6,7,8], Tavakkul M. Iskenderov[9], Igor V. Doronin[10], Duje Lisičić[11], Miguel A. Carretero[1,12,13]

[1] *CIBIO Research Centre in Biodiversity and Genetic Resources, InBIO, Universidade do Porto, Campus de Vairão, 4485-661, Vairão, Portugal*
[2] *Department of Zoology, Faculty of Science, Charles University, Vinićná 7, 12844, Prague, Czech Republic*
[3] *Evolutionary Ecology Group, Hungarian Department of Biology and Ecology, Babeş-Bolyai University, Clinicilor Street 5–7, 400006, Cluj Napoca, Romania*
[4] *"Milvus Group" Bird and Nature Protection Association, Tîrgu Mureş, 540445, Romania*
[5] *Faculty of Natural and Agricultural Sciences, Ovidius University Constanța, Aleea Universității 1, Campus - Corp B, 900470, Constanţa, Romania*
[6] *Asociația Chelonia România, 062082, Bucharest, Romania*
[7] *CEDMOG Center, Ovidius University Constanța, Tomis Avenue 145, Constanta, Romania*
[8] *Black Sea Institute for Development and Security Studies, Ovidius University Constanța, 900470, Constanța, Romania*
[9] *Institute of Zoology, National Academy of Sciences of Azerbaijan, AZ-1073, Baku, Republic of Azerbaijan*
[10] *Zoological Institute, Russian Academy of Sciences, Universitetskaya nab.,1, 199034, St. Petersburg Russia*
[11] *Division of Animal Physiology, Department of Biology, Faculty of Science, University of Zagreb, Roosveltov trg 6, HR, 10 000, Zagreb, Croatia*
[12] *Departamento de Biologia, Faculdade de Ciências da Universidade do Porto, R. Campo Alegre, s/n, 4169 - 007, Porto, Portugal*
[13] *BIOPOLIS Program in Genomics, Biodiversity and Land Planning, CIBIO, Campus de Vairão, 4485-661, Vairão, Portugal*
**Corresponding authors*. E-mail: sashaoskirko@gmail.com

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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).

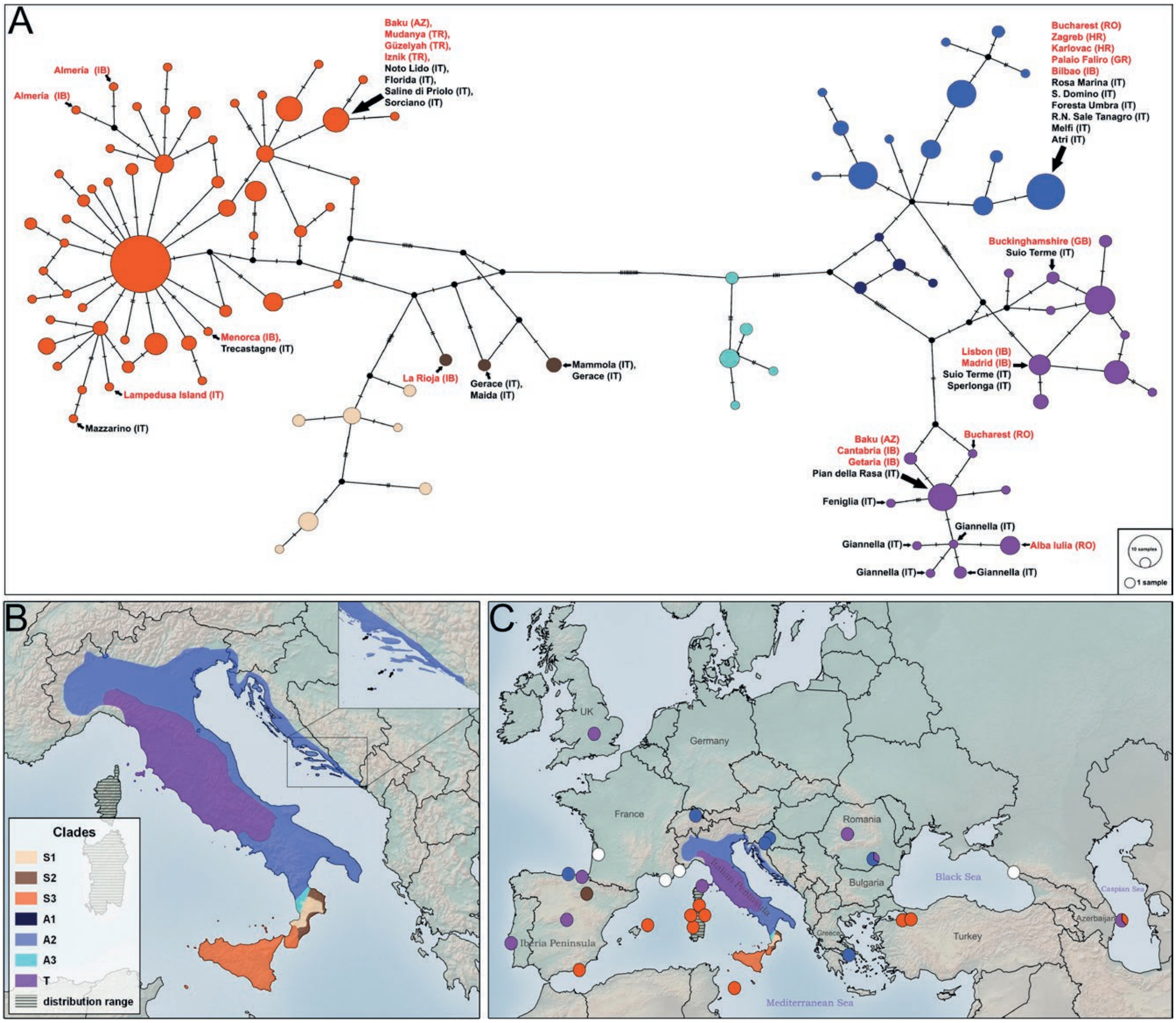

Fig. 1. Network and maps of the natural distribution and introduced populations of *Podarcis siculus* in Eurasia. **A.** Networks of the seven mtDNA clades identified by the phylogeny, colours according to Senczuk et al. (2017). Each circle size is proportional to their frequencies and each filled rectangle 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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