Genetic data reveal a multiple origin for the populations of the Italian wall lizard Podarcis sicula (Squamata: Lacertidae) introduced in the Iberian Peninsula and Balearic islands

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Genetic data reveal a multiple origin for the populations of the Italian wall lizard *Podarcis sicula* (Squamata: Lacertidae) introduced in the Iberian Peninsula and Balearic islands

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Abstract

Biological invasions have become a major cause of biodiversity loss. Tracing the origin of the populations of alien species is essential to infer the dispersal pathway and finally to set conservation policies aimed at preventing new introductions. The Italian wall lizard, *Podarcis sicula*, is one of the reptile species most widely introduced, with allochthonous populations occurring from the United States to Turkey. For some of them, instances of geographic expansion and competition/hybridization with autochthonous *Podarcis* sp. have been indicated. In the Iberian Peninsula, five introduced populations are known: Lisbon (W Portugal), Noja, Cantabria (N Spain), La Rioja (N Spain) and Almería (S Spain) and Sant Celoni, Barcelona (NE Spain) while the species now widely ranges Menorca Island in the Balearics. Here we assess the origin of four populations (Barcelona population will not be included) by comparing in a phylogenetic framework the cytochrome *b* gene sequences of specimens from the introduced and native populations. The results from this study provide evidence for distinct sources, pathways, and timing of introduction in Iberian Peninsula and Balearics by *P. sicula* from Tuscany, Calabria, Sicily, and Sardinia. This finding underpins the fact that *P. sicula* holds considerable potential invasiveness and advises for conservation strategies based on a global and preventive plan for avoiding new introductions as well as on eradication and control measures when prevention fails.

Keywords: Podarcis sicula*, biological invasions, cytochrome b, Iberian Peninsula, Menorca*

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 real epidemic 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 (Brown et al. 2007). Although the effects of an introduction are difficult to predict, several general impacts are now well-known.

In the particular case of reptiles, there is evidence for ecological, evolutionary and social effects, including biotic homogenization, disruptions of food-webs, hybridization, disease, behavioural changes, effects on human health, between other numerous negative impacts (Ficetola et al. 2009; Kraus 2009). Among the ecological effects, the negative impact on native biota is to be remarked. This is the case of the snake *Natrix maura* which is assumed to be responsible for the decline of the Mallorcan midwife toad (*Alytes muletensis*) through the predation of larvae and adults (Guicking et al. 2005; Kraus 2009; Álvarez et al. 2010). An example of negative social effect is the introduction of the brown tree snakes (*Boiga irregularis*) in Guam, which increase the susceptibility of humans to disease and even death (Fritts et al. 1994; Rodda et al. 1997).

The Italian wall lizard, *Podarcis sicula* constitutes an outstanding case of reptile species largely introduced worldwide. Its natural range is restricted to the Italian Peninsula and Sicily. However, an

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introduction of the species either in historical times or even more recently has been postulated for the Tyrrhenian Islands, Corsica and Sardinia, and in the islands and coastal areas of the eastern Adriatic Sea (Corti 2006; Isailovic et al. 2008 in IUCN 2011). Moreover, *P. sicula* is certainly introduced in the Iberian Peninsula, in Cantabria (Meijide 1981), Almería (Mertens & Wermuth 1960), Lisbon (González de la Vega et al. 2001), La Rioja (Valdeón et al. 2010) and nearby Barcelona (Rivera et al. 2011); in Southern France, in Toulon and Château d'If Island (Morgue 1924; Orsini 1984); in Turkey, in Istanbul and Marmara Islands (Basoglu & Baran 1977); in North Africa, in Tunisia and Tripoli (Arnold & Ovenden 2002); and in the United States, in Philadelphia, Kansas, and New York (Conant 1959; Behler & King 1979; Conant & Collins 1991; Deichsel & Miller 2000).

Several ecological and behavioural traits make *P. sicula* a highly effective invader in the majority of the regions where it is introduced. Experimental results have demonstrated behavioural interference with *P. melisellensis* (Downes & Bauwens 2002), which has gone extinct after introduction of *P. sicula* in Adriatic islets (Nevo et al. 1972). Furthermore, hybridization between *P. sicula* and other *Podarcis* species has been demonstrated by means of genetic data at least with the endemic *Podarcis* of Sardinia (*P. tiliguerta,* Capula 2002), of the Aeolian islands (*P. raffonei,* Capula et al. 2002) and of Sicily (*P. wagleriana,* Capula 1993).

Indeed, this species is very eclectic in habitat use being found in natural grassy areas, roadside verges, scrublands meadows and costal dunes, but also in agro environments, inside pine plantations, and associated to parkland urban areas, stone walls and buildings (Capula 1994; Oliverio et al. 2001; Biaggini et al. 2006; Corti 2006). The Italian wall lizard usually uses trees and human manufactures as a refuge, so its accidental transportation by humans and the availability of an anthropised environment are important factors for its spread. The use of planted trees and human manufactures as refuges enhances its accidental transportation while the availability of an anthropic environment seems important for its spread (Corti & Lo Cascio 2002).

This proneness of *P. sicula* both to be dispersed by humans and to produce negative effects on native species makes necessary to look closely for their pattern of invasiveness. By looking separately for each case of invasion we can obtain valuable information for the prevention and management of this biological invasion. Molecular evidence supporting the allochthonous status of *P. sicula* have been provided for populations occurring in United States (Deichsel & Miller 2000; Oliverio et al. 2001) and

Menorca (Podnar et al. 2005) and for the Iberian populations of La Rioja and Sant Celoni, Barcelona (Valdeón et al. 2010; Rivera et al. 2011). However, the origin of most of the introduced populations is still unclear. Though, tracing the origin of the introduced populations and identifying the pathways of their translocations constitute the baseline to forecast the colonization patterns, as well as to prevent new introductions (Pyron et al. 2008; Rodda et al. 2008; Dorcas et al. 2010).

In this work, we use a molecular marker to identify the geographic origin of all the introduced populations of *P. sicula* known from Iberian Peninsula and Balearic islands. We specifically aim to determine whether these geographically separated populations derive from a single event of introduction or if they have multiple origins. By tracing the origin of introduced populations we expect to infer the source(s) and pathway(s) of dispersal, crucial for developing conservation and management policies aimed at preventing negative effect on native species.

Material and methods

Populations sampled

Four introduced populations are known from mainland Iberian Peninsula (Figure 1): Lisbon (western Portugal, González de la Vega et al. 2001), Noja (Cantabria, northern Spain, Meijide 1981), Almería (southern Spain, Mertens & Wermuth 1960) and La Rioja (northern Spain, Valdeón et al. 2010) while the species is widespread in Menorca, Balearic Islands (Pérez-Mellado 2002). Very recently another population was found near Barcelona at Sant Celoni (Rivera et al. 2011). All Iberian localities, but La Rioja and Sant Celoni, are coastal, Lisbon and Almería being strictly urban whereas Noja is a sand dune area surrounded by urban habitat. La Rioja and Sant Celoni populations, located 100 km and 15 km inland respectively, are associated to old olive trees on sale in a plant nursery (Valdeón et al. 2010; Rivera et al. 2011).

Sampling and DNA extraction and amplification

The sampling includes all the Iberian and Balearic localities where the species has been reported to be introduced (Figure 1). Tail tips were collected from 23 samples (2-7 samples from each locality, Table I) and then stored in pure ethanol.

Total genomic DNA was extracted from tail tissue following the standard saline method (Sambrook et al. 1989). A fragment of 684 bp of the mitochondrial gene cytochrome *b* (cyt *b*) was amplified by PCR using the primers GluDG-A and
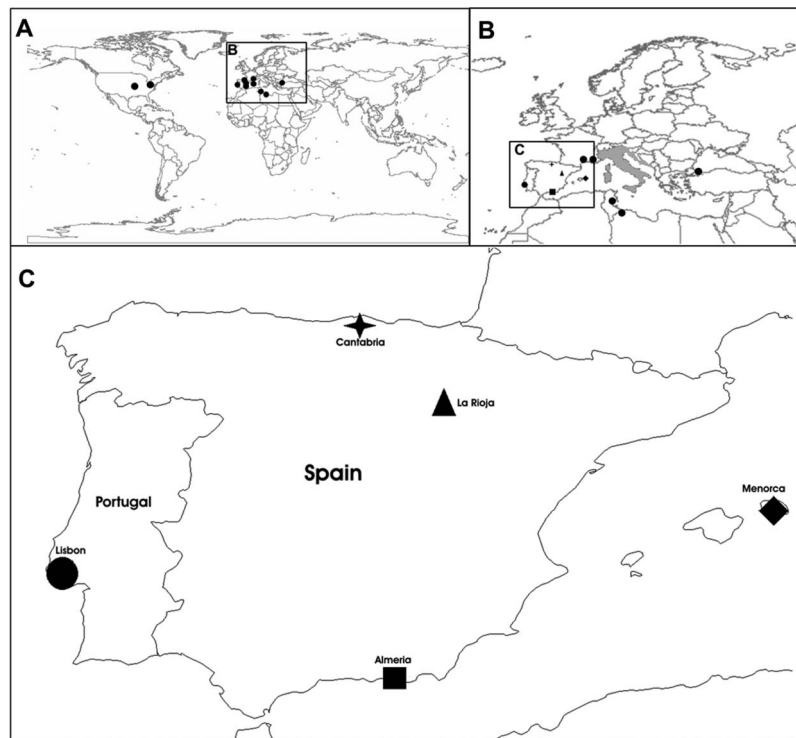
Figure 1. Introduced populations and native range of *Podarcis sicula*. **A**, introduced populations in the World – United States, North Africa and Europe (black dots); **B**, introduced populations in Europe (black) and native range (grey); **C**, introduced populations in the Iberian Peninsula and Menorca.

Table I. Iberian localities where the introduced *P. sicula* populations were sampled. Geographic coordinates and sample size are also reported.

	Locality	Country	Coordinates		Sample size
			Latitude	Longitude	
Almería	Parque de Nicolás Salmerón	Spain	36, 8348	2, 4651	2
Cantabria	Playa del Ris (Noja)	Spain	43, 4891	−3, 5326	7
La Rioja	Alfaro	Spain	42, 4891	−1, 7753	2
Lisbon	Gardens of Expo	Portugal	38, 7622	−9, 0945	5
	Es Mercadal		40, 0256	4, 1624	2
Menorca	Ferreries	Spain	39, 9776	3, 9715	3
	Ciutadella		40, 0039	3, 8689	2

cb3H (Palumbi 1991). The amplification conditions consisted of an initial step of denaturing of 3 min at 94°C, followed by 35 cycles of denaturation of 30 s at 94°C, annealing of 30 s at 51°C and extension of 50 s at 72°C and a final extension step of 5 min at 72°C. The PCR products were purified and sequenced by an external service (by the company Macrogen® Korea).

Sequence data and phylogenetic analysis

Mitochondrial cytochrome b sequences from 39 individuals of *P. sicula* from the native range generated by Podnar et al. (2005) were downloaded from GenBank (accession numbers AY185095, AY185094, AY770869–AY77090). Three additional sequences from (*Podarcis muralis* and *P. melisellensis*) were also downloaded from GenBank (accession numbers AY185096, AY185029 and AY185057) and included in the phylogenetic analyses as outgroups (following Podnar et al. 2005). We maintained geographic and haplotype designations as in Podnar et al. (2005), in order to facilitate the comparison between our samples and those from previous studies. These sequences were aligned with those generated in the present study (accession numbers JX072938-JX072960) using the ClustalW software (Thompson et al. 1994).

We carried out a Maximum Likelihood (ML) phylogenetic analysis to infer the relationships between the Iberian and Balearic samples and those from the native range of *P. sicula* analyzed by Podnar et al. (2005). The best model of sequence evolution was estimated by Mega 5 (Tamura et al. 2011) under the Bayesian Information Criterion (BIC) as the model HKY + Gamma (Hasegawa et al. 1985; Yang 1993). The ML tree was performed in Mega 5 with the heuristic search mode and the node support was calculated over 1000 bootstrap replicates. In addition to the tree-building method, we analyzed the genealogical relationships among native and non-native haplotypes by means of a statistical parsimony network using the program TCS 1.21 (Clement et al. 2000).

Results

The final alignment includes 65 sequences and 687 base pairs. A total of 42 haplotypes were identified among which 35 from the sequences from Podnar et al. (2005) and eight from the five Iberian and Balearic introduced populations here studied. In particular, in Almería, La Rioja and Lisbon populations, two different haplotypes each were found, while in Cantabria and Menorca one haplotype occurred.

ML tree shows that lizards from Noja (Cantabria) are closely related to those from the "Tuscany" clade in Podnar et al. (2005) (Figure 2). Also, the lizards from Lisbon cluster together with Tuscanian samples, yet being fairly differentiated from them. Haplotypes from Almería and Menorca are related with those from Sicily and Sardinia and included within the "Sicula" haploclade. Moreover, the five Menorcan samples analyzed in this study showed the same haplotype as the single Menorcan sample analyzed by Podnar et al. (2005). The two haplotypes found in the specimens from La Rioja cluster in a distinct clade, which is related to the "Monasterace" (Southeast Calabria) and "Sicula" haploclades. All the relationships above reported were highly supported by bootstrap analysis with BP values ranging from 86 to 99. Based on these relationships, the putative origin of each introduced population is discussed in the next section and depicted in Figure 3.

The network analysis shows 11 distinct networks and the maximum number of mutational steps allowing for a 95% parsimonious connection between haplotypes was estimated to be 11 (Figure 4). The relationships between haplotypes as depicted by the networks corroborate the results from ML analysis. Cantabria lizards cluster together with those from Tuscany, Almería and Menorca samples group with samples from the Sicula clade, and La Rioja and Lisbon samples constitute two independent groups.

Discussion

Origin, pathways and timing of the introduction

Phylogenetic analysis clearly indicates that the introduced Iberian and Balearic populations of *P. sicula* are related to native populations from distinct Italian regions, and not from other non-native (Iberian) populations. In turn, this result suggests multiple and independent introductions of the Italian wall lizard in the Iberian Peninsula and Menorca (Figure 3).

As a whole, *P. sicula* from Ibero-Balearic localities clustered in two principal haploclades recovered by Podnar et al. (2005), the Tuscany haploclade and the Sicula haploclade, which cluster haplotypes belonging to the *P. s. campestris* and the *P. s. sicula+P. s. cetii* subspecies respectively. On the other hand, haplotypes found in the population from La Rioja constitute an independent haploclade and are not included in any of those reported by Podnar et al. (2005).

The Italian wall lizards occurring in Lisbon and Cantabria are likely originated from a Tuscan stock. In particular, Cantabrian lizards seem to be introduced from the Tuscany itself, and perhaps from Florence area since their haplotypes differ in only one-two nucleotide position from the haplotypes found by Podnar et al. (2005) in the native populations from Florence. On the other hand, the lizards sampled in Lisbon were probably introduced from a proximate area to Tuscany as they are closely related to the lizards form Tuscany but fairly differentiated from them (Figure 2).

Both Almería and Menorca lizards are related to the Sicilian and Sardinian populations of *P. sicula*. In both cases, it is difficult to disentangle whether the origin of these allochthonous populations is from Sicily or Sardinia, or even if Almería population derives from the Menorcan stock. Indeed, according to Podnar et al. (2005), the Sardinian samples (assigned to the subspecies *P. s. cetti*) only differ from the Sicilian samples (assigned to the subspecies *P. s. sicula*) by three substitutions in a gene fragment of 687 nucleotides, which suggest and support the hypothesis of a historical colonization of Sardinia from Sicily (Lanza 1982). Thus, Menorcan samples could have been originated either from Sicily as suggested by Greca and Sacchi (1957) or from Sardinia as suggested by Müller (1905) and Eisentraut (1950). Although Podnar et al. (2005) would support the hypothesis of an introduction
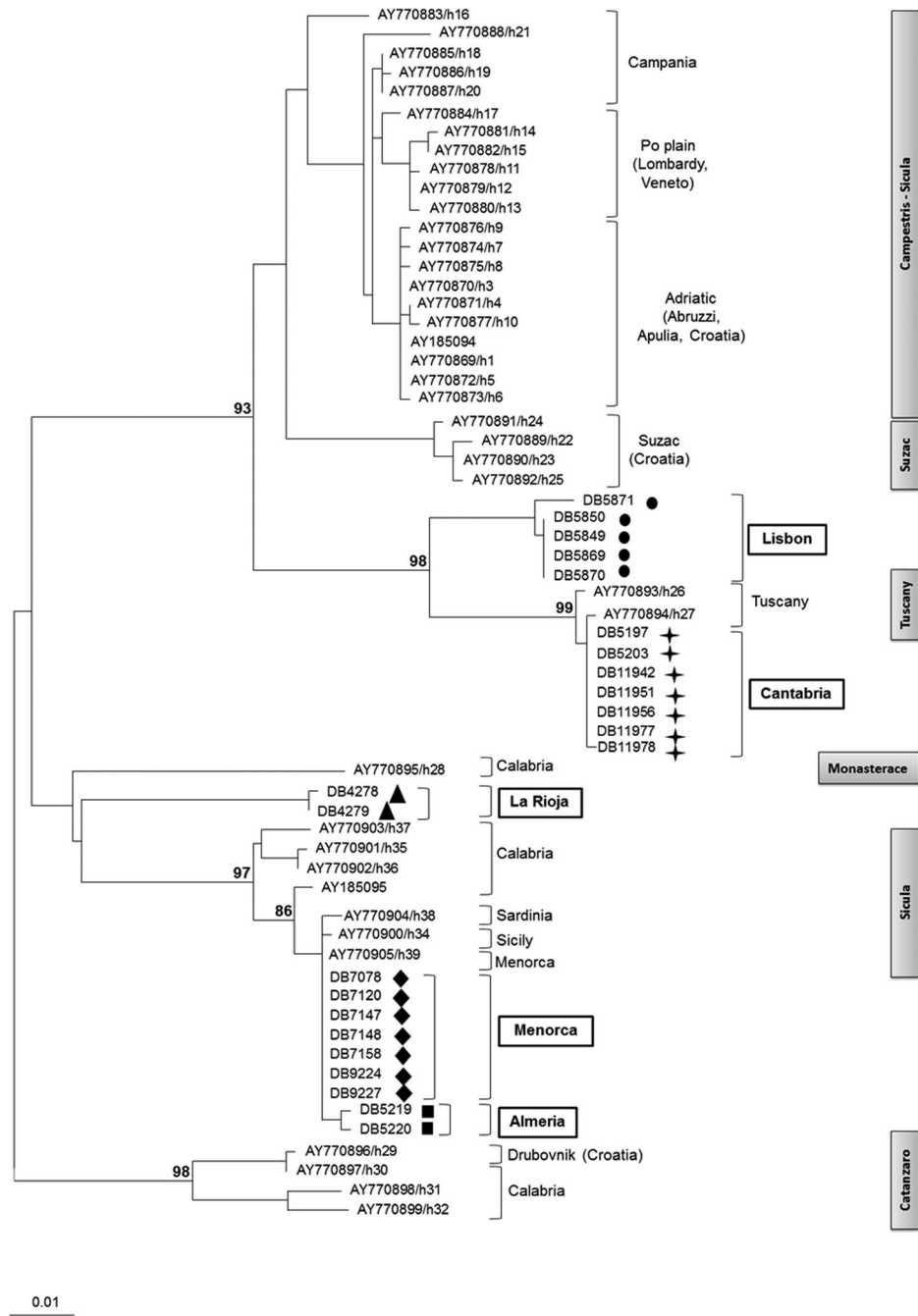
Figure 2. ML phylogenetic tree depicting the relationships between cytochrome b, haplotypes from native *Podarcis sicula* populations (Podnar et al. 2005) and those from the Iberian and Balearic introduced populations: Almeria, Cantabria, La Rioja, Lisbon, and Menorca. Specimens' localities are reported along with *P. Sicula* haploclades (grey boxes) as in Podnar et al. (2005). Bootstrap support is indicated above the nodes of interest.

from Sicily as Menorcan haplotypes are some-what closer to the Sicilian than to the Sardinian haplotypes (as the nucleotide differences between Menorcan samples and Sicilian/Sardinian samples are 2/3 respectively), the gene fragment they used does not have enough resolution for supporting this statement and the haplotype comparison is based

only on single specimens from Menorca, Sicily and Sardinia. Additionally, the morphological inspections of Menorcan lizards (unpublished data from the authors) show a perfect match with the *cetii* morpho-type occurring in Sardinia rather than with the *sicula* morphotypes observed in Sicily. Thus, taking into account this evidence, we would tentatively suggest
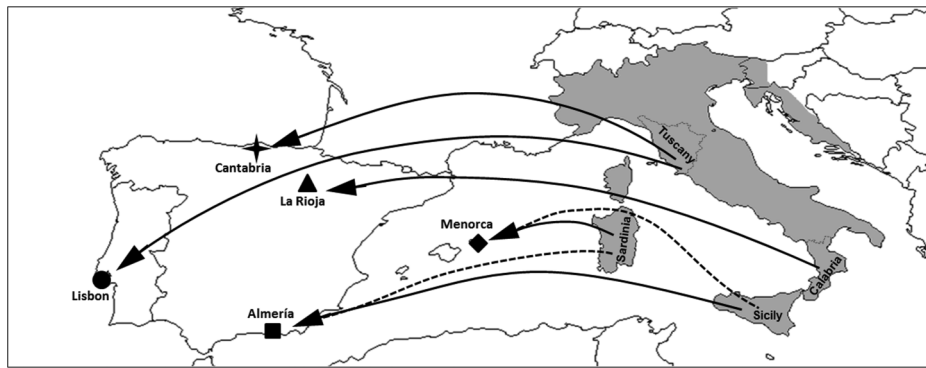
Figure 3. Origin of the Iberian and Balearic populations of *Podarcis sicula* as inferred from genetic analyses.

a Sardinian origin for the Menorcan population of *P. sicula*, although the large spectrum of morphological variation of *P. sicula* advices for caution in the use of morphological diagnosis when inferring the origin of populations. Following a similar reasoning and based on morphological data, a tentative hypothesis of a Sicilian origin can be drawn for the lizards introduced in Almería.

Lizards from La Rioja constitute an independent clade, which is related to Monasterace and Sicula haploclades of Podnar et al. (2005), although well differentiated from them. This result only partially agrees with those reported by Valdeón et al. (2010), who also found La Rioja samples to be related with Catanzaro, Monasterace, and Sicula haploclades, but with a closer relationship with the former rather constituting an independent clade. However, it is important to notice that given the high evolutionary rate of the mitochondrial gene fragment analysed in this study, it provided a higher resolution than those used by those authors (12S and 16S genes combined). This evidence suggests not only that the source population of La Rioja would be located in an area close to Calabria (and perhaps in between Monasterace and Reggio Calabria), but also that the genetic diversity of *P. sicula* in the southern portion of its range has not been completely sampled, yet.

The pathways of introduction are known for some of the studied populations. Evidence for an anthropogenic introduction through the trade of old olive trees from southern Italy to Spain has been reported by Valdeón et al. (2010) and Rivera et al. (2011) for La Rioja and Sant Celoni populations respectively. Lisbon population appears to be introduced through the transport of several materials and ornamental plants during the Expo'98 event (González de la Vega et al. 2001). As already mentioned, the Italian wall lizard often uses trees and human manufactures as refuge and thermoregulatory

site. Similarly the pathways of introduction for the other Iberian populations could have followed the human translocations of cargo in the maritime trade. Both Almería and Cantabrian populations maintained a route of maritime traffic with Italy during the Spanish Civil War between 1936 and 1939 (Rivera & Arribas 1993). Noja population has not a seaport, but may derive from the already extinct Santander population, which was closer to a seaport (Pleguezelos 2004). So it is possible that the primary pathway of introduction in Cantabria region was also derived from the development of maritime trade.

Even if based on circumstantial historical evidence, the timing of these introductions seems different from case to case. Noja (Cantabria) and Almería populations are considered to be introduced in the late XIX century or early XX century when an intensification of maritime traffic occurs (Mertens & Wermuth 1960; Meijide 1981; Henle & Klaver 1986; Olmedo 1997). Lisbon, La Rioja and Sant Celoni populations would be even more recent due to ornamental plants transportation (end of 90s and during the 2008/2009 period respectively; González de La Vega et al. 2001; Valdéon et al. 2010; Rivera et al. 2011). Finally, the origin of Menorcan population is probably dated to a more ancient time, likely back to the Middle Age (Mayol 1985) when the traffic between Italian Republics and Balearic Islands was intense, as the species is widespread across the entire islands (Pleguezuelos 2004).

Conservation implications

The results from this study provide evidence for distinct sources, pathways and timing of introduction in Iberian Peninsula and Menorca by *P. Sicula* from the central and the southern portion of its native distribution range. This evidence is crucial to design conservation strategies.
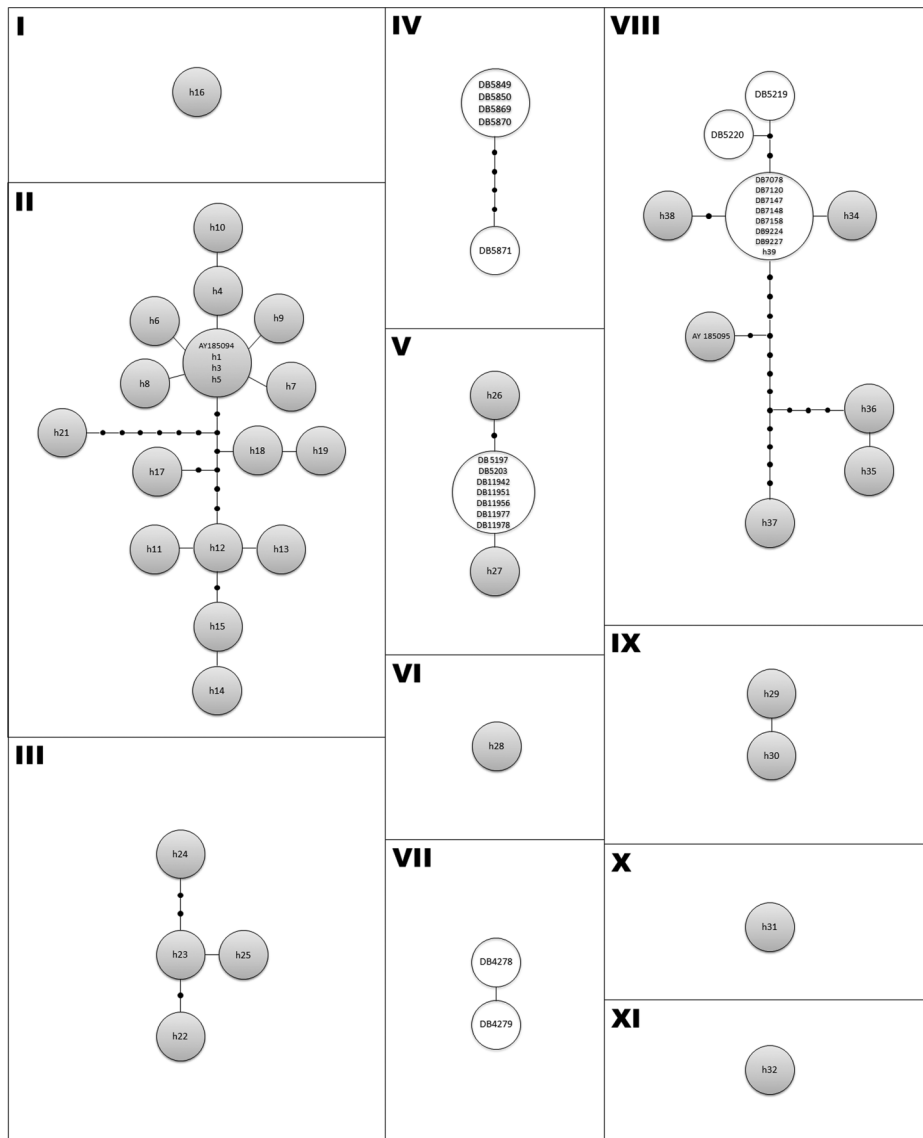
Figure 4. Statistical parsimony haplotype network depicting the genealogical relationships between native and non-native *P. sicula* haplotypes. See Table I and Figure 2 for details on sampling localities and haplotypes codes. Circle size is proportional to the number of samples with the same haplotype. Black circles represent missing haplotypes. The eleven networks include samples from: (I) Campania - *Campestris Sicula clade*; (II) Po Plain + Campania + Adriatic – *Campestris-Sicula clade*; (III) Suzac (Croatia) - *Suzac clade* (IV); Lisbon (non-native); (V) Cantabria (non-native) + Tuscany - Tuscany clade; (VI) Calabria - *Monasterace clade*; (VII) La Rioja (non-native) (VIII) Almería (non-native) + Menorca (non-native) + Sardinia + Sicily + Calabria - *Sicula clade*; (IX) Drubovnik (Croatia) - *Catanzaro clade*; (X) Calabria - *Catanzaro clade*; and (XI) Calabria - *Catanzaro clade*.

If introduction would come from a single source, conservation strategies would involve controlling in the original region/locality, which could be a feasible target. Since, in contrast, multiple independent events of introduction have been found here, a realistic conservation planning should be more global and preventive, addressed to the detection and control of alien lizards when arrived. The prevention of the introduction is, in fact, the most effective and cheaper way to avoid it and can be done by implementing inspection and quarantine measures of the

commercial cargo and trade, since these two means are the main pathways of introduction.

When the prevention fails, an eradication and control program should be implemented, paying attention to both biological data, population size and distribution, and specific educational effort for the general public are needed for such programs to be successful. Introduced populations of *P. sicula* have been successfully eradicated from La Rioja (Valdeón et al. 2010) and Mallorca (Pinya & Carretero 2011; Mateo JA, pers. comm.) and it could be possible also

for the Noja (Cantabria) population as its presence is localized. Conversely, the populations in Almería and Lisbon have already reached a size, which makes the full eradication not feasible (Pleguezelos 2004; Loureiro et al. 2008; personal observations), thus control measures should be taken to keep the population size stable and avoid its expansion. In the case of Menorca, *P. sicula* is naturalized since a long time, being nowadays the dominant species in the main island, so it is almost impossible to take an effective action. However, a substantial effect must be kept to prevent translocations toward the small surrounding islets of Menorca (as well as to other Balearic Islands) where it could be harmful for the autochthonous and endangered *Podarcis lilfordi* (Pinya & Carretero 2011).

Finally, on a general ground besides investigating the origin and the pathways of the introductions, resources have to be invested also for collecting more data about: (i) the current demographic status and spatial distribution of the introduced populations; (ii) the factors limiting or promoting the successful establishment after of the introduction and expansion (adopting Species Distribution Models approach to predict the invasion risk (Schulte et al. 2011)), as well as on (iii) the impacts of this species on native lizards. The integration of all this information would allow a deep comprehension and a successful management of this biological invasion.

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