Conserv Genet (2014) 15:295–304
DOI 10.1007/s10592-013-0538-z

RESEARCH ARTICLE

Genetic diversity and historical biogeography of the Maltese wall lizard, *Podarcis filfolensis* (Squamata: Lacertidae)

Virginia Rodríguez · Richard P. Brown · Bàrbara Terrasa · Valentín Pérez-Mellado · Antònia Picornell · José A. Castro · Cori Ramon

Received: 28 May 2013 / Accepted: 30 September 2013 / Published online: 16 October 2013

Abstract *Podarcis filfolensis* is an endemic lizard from the Maltese archipelago. There is evidence of human-mediated decline and even extirpation of some insular populations of this species. However, information about the intraspecific genetic diversity and phylogeographic patterns of this species is limited. Here we analyze genetic markers from a multi-locus dataset (mtDNA, 2,533 bp; nuclear *c-mos* gene, 353 bp; 11 microsatellites) for individuals from extant populations of *P. filfolensis*. Despite generally low genetic variability, two main mitochondrial groupings were clearly identified. In general, individuals from the main island of Malta were genetically distinct from those from Gozo, Comino, Cominotto and Small Blue Lagoon Rock, and also from Linosa and Lampione individuals. Three genetic clusters were detected based on microsatellite data: one was found at higher frequency on Malta, while the other two included samples from the remaining islands, showing some concordance with the mtDNA pattern. A time-calibrated Bayesian tree for the principal mitochondrial lineages indicated strong statistical support for two *P. filfolensis* lineages that originated in the Pleistocene (105.4–869 Ka). We show that these lineages largely meet the criteria for recognition as evolutionary significant units despite some recent admixture (possibly due to recent translocations between islands). Human disturbance, low genetic variability, evidence of bottlenecks and extirpation on one island indicate that a thorough review of the current conservation status of *P. filfolensis* would be timely.

Keywords Conservation · Maltese archipelago · Microsatellites · Mitochondrial DNA · *Podarcis filfolensis* · Species tree

V. Rodríguez · B. Terrasa · A. Picornell · J. A. Castro · C. Ramon (✉)
Laboratori de Genètica, Departament de Biologia, Universitat de les Illes Balears, Ctra Valldemossa km 7.5, 07122 Palma de Mallorca, Spain
e-mail: cori.ramon@uib.es

R. P. Brown
School of Natural Sciences & Psychology, Liverpool John Moores University, Liverpool, UK

V. Pérez-Mellado
Departamento de Zoología, Universidad de Salamanca, Salamanca, Spain

Introduction

A variety of man-mediated effects have had major impacts on patterns of species richness and biogeography of islands. In several Mediterranean islands, the ongoing decline and extinction of reptile species is directly related to human influence (Ficetola and Padoa-Schioppa 2009). This appears to be the case in the Maltese archipelago (Schembri 1993; Cassar et al. 2008). Unrelenting anthropogenic pressures are severely affecting the biodiversity of the Maltese islands, with the Island Directory from the United Nations Environment Program rating Malta as being 4th (of 234 islands assessed) for human impact (http://islands.unep.ch/Tihi.htm) and 3rd for terrestrial conservation importance (http://islands.unep.ch/Tici.htm).

The Maltese archipelago consists of three main islands: Malta, Gozo and Comino and a number of small uninhabited islets: Cominotto, Filfla, St. Paul's Island, Fungus Rock (General's Rock) and a few minor rocks (Schembri

1994). These islands are part of the Pelagian Platform (Micallef et al. 2012), a large area located between Sicily and North Africa, and emerged 7–5 Ma ago as a result of tectonic plates collision. The Maltese region would have been colonized by species from both Europe and Africa (Cassar et al. 2008) as a consequence of the drying out of the Mediterranean basin during the Messinian Salinity Crisis (MSC) 5.33 Ma ago (Krijgsman et al. 1999) when terrestrial organisms would have been able to migrate to the area. At the end of the MSC, the Mediterranean basin reflooded and the Maltese islands were isolated, although some reconnections with Sicily were occasionally re-established during Pleistocene glaciations (Thake 1985; Hunt and Schembri 1999). Sicily and the Maltese islands were last joined during the last glacial maximum (MIS 2, 24 Ka), when sea levels dropped 120–130 m. Within the Maltese islands, the largest islands of Gozo and Malta only recently became fully isolated 7.2–7 Ka (Furlani et al. 2013). Progressive colonization of the islands during historical changes in sea–level seems to have led to the current biota, a similar scenario to other Mediterranean islands and species (Podnar et al. 2005; Brown et al. 2008; Agulló et al. 2011; Bauza-Ribot et al. 2011).

The Maltese wall lizard, *Podarcis filfolensis* (Bedriaga 1876), is a medium-sized lizard endemic to the Maltese islands (Savona Ventura 1983; Corti and Lo Cascio 1999; Schüter 2005). The species is also believed to have been introduced into two of the Pelagian Islands (situated in the Channel of Sicily), Linosa and Lampione, although it is not clear when this introduction took place (Corti and Lo Cascio 1999; Capula 2006; Lo Cascio and Corti 2008). *P. filfolensis* is one of the least-studied species of the genus. Five subspecies have been recognized: four of them in the Maltese islands, *P. f. maltensis* on Malta, Gozo and Comino islands; *P. f. kieselbachi* on St. Paul's Island; *P. f. filfolensis* on Filfla Island and *P. f. generalensis* on Fungus Rock, and one subspecies in the Pelagian Islands, *P. f. laurentiimuelleri* (Scalera et al. 2004; Sciberras and Schembri 2008). Specimens from Cominotto were also considered as a new subspecies by Savona Ventura (1983), but he did not described them formally. The named subspecies differ mainly in mean body size and coloration, especially with respect to the gular region of males and the degree of dark markings on the black, flanks and ventral region of the neck (Sciberras and Schembri 2008).

Like many lacertids from Mediterranean islands, *P. filfolensis* was formerly very common throughout the Maltese islands; including Malta and Gozo (see, for example, Despott 1915). Today, however, the species has a patchy distribution on Malta and Gozo, and is common only on some coastal islets (pers. obs.). Maltese wall lizards currently face several threats. On Cominotto, lizards are frequently predated by *Chalcides ocellatus* and possibly by the recently introduced *Chamaeleon chamaeleon* (Sciberras 2007). Maltese lizards are also predated by birds such as woodchat shrikes (*Lanius senator*), kestrels (*Falco tinnunculus*) (Sultana and Guaci 1971), short-toed eagles (*Circaetus gallicus*) (Bannerman and Vella-Gaffiero 1976) and blue rock thrushs (*Monticola solitarius*) (Sultana and Guaci 1971), as well as snakes such as the cat snake (*Telescopus fallax*), the black whip snake (*Hierophis viridiflavus*), the Algerian whip snake (*Hemorrhois algirus*) and the leopard snake (*Rhinechis situla*) (Lanfranco 1955, 1970; Savona Ventura 1979; Lanza 1987; Borg 1989). The St. Paul's Island population now appears to have been extirpated, possibly due, in part, to rat predation (Sciberras and Schembri 2008), but may also be as a result of human interference (pers. obs.). The huge increase in the human population on Malta during the last three decades has resulted in large-scale habitat destruction (Borg 1989). Malta exhibits the highest population density (1,300 persons per km^2) in the European Union (Eurostat population, 2011), which is further-increased by more than 1 million tourists per year (Cassar et al. 2008; Deidun 2010).

Phylogenetic relationships between *P. filfolensis* and other *Podarcis* have proved controversial: allozyme data suggested *P. filfolensis* is closely related to *P. sicula* (predominantly southern eastern species), and to *Podarcis melissensis* (a species from the east Adriatic coast; Capula 1994), while analyses of mitochondrial (mtDNA) data suggested that it was a sister species to *P. wagleriana* (endemic to Sicily and the Egadi Islands), representing an ancient clade closely related to *P. muralis* (Oliverio et al. 2000). More recent studies suggest that *P. filfolensis* belongs to an evolutionary clade containing three other Mediterranean island species of *Podarcis*: *P. tiliguerta*, *P. lilfordi* and *P. pityusensis* (Arnold et al. 2007).

Preliminary intraspecific analyses of mtDNA genes ($tRNA_{Phe}$ and 12S rRNA) and proteins (electrophoretic analysis of 26 presumptive gene loci), using *P. filfolensis* from three localities (Malta, Filfla and Linosa islands), showed that the Maltese specimens (Malta and Filfla) are closely related to each other, and that these are genetically differentiated from those from Linosa Island, which showed high levels of genetic variability (Scalera et al. 2004).

Our primary aim is to establish patterns of genetic diversity within *Podarcis filfolensis*. We achieve this, using sensitive nuclear microsatellite loci, together with mtDNA sequences as an alternative marker. Establishment of genetic structuring within the Maltese archipelago is the first step in determining management units that will underpin future conservation strategies.

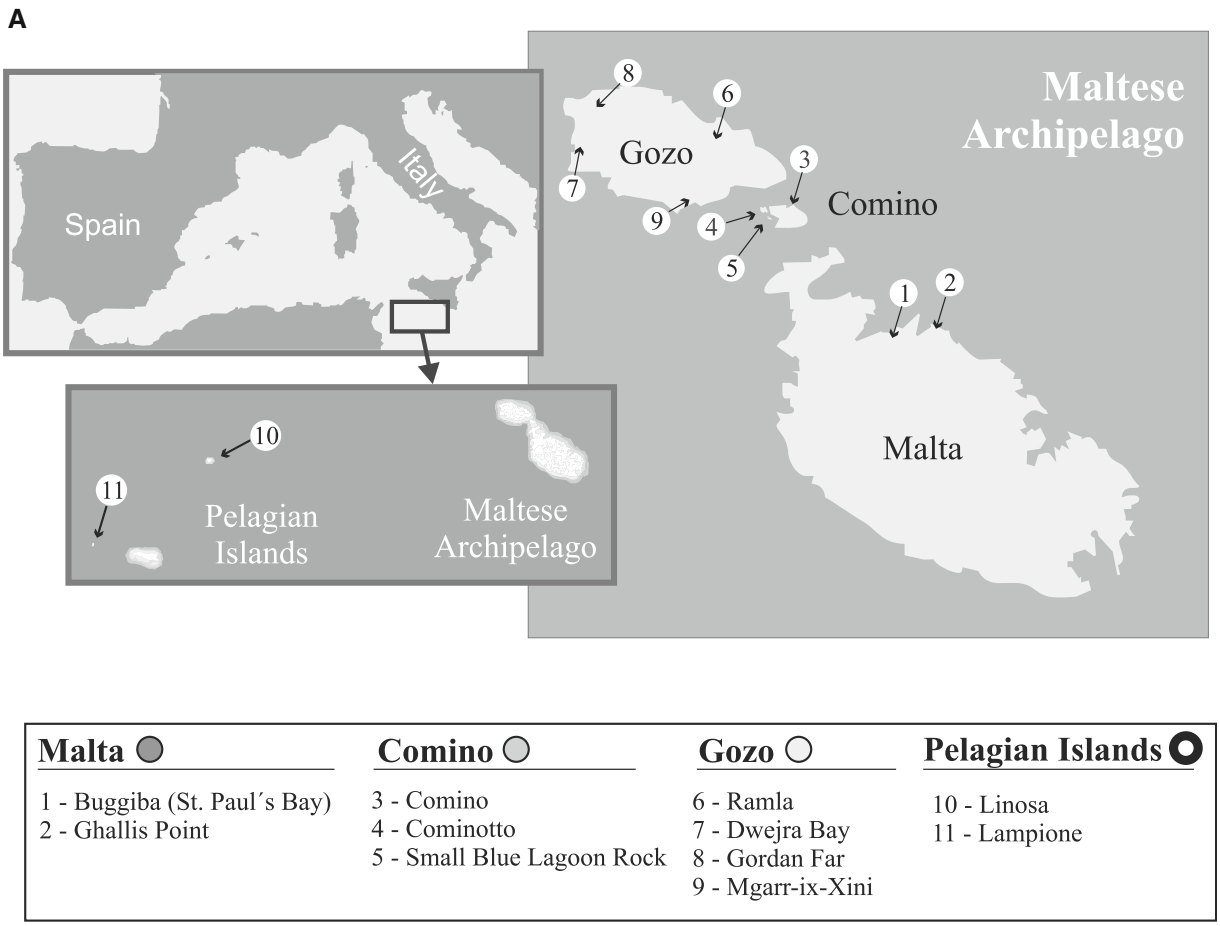

Fig. 1 Map of the Maltese archipelago and the Pelagian Islands; localities sampled in *Podarcis filfolensis* are indicated (**A**). MtDNA haplotype network for *Podarcis filfolensis*, including samples from the Pelagian Islands (**B**)

Materials and methods

Samples and DNA isolation

Thirty-three *Podarcis filfolensis* were captured by noosing in 2007/2008, under official licenses from MEPA (Environment Protection Directorate of Malta; permit: NP00051/07), and tail-tips were removed before releasing the individual at the site of capture. Tissue samples were stored in 100 % ethanol. DNA was subsequently extracted using a phenol–chloroform protocol similar to that described by Sambrook et al. (1989). The *P. filfolensis* were from Malta ($n = 7$; Fig. 1A, localities 1, 2), Comino ($n = 5$; Fig. 1A, locality 3), Cominotto ($n = 5$; Fig. 1A, locality 4), Small

Blue Lagoon Rock ($n = 5$; Fig. 1A, locality 5) and Gozo ($n = 9$; Fig. 1A, localities 6–9) from the Maltese archipelago, as well as Linosa and Lampione (one each) in the Pelagian Islands (Fig. 1A, localities 10, 11). The low population density on Malta Island (Grech 1999 and personal observations) only allowed samples to be collected at two locations (Buggiba and Ghallis Point). Samples from the rocky islets of Filfla (5 km south of Malta) and Fungus Rock (at the mouth of a small bay on the west coast of Gozo) were not collected because the local authorities did not grant authorization. This was also the case for St. Paul's Island, although we visited this island twice to assess lizard densities (September 2007 and May 2008).

Microsatellite data

We genotyped *P. filfolensis* specimens with eleven microsatellite loci that had been isolated from the closely-related *P. lilfordi* (Bloor et al. 2011). PCRs were run in a GeneAmp PCR System 2700 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) using primers and conditions described in Bloor et al. (2011). Fluorescently labeled PCR products were run on an ABI 3130 DNA Sequencer (Applied Biosystems, Foster City, CA, USA) with GeneScan-500 (LIZ) internal size standard, and fragment length was assigned with GeneMapper software v. 3.2 (Applied Biosystems, Foster City, CA, USA). Allele assignment was the same as those described by Bloor et al. (2011).

General statistics of microsatellite diversity and a population bottleneck index, M (Garza and Williamson 2001), were obtained using Arlequin v.3.1.1 (Excoffier et al. 2005). An analysis of molecular variance (AMOVA) was also computed to determine genetic structure between geographic populations (Malta vs. Gozo, Comino and adjacent islets). The number of genetically distinct clusters was estimated using Structure v.2.3.3 (Pritchard et al. 2000) and Structure harvester (Earl and vonHoldt 2012). Structure was run twenty times, each run comprising 400,000 steps (200,000 discarded as burn-in), using an admixture model and correlated allele frequencies among populations for all values of K from 1 to 10. The programs Structure and CLUMPP v.1.1.2 (Jakobsson and Rosenberg 2007) were used to determine the estimated membership coefficient (Q), and assign individuals to populations. We applied a threshold value of 0.20, as this provides a greater efficiency and accuracy to differentiate between purebreds and hybrids (Vähä and Primmer 2006): Q values between 0.2 and 0.8 may indicate hybridization between individuals from different clusters.

Nuclear gene sequence

Partial sequences from the proto-oncogene *c-mos* were amplified and sequenced following primers and conditions described by Terrasa et al. (2009). Sequences were edited and aligned by BioEdit v.7.0.5.2 (Hall 1999), and TCS v.1.21 (Clement et al. 2000) was used to construct a haplotype network.

Mitochondrial data

Five mtDNA fragments were amplified for each specimen and sequenced to provide partial genes sequences from the following regions: (i) partial 12S rRNA, (ii) short partial cytochrome *b* (cytb), (iii) long partial cytb and partial $tRNA_{Thr}$, (iv) partial control region, (v) two partial subunits of the NADH dehydrogenase gene and associated tRNAs (referred to as ND1, ND2, $tRNA_{Ile}$, $tRNA_{Gln}$, and $tRNA_{Met}$). Primers and amplification conditions are the same as those used for *P. lilfordi* (Terrasa et al. 2009) and *P. pityusensis* (Rodríguez et al. 2013). Both heavy and light strands were sequenced on an automated ABI 3130 sequencer (Applied Biosystems, Foster City, CA, USA) using a BigDye® Terminator v. 3.1 Cycle sequencing kit (Applied Biosystems, Foster City, CA, USA), and edited and aligned using BioEdit v.7.0.5.2 (Hall 1999).

Basic genetic diversity indices and neutrality statistical tests were calculated using DnaSP v.5.10.01 (Librado and Rozas 2009), and an analysis of molecular variance (AMOVA) was conducted in the same way as for microsatellite data. Bayesian inference of population structure was determined using BAPS v.5.3 (Corander et al. 2003), with an upper bound of $K = 30$, and without prior information on geographic location.

A statistical parsimony network, with a 95 % connection limit, was constructed using mtDNA sequences from both the Maltese archipelago and the Pelagian Islands using TCS v.1.21 (Clement et al. 2000), in order to examine mtDNA relationships.

Species phylogeny and divergence times

Divergence times and phylogenetic relationship between the main *P. filfolensis* mitochondrial lineages, identified by BAPS, and main lineages from the related species *P. pityusensis* (Rodríguez et al. 2013) and *P. lilfordi* (Brown et al. 2008) were estimated using the multispecies coalescent approach (program: *BEAST v. 1.7.4; Heled and Drummond 2010; Drummond et al. 2012). The major advantage of this method is that it allows estimation of divergence times while taking into account within-population ancestral polymorphism (of mtDNA in this case). We use the term 'population tree', to describe the resultant tree which shows the historical relationships among potentially noninterbreeding *Podarcis* populations (rather than recognized species). We used nine populations in the analysis. These were: *P. lilfordi* from (i) Menorca, (ii)

West Mallorca, (iii) N & S Mallorca & N Cabrera, (iv) South Cabrera and (v) Cabrera (see Brown et al. 2008); *P. pityusensis* from (i) Formentera, Freus islands and Ibiza and (ii) Ibizan islands (see Rodríguez et al. 2013); *P. filfolensis* from (i) Malta, and (ii) remaining islands, as described here. We calibrated the (*P. lilfordi*, *P. pityusensis*) species nodes on the tree using the normal distribution $N(5.325, 0.0001)$, based on the end of the MSC (5.33 Ma; see Brown et al. 2008 and Rodríguez et al. 2013 for more details). The time of the most recent common ancestor (MRCA) of all *P. pityusensis* was loosely constrained using the Gamma distribution as $G(5.8, 0.2)$ and the MRCA of *P. lilfordi* was loosely constrained as $G(3.48, 0.5)$ to provide a slightly greater prior density around divergence times that are within the same ballpark as the posteriors obtained in our previous analyses (Brown et al. 2008; Rodríguez et al. 2013).

We analysed eight partitions: (i) 12S rRNA, (ii) control region, (iii) all tRNAs, (iv) 1st and 2nd codon positions of the ND1/ND2 sequences, (v) 3rd codon position of ND1/ND2, (vi) 1st codon position of cytb, (vii) 2nd codon position of cytb, (viii) 3rd codon position of cytb, under evolutionary models identified using MrAIC v. 1.4.4 (Nylander 2004), following AICc (Akaike Information Criterion corrected for small samples). When the "best" model was not available in *BEAST, we used the next most complex model.

All *BEAST (Heled and Drummond 2010) analyses started with a random tree, with the MCMC chain being run for 300 million generations, sampled every 10,000 steps. A strict clock model was applied (see Brown and Yang 2011) and the tree prior specified using a Yule process. Visual examination of posterior samples that were plotted using Tracer v. 1.5 (Rambaut and Drummond 2007) indicated that more than enough initial samples were discarded to ensure burn-in of the chain. TreeAnnotator (BEAST package) was used to combine and analyse the posterior tree samples at stationarity (trees were combined using the maximum sum of clade credibilities criterion).

Results

Microsatellite data: variability and population structure

Thirty-one individuals from the Maltese archipelago were successfully genotyped for all 11 microsatellite loci. Numbers of alleles per microsatellite locus ranged from 2 (Pli10) to 20 (Pli17), with an average number of 10.8 alleles per locus (Table 1). Observed and expected heterozygosities ranged from 0.065 to 0.864 and 0.063 to 0.957, respectively (Table 1). Smaller values for observed compared with expected heterozygosities were indicative of population structuring. Low values for the Garza and Williamson test statistic (M) (0.051–0.351) supported recent size reductions. Pre-defined geographic populations (Malta vs. remaining archipelago) showed that a greater proportion of the variation was within (99.15 %) as opposed to between (0.85 %) populations ($F_{ST} = 0.00851$; $P = 0.18$).

Table 1 Genetic variability for microsatellite loci in *P. filfolensis*. Locus names and allele assignment correspond to those in Bloor et al. (2011)

Locus	Num. alleles	Allelic range	Obs. Het.	Exp. Het.	M
Pli2	13	256–310	0.524	0.909	0.236
Pli3	6	247–271	0.286	0.801	0.240
Pli4	16	376–460	0.733	0.928	0.188
Pli6	10	382–420	0.621	0.862	0.256
Pli9	4	331–361	0.267	0.370	0.129
Pli10	2	227–265	0.065	0.063	0.051
Pli12	15	188–254	0.370	0.892	0.224
Pli17	20	233–289	0.864	0.957	0.351
Pli18	13	115–163	0.742	0.893	0.265
Pli21	13	114–196	0.741	0.868	0.157
Pli22	7	103–127	0.355	0.533	0.280

Garza and Williamson statistic (2001)

Three genetically distinct clusters (highest value of $\Delta K = 0.734$; Fig. 2A) were evidenced with Structure analyses and Evanno's statistic (Evanno et al. 2005). Groups are associated with geographic regions: one of them (cluster I) was distributed in Malta, but also included three admixed individuals (two from Gozo and one from Comino, with Q ranging from 0.41 to 0.5); the others clusters (clusters II and III) contained samples from Gozo, Comino, Cominotto and Small Blue Lagoon Rock, along with one admixed individual from Buggiba, Malta (Fig. 1A, locality 1; $Q = 0.46$). In general, samples are poorly assigned to clusters (with the exception of Ghallis Point specimens from Malta: Fig. 1A, locality 2), with estimated membership coefficients ranging from 0.38 to 0.58.

Nuclear gene sequence

The *c-mos* gene was sequenced in 26 individuals (GenBank accession numbers: KF022080–85), providing a 353 bp alignment. However, this sequence was found to be relatively uninformative, with only five variable sites resulting in six haplotypes. The haplotype network showed a star-like structure (figure not shown), with the most frequent haplotype found in 12 specimens from different localities, including two individuals from one Malta locality (Buggiba; Fig. 1A, Site1). Individuals from the other Malta locality (Ghallis Point; Fig. 1A, Site 2), had a unique haplotype.

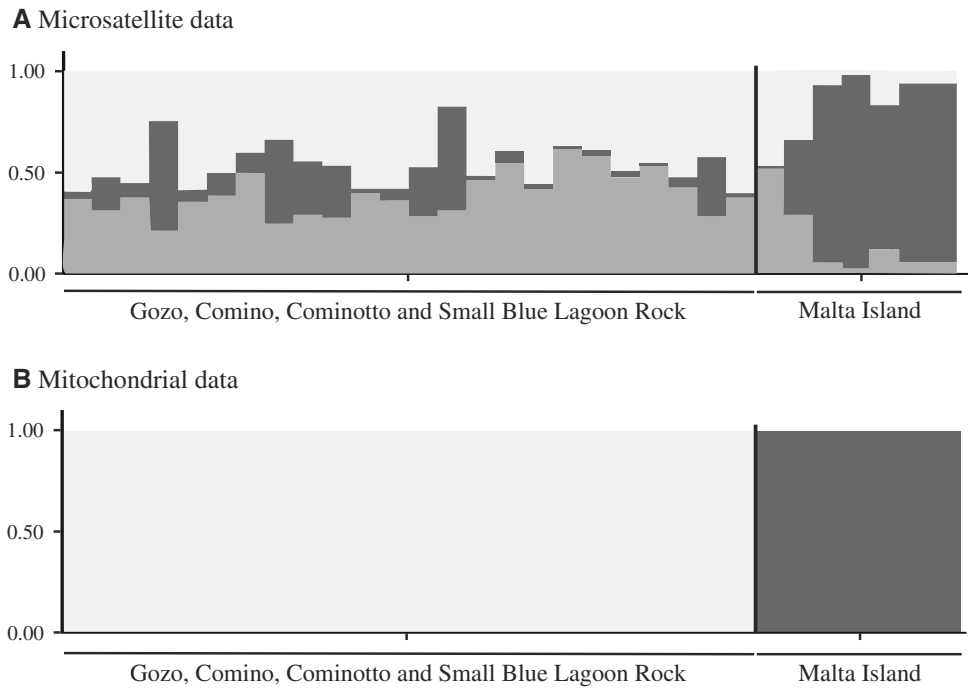

Fig. 2 Genetic structure inferred from microsatellites and mtDNA for the two main geographic regions used in this study. **A** Microsatellite-based Bayesian assignments identified three main groups (*cluster I: dark grey*, *cluster II: light grey*, *cluster III: grey*). The *bar plot* shows estimated membership coefficient (***Q***); vertical bars represent individuals and assignment probabilities. **B** Mixture analyses, for mitochondrial data, estimated by BAPS software identified two clusters (*cluster A: dark grey* and *cluster B: light grey*). In the Bar plot, vertical bars represent individuals and proportions of admixture, with different colours corresponding to different ancestral sources

Mitochondrial data: diversity and population structure

Thirty-three individuals were analyzed for all five mtDNA fragments. Sequences have been deposited in GenBank under accession numbers: JX852109, JX852112, JX852115, JX852138 and KF022049–79. Analyses of Maltese archipelago specimens (31) revealed 20 different haplotypes in the 2,533 bp of concatenated sequence, defined by 41 polymorphic sites, with a mean uncorrected pairwise difference of 9.4 bp (Table 2). Genetic structuring among geographic areas (i.e., Malta vs. rest of archipelago) was examined (AMOVA), showing that most of the variation was among (81.74 %) as opposed to within (18.26 %) regions ($F_{ST} = 0.8174$; $P < 0.001$). Mixture analyses using BAPS indicated that partitioning into two clusters gave the highest Log likelihood (−344.0553; Fig. 2B). The first cluster (A) contained all samples from Malta, while the rest of islands in the Maltese archipelago (Gozo, Comino, Cominotto and Small Blue Lagoon Rock) were included in the second cluster (B). These results show that mtDNA genetic diversity is strongly geographically structured. Inclusion of Pelagian Island specimens in the analyses showed their similarity with specimens from Gozo, Comino, Cominotto and Small Blue Lagoon Rock (cluster B in BAPS). Figure 1B shows the TCS haplotype network.

Species phylogeny and divergence times

For these analyses we used 2,371 bp of mtDNA sequence, that was homologous with that available from previous studies. We obtained strong posterior support ($P = 1.00$) for the most basal *Podarcis* nodes. The ancestral species node for the *Podarcis* species analysed was dated at 10.41 Ma (95 % highest posterior density, HPD: 5.61–13.61 Ma) and the mean posterior divergence time between the two *P. filfolensis* lineages was 417 Ka (95 % HPD: 105–869 Ka) (Fig. 3). The mtDNA genealogy for *P. filfolensis* is shown in Fig. 4.

Discussion

Our analyses suggest low genetic diversity within Maltese lizards compared with other *Podarcis*. There are relatively few alleles at the microsatellite loci used here, although the fact that these loci were identified in a different species is likely to be important. Recent size reductions were inferred in our analyses and are a potential cause of this low genetic diversity. MtDNA diversity is also quite low. The closely-related species, *P. pityusensis* has been studied in detail, and two mtDNA clades detected. Given that the divergence time of these *P. pityusensis* clades appears similar to *P. filfolensis* (Rodríguez et al. 2013) it is notable that the percentage of within-group mtDNA diversity is much lower in *P. filfolensis*.

Clustering analyses for both mtDNA and microsatellites identified two main groupings that broadly corresponded to distinct geographical areas, i.e., Malta Island versus the remaining islands (Gozo, Comino, Cominotto and Small Blue Lagoon Rock), but this pattern is not entirely supported by nuclear DNA. Samples from the four islands were subdivided into two clusters that were not

Table 2 Diversity parameters for the concatenated mtDNA fragment in *P. filfolensis* compared with *P. lilfordi* and *P. pityusensis*

Species	No	V	No hap	Nucleotide diversity	K	Fu's (1997) Fs	Fu and Li's (1993) D	Fu and Li's (1993) F	Fay and Wu's (2000) H	Tajima's (1989) D
P. lilfordi (2,382 bp[a])	117	189	62	0.019 ± 0.005	45.067 ± 19.503	−1.129[ns]	0.652[ns]	0.860[ns]	−17.572[ns]	0.770[ns]
P. pityusensis (2,430 bp[a])	73	90	60	0.004 ± 0.001	9.083 ± 4.17	−56.983***	−2.432**	−2.589**	−25.341**	−1.745*
P. filfolensis (2,533 bp)	31	41	20	0.004 ± 0.001	9.355 ± 4.27	−4.097*	−0.681[ns]	−0.668[ns]	−12.409**	−0.325[ns]

No number of individuals sampled; *V* variable position; *No hap* number of haplotypes; *K* average number of pairwise differences

[ns] Not significant

*$P < 0.1$; **$P < 0.05$; ***$P < 0.001$

[a] Obtained from data in Terrasa et al. (2009) and Rodríguez et al. (2013)

geographically structured. Also, one of the Malta localities (Buggiba) showed recently admixed individuals that had affinities with the other islands (evidenced in both microsatellites and *c-mos*). This is likely to indicate recent introductions, particularly in view of the categorical structuring of mtDNA lineages between these regions, and may explain why the between region structuring was not significant for the microsatellites.

Specimens from Linosa and Lampione islands grouped with Gozo, Comino, Cominotto and Small Blue Lagoon Rock (Fig. 1B), indicating these two introduced populations may have originated from an ancestor belonging to the same mitochondrial lineage that Gozo, Comino, Cominotto and Small Blue Lagoon Rock. Previously, this similarity between samples from the Pelagian Islands and the Maltese archipelago led Capula (1994) to hypothesize that *P. filfolensis* probably reached the former by human-mediated dispersals events.

It is worthwhile considering the geophysical events that may have played a role in generating the described genetic diversity. Maltese endemics probably reached the archipelago during the MSC, and were isolated after flooding of the Mediterranean basin some 5.33 Ma (Cassar et al. 2008). A subsequent rise in sea level around 600 Ka (Emig and Geistdoerfer 2004) may be the origin of divergence of the two principal founding lineages of *P. filfolensis*, which shared a common ancestor (MRCA) around 105.4–869 Ka. This suggests a mid-late Pleistocene intraspecific divergence, which coincides with that observed in *P. pityusensis* (a species that also comprises two main mtDNA and two microsatellite clusters: Brown et al. 2008; Rodríguez et al. 2013). Sea depths between the Maltese islands are very shallow (15–20 m), so regular connections will have been possible when sea levels dropped.

Under widely accepted criteria, a significant difference, together with the results of the analysis based on mtDNA data, would have provided very strong support for assignment of two evolutionary significant units (ESUs) (Moritz 1994). Nevertheless, given the small sample sizes and the likelihood of between-region introductions lowering the apparent levels of differentiation, we propose that two ESUs are valid for *P. filfolensis* and that these need to be considered in any future management plan. Taken together, the low population density on Malta itself (pers. obs.), the likely extirpation on St. Paul's Island close to Malta, and the evidence of reduced genetic diversity and small population sizes suggests that, at the very least, our proposed Malta ESU merits urgent recognition as a threatened species.

Previous authors have recognized five subspecies. *P. f. filfolensis* and *P. f. generalensis* were not included in our analysis, nor *P. f. kieselbachi* that seems to have been extirpated. Here, we analyse only two subspecies (*P. f.*

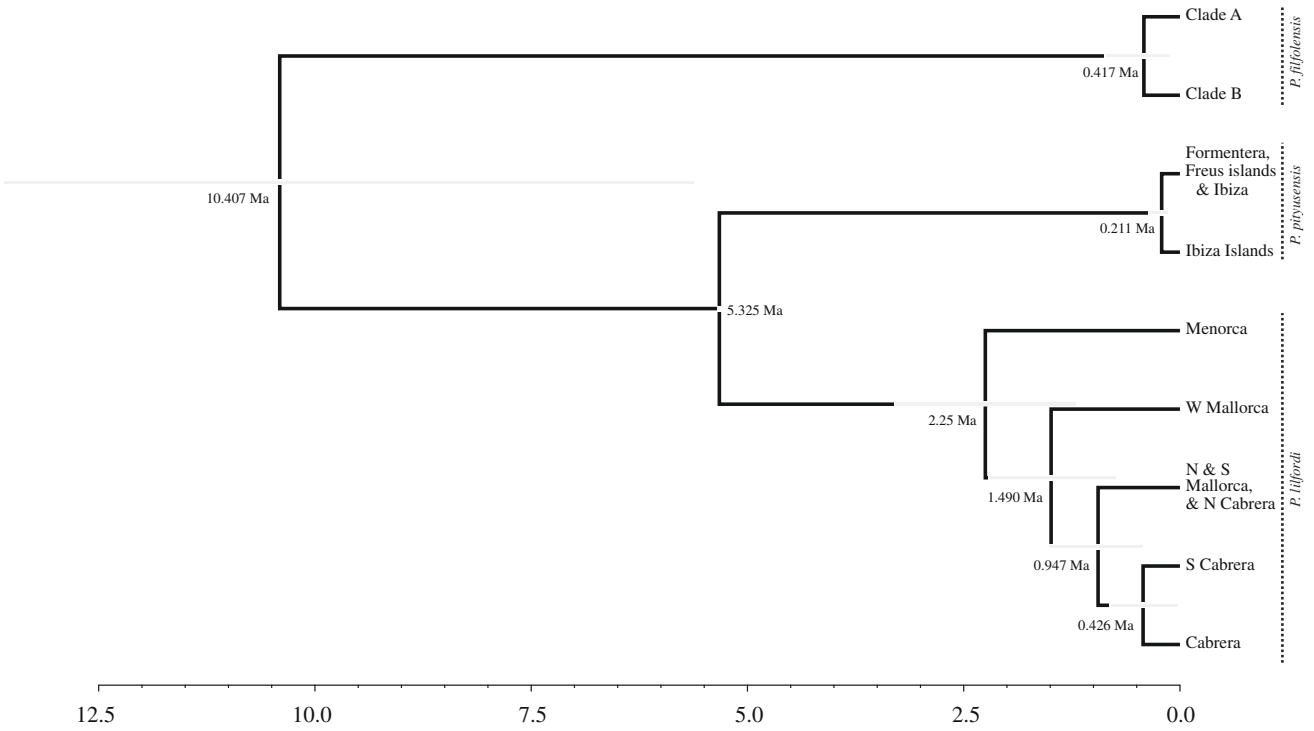

Fig. 3 Populations tree chronogram estimated by *BEAST. *P. filfolensis* lineages corresponded to clusters assigned by BAPS software (Fig. 2B). *P. lilfordi* and *P. pityusensis* lineages are those identified by Brown et al. (2008) and Rodríguez et al. (2013), respectively. *Grey bars* correspond to the 95 % highest posterior density intervals for each divergence time

maltensis and *P. f. laurentiimuelleri*; as well as the specimens from Cominotto) but their distribution does not match with the two lineages we found. The disparity between the subspecies recognition and our proposal of two ESUs needs to be resolved. An additional problem is that a range of morphological forms overlap between populations, so subspecies assignment would require considerable weighting on geographical location (Sciberras and Schembri 2008). It is important to conserve unique phenotypic and/or ecological diversity, but the enormous variability of *Podarcis* species led to a large number of subspecies in the past. Re-evaluation and the re-assignment of morphological subspecies is therefore needed (Oliverio et al. 2000).

Despite being currently assigned to the "Least Concern" category by the IUCN (Corti et al. 2009), the long-term viability of *P. filfolensis* populations in the Maltese archipelago appears rather precarious. Corti et al. (2009) justified this category because, although its extent of occurrence is less than 5,000 km^2, it is common, adaptable, and did not appear to be in decline. Nevertheless, *P. filfolensis* is protected both nationally, by the Environment Protection Act (CAP. 435)-Development Planning Act (CAP. 356)-Flora, Fauna and Natural Habitats Protection Regulations, 2006 (L.N. 311 of 2006; Malta Environment & Planning Authority; MEPA), as a "species of national interest whose conservation requires the designation of special areas of conservation", and internationally by the Habitats Directive (Council Directive 92/43/EEC of 21 May 1992 on the conservation of natural habitats and of wild fauna and flora; European Commission Environment), where it is listed in Annex IV "species of Community interest in need of strict protection" and by the Bern Convention (Council of Europe), where it is listed in Appendix II "strictly protected species of fauna". The revision of their conservation status and the promotion of initiatives to protect habitats, especially in Malta, would hopefully ensure the future of this species. A unique island form, the subspecies *P. filfolensis kieselbachi* found on St. Paul's Island seems to have been extirpated; no specimens were observed despite intensive searching during two visits. Furthermore, population densities appear to be low in other Maltese islands, when compared with other Mediterranean island *Podarcis*. Grech (1999) recorded lizard densities of 14–33 individuals per hectare: values that are far below those recorded in Western Mediterranean islands (Pérez-Mellado et al. 2008). Individuals on the islands of Gozo, Comino, and surrounding islets were relatively easy to observe, but the distribution and abundance on the main island of Malta, was found to be patchy and generally low. Suitable habitats for lizards were strongly degraded and, although intensive searches in large areas of the island were conducted, we

Fig. 4 MtDNA phylogenetic tree estimated by *BEAST showing relationships among *P. filfolensis* haplotypes. Principal clusters (A, B), geographical localization of each specimen and Bayesian posterior probabilities (>0.9) are indicated on the tree

only found specimens in two localities. Therefore, sparse sampling on Malta may have led to extant lineages remaining undetected. However, this would seem rather unlikely given the small size and relatively uniform topography/ecology of the island.

In conclusion, molecular analyses revealed low genetic diversity, but indicated that two ESUs could be recognized in *P. filfolensis*. This finding, together with small population sizes and high levels of human disturbance, lead us to suggest a revision of the conservation status of this lineage by the Red List of Threatened Fauna of the International Union for Conservation of Nature. This should lead to governmental policy initiatives to protect these ESUs.

Acknowledgments We are grateful to Arnold Sciberras, for specimens from Linosa and Lampione islands. This work was financed by the research Grants CGL2009-12926-C02 and CGL2012-39850-C02 of the Ministerio Español de Economía y Competitividad and European Regional Development Fund (ERDF). V. R. was funded by an FPI fellowship from the Conselleria d'Educació, Cultura i Universitats (Govern de les Illes Balears), co-financed by the European Social Fund.

References

Agulló J, Juan A, Guilló A, Alonso M, Crespo M (2011) Genetic diversity and phylogeographical assessment of *Helianthemum caput–felis* Boiss.(Cistaceae) based on AFLP markers. Paper presented at the Biodiversity Hotspots in the Mediterranean Area: species, communities and lanscape level, 45th International Congress of SISV & FIP, Cagliari

Arnold EN, Arribas Ó, Carranza S (2007) Systematics of the Palaearctic and Oriental lizard tribe Lacertini (Squamata: Lacertidae: Lacertinae), with descriptions of eight new genera. Zootaxa 1430. Magnolia Press, Auckland

Bannerman DA, Vella-Gaffiero JA (1976) Birds of the Maltese archipelago. Malta Museums Department

Bauza-Ribot M, Jaume D, Fornos J, Juan C, Pons J (2011) Islands beneath islands: phylogeography of a groundwater amphipod crustacean in the Balearic archipelago. BMC Evol Biol 11(1):221

Bedriaga J (1876) Die Faraglione-Eidechse und die Entstehung der Farben bei den Eidechsen. Heidelberg: Carl Winter:1–21

Bloor P, Rodríguez V, Terrasa B, Brown R, Pérez-Mellado V, Castro J, Picornell A, Ramon M (2011) Polymorphic microsatellite loci for the Balearic Island Lizard *Podarcis lilfordi* (Squamata: Lacertidae). Conserv Genet Resources 3(2):323–325

Borg MJ (1989) Aspects of the biology of *Podarcis filfolensis*. University of Malta

Brown RP, Yang Z (2011) Rate variation and estimation of divergence times using strict and relaxed clocks. BMC Evol Biol 11(1):271

Brown RP, Terrasa B, Perez-Mellado V, Castro JA, Hoskisson PA, Picornell A, Ramon MM (2008) Bayesian estimation of post-Messinian divergence times in Balearic Island lizards. Mol Phylogenet Evol 48(1):350–358

Capula M (1994) Evolutionary relationships of *Podarcis* lizards from Sicily and the Maltese Islands. J Zoology, Syst Evolut Res 32:180–192

Capula M (2006) *Podarcis filfolensis* (Bedriaga, 1876). In: Sindaco R DG, Razzetti E, Bernini F (ed) Atlante degli Anfibi e dei Rettili d'Italia. Polistampa, Firenze, pp 466–469

Cassar LF, Conrad E, Schembri PJ (2008) The Maltese Archipelago. In: Vogiatzakis I, Pungetti G, Mannion AM (eds) Mediterranean Island Landscapes, vol 9. Landscape Series, Spinger, pp 297–322

Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. Mol Ecol Notes 9:1657–1659

Corander J, Waldmann P, Sillanpää MJ (2003) Bayesian analysis of genetic differentiation between populations. Genetics 163(1):367–374

Corti C, Lo Cascio P (1999) I lacertidi italiani. L'epos

Corti C, Pérez-Mellado V, Sindaco R, Romano A (2009) *Podarcis filfolensis*. IUCN Red List of Threatened Species. Version 2012.2. www.iucnredlist.org

Deidun A (2010) Challenges to the conservation of biodiversity on small islands: the case of the Maltese Islands. Inter J Arts Sci 3:175–187

Despott G (1915) The reptiles of the Maltese Islands. The Zoologist 19(891):821–827

Drummond AJ, Suchard MA, Xie D, Rambaut A (2012) Bayesian phylogenetics with BEAUti and the BEAST 1.7. Mol Biol Evol 29:1969–1973

Earl D, vonHoldt B (2012) Structure Harvester: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conserv Genet Resour 4(2):359–361

Emig CC, Geistdoerfer P (2004) The Mediterranean deep-sea fauna: historical evolution, bathymetric variations and geographical

changes. Carnets de Géologie/Notebooks on Geology, Article 2004/01 (CG2004 A01 CCE-PG)
Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software structure: a simulation study. Mol Ecol 14(8):2611–2620
Excoffier L, Laval G, Schneider S (2005) Arlequin (version 3.0): an integrated software package for population genetics data analysis. Evol Bioinform Online 1:47–50
Fay JC, Wu CI (2000) Hitchhiking under positive Darwinian selection. Genetics 155(3):1405–1413
Ficetola GF, Padoa-Schioppa E (2009) Human activities alter biogeographical patterns of reptiles on Mediterranean islands. Glob Ecol Biogeogr 18(2):214–222
Fu YX (1997) Statistical tests of neutrality of mutations against population growth Hitchhiking and background selection. Genetics 147(2):915–925
Fu YX, Li WH (1993) Statistical tests of neutrality of mutations. Genetics 133(3):693–709
Furlani S, Antonioli F, Biolchi S, Gambin T, Gauci R, Lo Presti V, Anzidei M, Devoto S, Palombo M, Sulli A (2013) Holocene sea level change in Malta. Quatern Int 288:146–157
Garza JC, Williamson EG (2001) Detection of reduction in population size using data from microsatellite loci. Mol Ecol 10(2):305–318
Grech R (1999) Aspects of the behaviour of the Maltese wall lizard. University of Malta, Podarcis filfolensis in coastal areas
Hall T (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleid Acids Symp Series 41:95–98
Heled J, Drummond AJ (2010) Bayesian inference of species trees from multilocus data. Mol Biol Evol 27(3):570–580
Hunt CO, Schembri PJ (1999) Quaternary environments and biogeography of the Maltese Islands. Á In: Mifsud, A and Savona Ventura, C(eds), Facets of Maltese prehistory The Prehistoric Society of Malta:243
Jakobsson M, Rosenberg NA (2007) CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. Bioinformatics 23(14):1801–1806
Krijgsman W, Hilgen FJ, Raffi I, Sierro FJ, Wilson DS (1999) Chronology, causes and progression of the Messinian salinity crisis. Nature 400(6745):652–655
Lanfranco G (1955) Reptiles, amphibians of the Maltese Islands. Malta Year Book, Malta, pp 198–203
Lanfranco G (1970) Notes on Coluber florulentus algirus Maltese Naturalist 1(1):13–15
Lanza B (1987) Tutti i serpenti italiani. Silva 2:48–69
Librado P, Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25(11):1451–1452
Lo Cascio P, Corti C (2008) Indagini sull'ecologia dei rettili sauri della RNO e del SIC "Isola di Lampedusa". Naturalista sicil 32:319–354
Micallef A, Foglini F, Le Bas T, Angeletti L, Maselli V, Pasuto A, Taviani M (2012) The submerged paleolandscape of the Maltese Islands: Morphology, evolution and relation to Quaternary environmental change. Marine Geology
Moritz C (1994) Defining "evolutionarily significant units" for conservation. Trends Ecol Evol 9(10):373–375
Nylander JAA (2004) MrAIC.pl. Evolutionary Biology Centre, Uppsala University
Oliverio M, Bologna MA, Mariottini P (2000) Molecular biogeography of the Mediterranean lizards *Podarcis Wagler*, 1830 and *Teira Gray*, 1838 (Reptilia, Lacertidae). J Biogeogr 27(6): 1403–1420
Pérez-Mellado V, Hernández EJA, García DT, Terrassa B, Ramon MM, Castro J, Picornell A, Martín-Vallejo J, Brown R (2008) Population density in *Podarcis lilfordi* (Squamata, Lacertidae), a lizard species endemic to small islets in the Balearic Islands (Spain). Amphib-Reptilia 29(1):49–60
Podnar M, Mayer W, Tvrtkovic N (2005) Phylogeography of the Italian wall lizard, *Podarcis sicula*, as revealed by mitochondrial DNA sequences. Mol Ecol 14(2):575–588
Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155(2):945–959
Rambaut A, Drummond AJ (2007) Tracer v. 1.5
Rodríguez V, Brown RP, Terrasa B, Pérez-Mellado V, Castro JA, Picornell A, Ramon MM (2013) Multilocus genetic diversity and historical biogeography of the endemic wall lizard from Ibiza and Formentera, Podarcis pityusensis (Squamata: Lacertidae). Mol Ecol 22:4829–4841
Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, vol 2. Cold Spring Harbor Laboratory Press, New York
Savona Ventura C (1979) Reptiles and amphibians in Maltese ecology. Potamon 1(2):14–16
Savona Ventura C (1983) The herpetofauna of Comino and satellite islets with a note on the colouration of *Podarcis filfolensis*. Animalia 10(1/3):87–93
Scalera R, Capula M, Fornasari L, Zava B, Bombi P, Mariottini P, Bologna MA (2004) Population structure, genetics and conservation of the Maltese wall lizard, *Podarcis filfolensis*, on Linosa Island (Reptilia, Lacertidae). Ital J Zool 71 (sup002):153–159
Schembri PJ (1993) Physical geography and ecology of the Maltese Islands: A brief overview. In: Busuttil S. LF, Mizzi L. (ed) Malta: Food, agriculture, fisheries and the environment. Options Méditerranéennes : Série B. Etudes et Recherches; n. 7. CIHEAM, Montpellier, pp 27–39
Schembri PJ (1994) Malta's natural heritage. In: Friggieri O (ed) Frendo H. Malta culture and identity, Malta Ministry of Youth and the Arts, pp 105–124
Schüter U (2005) Die Malta-Eidechse-*Podarcis filfolensis* (Bedriaga, 1876). Die Eidechse 16(1):1–10
Sciberras A (2007) The presence of *Chamaleo chamaleon* (Reptilia) on the Maltese islands, with a note on the occurence of this species on Cominotto Island and its possible effects on the endemic local lizard. Herpetol Bull 102:38–40
Sciberras A, Schembri PJ (2008) Conservation status of the St Paul's Island wall lizard (*Podarcis filfolensis kieselbachi*). Herpetol Bull 105:28–34
Sultana J, Guaci C (1971) The breeding birds of Malta. Malta Year Book, Malta, pp 329–346
Tajima F (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123(3):585–595
Terrasa B, Pérez-Mellado V, Brown RP, Picornell A, Castro JA, Ramon MM (2009) Foundations for conservation of intraspecific genetic diversity revealed by analysis of phylogeographical structure in the endangered endemic lizard *Podarcis lilfordi*. Divers Distrib 15(2):207–221
Thake M (1985) The biogeography of the Maltese islands, illustrated by the Clausiliidae. J Biogeogr 12:269–287
Vähä JP, Primmer CR (2006) Efficiency of model-based Bayesian methods for detecting hybrid individuals under different hybridization scenarios and with different numbers of loci. Mol Ecol 15(1):63–72