Evolutionary history of the Maltese wall lizard *Podarcis filfolensis*: insights on the 'Expansion–Contraction' model of Pleistocene biogeography

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Abstract

The expansion–contraction (EC) model predicts demographic and range contraction of temperate species during Pleistocene glaciations as a consequence of climate-related habitat changes, and provides a paradigm for explaining the high intraspecific diversity found in refugia in terms of long-term demographic stability. However, recent evidence has revealed a weak predictive power of this model for terrestrial species in insular and coastal settings. We investigated the Pleistocene EC dynamics and their evolutionary consequences on temperate species using the Maltese archipelago and its endemic lizard *Podarcis filfolensis* as a model system. The evolutionary and demographic history of *P. filfolensis* as inferred from mitochondrial and nuclear sequences data does not conform to the EC model predictions, supporting (i) demographic and spatial stability or expansion, rather than contraction, of the northern and southern lineages during the last glacial period; and (ii) a major role for allopatric differentiation primed by sea-level dynamics, rather than prolonged demographic stability, in the formation of the observed genetic diversity. When combined with evidence from other Mediterranean refugia, this study shows how the incorporation of Pleistocene sea-level variations in the EC model accounts for a reverse demographic and range response of insular and coastal temperate biotas relative to continental ones. Furthermore, this cross-archipelago pattern in which allopatric diversity is formed and shaped by EC cycles resembles that seen between isolated populations within mainland refugia and suggests that the EC model, originally developed to explain population fluctuations into and out-of refugia, may be appropriate for describing the demographic and evolutionary dynamics driving the high genetic diversity observed in these areas.

Keywords: Allopatric divergence, genetic diversity, glacial expansion, historical demography, Mediterranean islands, phylogeography

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Introduction

Understanding the relationships between geographical patterns of distribution of biological diversity and evolutionary processes is a central and longstanding goal for evolutionary biogeography. In the last decades, the combination of palaeontological, palaeoclimatic and phylogeographical studies has provided important insights in this respect, highlighting the key role of Pleistocene climatic oscillations in structuring the present-day biodiversity patterns at the regional and global scale (Hewitt 1996, 2000, 2004; Taberlet *et al.* 1998; Schmitt 2007; Hickerson *et al.* 2010).

During the Pleistocene climatic cycles, species were greatly affected both by the advance and retreat of the continental ice sheets and consequent climate-related changes in habitat, and by the sea-level fluctuations associated with global ice-volume changes. These climate-driven changes imposed a cyclic contraction–expansion

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of terrain and habitats, with a consequent and parallel contraction and expansion of species' ranges. This 'expansion–contraction' (EC) model (Provan & Bennet 2008) has been empirically corroborated for the terrestrial flora and fauna of North America and Europe by a vast body of evidence from fossils, pollen records and comparative phylogeographical studies (see Huntley & Birks 1983; Bennett *et al.* 1991; Williams *et al.* 1998; for a review see Hewitt 2004; Schmitt 2007). For temperate species (*sensu* Stewart & Dalén 2008; including 'Continental' and 'Mediterranean' elements *sensu* Schmitt 2007), this model provides a demographic scenario of size and range contraction with a shift to southern refugia during glacials, followed by postglacial population growth and northwards recolonization (Hewitt 1996; Provan & Bennet 2008).

However, although the basic mechanism of the EC model is straightforward, the application of this model in regional contexts has revealed a complexity of additional factors shaping population demography and species ranges. Over the last decade, a plethora of patterns of diversity and phylogeographical paradigms have been invoked to account for such a complexity of EC processes allowing the refining and extension of earlier models and scenarios of Pleistocene EC dynamics (reviewed in: Stewart & Lister 2001; Gómez & Lunt 2007; Schmitt 2007; Provan & Bennet 2008; Stewart *et al.* 2010; Nieto Feliner 2011; Schmitt & Varga 2012).

In particular, two outstanding issues have arisen from studies on the evolutionary and demographic dynamics of populations from coastal areas and refugia. First, it has been suggested that some temperate species inhabiting Mediterranean coastal areas could have maintained stable populations or even experienced range/demographic expansions during glacials as a consequence of the synergistic effect of the increased availability of terrestrial habitat brought about by drops in the sea-level and the mild coastal microclimates due to the buffering action of the sea (Canestrelli *et al.* 2007; Canestrelli & Nascetti 2008; Bisconti *et al.* 2011). Second, the complex topography and palaeogeographical development of many southern refugia suggested that contraction–expansion of habitat may have operated at a regional scale inside refugia throughout the palaeoclimatic and palaeogeographical evolution of these areas (Gómez & Lunt 2007; Canestrelli *et al.* 2010; Nieto Feliner 2011). These EC processes have led to population isolation and divergence, thus enhancing the formation of intraspecific genetic diversity (Canestrelli *et al.* 2010; Vega *et al.* 2010; Nieto Feliner 2011). Understanding the temporal context of the EC dynamics in coastal settings and their contribution at a regional scale in the

formation of this genetic diversity has important consequences for our understanding of Pleistocene biogeography and current patterns of biological diversity.

The investigation of both 'glacial expansion' of coastal populations and regional EC dynamics is often problematic in continental species for several reasons, including (i) the need for intense sampling in coastal areas and putative refugia, which is not always feasible in studies at the scale of the whole species range, (ii) the situation when entire coastal and refugial areas that existed during glacial periods were re-flooded during the following interglacial, and (iii) the occurrence of secondary contact and admixture between lineages from different refugia, which can erase any local genetic imprints of past demographic expansion and EC dynamics.

Mediterranean islands constitute a promising setting for investigating the occurrence of (coastal) glacial expansions of temperate species and the evolutionary consequences of EC dynamics because (i) climate change was attenuated (Thiede 1978), thus allowing the survival of at least a portion of the insular biota also during glacial cooling, (ii) islands have a broad expanse of coastline along which the availability of terrestrial habitats dramatically increased during glacial sea-level lowstands, and (iii) sea-level glacial/interglacial oscillations encapsulate a pure EC dynamic of insular habitats due to fluctuating island size. In this respect, terrestrial species inhabiting archipelagos where islands are separated by short stretches of shallow water provide a controlled, natural experiment on the microevolutionary and demographic processes primed by the climate-related Pleistocene changes. Species ranges followed cycles where the population occupied a single unit of land as individual islands became connected during the sea-level lowstands during glacials, but then became fragmented into smaller populations on single islands genetically isolated by a uniform physical barrier to gene flow (the surrounding sea) as sea-levels rose during interglacial periods (see Jordan *et al.* 2005; Papadopoulou *et al.* 2009).

The Maltese archipelago and its endemic lizard *Podarcis filfolensis* offer an optimal model system for investigating the demographic and evolutionary consequences of past climate-related habitat contractions and expansions. The Maltese archipelago is located 96 km south of the Sicilian land mass and 300 km north of the North African coast and comprises two major islands, Malta (246 km^2) and Gozo (67 km^2) and with a smaller island Comino (3 km^2) between, each surrounded by numerous islets and offshore rocks (Fig. 1). Palaeontological and palaeogeographical studies have established that the past changes in sea level
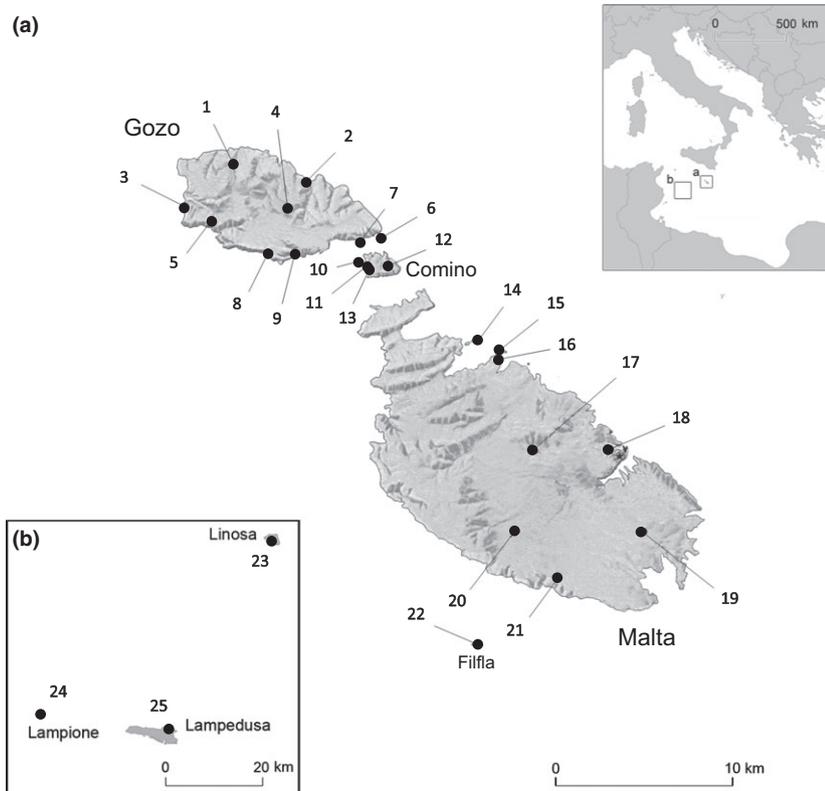
Fig. 1 Map of the study area showing sampling locations of the Maltese wall lizard, *Podarcis filfolensis*, on the Maltese (a) and the Pelagian (b) archipelagos. Detailed information on sampling localities and specimens is found in Table 1.

that started following the Zanclean transgression at the end of the Messinian, approximately 5.33 Mya, and continued during the Plio-Pleistocene glacial/interglacial cycles, have been responsible for the assembly and evolution of the Maltese biota (reviewed in Hunt & Schembri 1999; Pedley *et al.* 2002, 2007; Schembri 2003). Indeed, the southern Mediterranean location of the Maltese Islands and the surrounding sea buffered considerably the Maltese climate during periods of glacial cooling (Thiede 1978; Schembri 1997; Hunt & Schembri 1999). Therefore, climate probably played a subordinate role in biogeographical and evolutionary dynamics of the Maltese biota relative to eustatic sea-level changes, while the contribution of tectonic phenomena can be ruled out since major tectonic movement of the Maltese archipelago ceased around 1.6 Mya (Pedley 2011), and regional uplift completed by 125 kya during the MIS 5 (Pedley *et al.* 2002; Pedley 2011). Past sea-level changes caused repeated connection and separation of individual Maltese islands and pronounced change in their size and shape (Pedley *et al.* 2002; Micallef *et al.* 2013). The Maltese wall lizard *P. filfolensis* with its very limited over-sea dispersal capacity is especially suitable for studying the demographic and evolutionary effects of such dynamics as it occurs on all islands and most of the islets of the Maltese archipelago, as well as on the adjacent

Pelagian Islands (Lampedusa, Linosa and Lampione), which are part of the same geological unit as the Maltese Islands and, except for the volcanic Linosa, share the same geological and geographical history (Pedley *et al.* 1978; Pedley 1990; Gatt 2007). Moreover, the phylogenetic relationships between Sicilian and Maltese *Podarcis* species and the palaeogeographical connections between the Maltese Islands and Sicily are well established (Pedley *et al.* 2002; Harris *et al.* 2005), allowing inferences to be made regarding tempo and mode of the biogeographical origin of *P. filfolensis*. Furthermore, a study of the allozyme variation of five populations of this species (Capula 1994a) revealed high genetic polymorphism but low interpopulation divergence, which may be consistent with a Pleistocene evolutionary scenario.

In this study, we investigated the phylogeography and historical demography of the Maltese wall lizard using both mitochondrial and nuclear genetic markers. Our aims were (i) to establish the temporal and spatial context of population EC dynamics, (ii) to assess whether diversity patterns in this Mediterranean insular system have been shaped mainly by climatic or sea-level Pleistocene oscillations and (iii) to discuss our findings in the context of the formation of patterns of genetic diversity and distribution of temperate biota in coastal and refugial areas.

Materials and methods

Study species

The Maltese wall lizard, *Podarcis filfolensis* (Bedriaga 1876), is a polytypic species endemic to the Maltese and Pelagian archipelagos (see Fig. 1 and Table 1). Five nominal subspecies of *P.filfolensis* are currently recognized: *P. f. filfolensis* (Bedriaga 1876) on Filfla islet (off Malta), *P. f. maltensis* Mertens, 1921 on Malta, Gozo (=Ghawdex) and Comino (=Kemmuna), *P. f. generalensis* (Gulia 1914) on Fungus Rock islet (=General's Rock = Il-Hagra tal-General) (off Gozo), *P. f. kieselbachi* (Fejervary 1924) on Selmunett islet (=St Paul's Islands = Il-Gzejjer ta' San Pawl), and *P. f. laurentiimuelleri* (Fejervary 1924) originally on the Pelagian islands Linosa and Lampione, where it may not be native (Capula 1994a), and recently introduced to Lampedusa (Lo Cascio *et al.* 2005). The population on Cominotto islet (= Kemmunett) (off Comino) has been regarded as a distinct subspecies, but has not been named (Savona Ventura 1983).

Sample collection and laboratory procedures

In total, 98 Maltese wall lizards were collected from 25 sampling sites spanning all the main islands of the Maltese and Pelagian archipelagos and every islet and offshore rock where this species occurs. Thus, our sampling includes all the five named subspecies, the subspecies inquirenda from Cominotto and the supposedly introduced populations from the Pelagian Islands of Lampedusa, Lampione and Linosa. The geographical location of each sample is reported in Table 1 and shown in Fig. 1, and the sample size for each analysed gene is reported in Table 2. In addition, two samples of *P. wagleriana* from mainland Sicily and of *P. raffonei* from the Aeolian archipelago (Sicily) were included in the analyses. Tail tips were removed from field-collected lizards and stored in 96% ethanol; afterwards, all the lizards were released at the capture point.

Total genomic DNA was extracted from tail muscle following standard high-salt protocols (Sambrook *et al.* 1989). Portions of four genes were amplified by polymerase chain reaction: the mitochondrial NADH dehydrogenase subunit 4 with flanking tRNAs tRNA[Ser], tRNA[His] and tRNA[Leu] (hereafter *nd4*), and three nuclear fragments, the Melanocortin receptor 1 (hereafter *mc1r*) and the anonymous loci *pod15b* and *pod55* (Pereira *et al.* 2013). The primers used for amplification and respective references are reported in Table 3. Amplifications were conducted following conditions described in previous studies [*nd4*: Salvi *et al.* (2010); *mc1r*: Pinho *et al.* (2010);

Table 1 Geographical location and taxonomy of the 25 populations of *Podarcis filfolensis* used in this study

Sample	Subspecies	Locality*	Longitude	Latitude
1	*P. f. maltensis*	Wied il-Ghasri, Gozo Island	14.225	36.072
2	*P. f. maltensis*	Ramla l-Hamra, Gozo Island	14.281	36.060
3	*P. f. generalensis*	Fungus Rock Islet	14.188	36.046
4	*P. f. maltensis*	Ggantija, Gozo Island	14.266	36.045
5	*P. f. maltensis*	Ta' Sarraflu, Gozo Island	14.209	36.037
6	*P. f. maltensis*	Gebla tal-Halfa Islet	14.337	36.027
7	*P. f. maltensis*	Tac-Cawl Islet	14.321	36.024
8	*P. f. maltensis*	Ta' Cenc, Gozo Island	14.251	36.018
9	*P. f. maltensis*	Mgarr ix-Xini, Gozo Island	14.272	36.018
10	*P. f. subsp. inquirenda*	Cominotto Islet (Kemmunett)	14.320	36.013
11	*P. f. maltensis*	Il-Bibien (larger) Islet	14.326	36.011
12	*P. f. maltensis*	Comino Island (Kemmuna)	14.342	36.011
13	*P. f. maltensis*	Il-Bibien (smaller) Islet	14.328	36.009
14	*P. f. kieselbachi*	Selmunett Islet (St. Paul's Islet)	14.407	35.966
15	*P. f. maltensis*	Ta' Fra Ben, Malta Island	14.425	35.960
16	*P. f. maltensis*	Qawra, Malta Island	14.425	35.955
17	*P. f. maltensis*	Balzan, Malta Island	14.450	35.901
18	*P. f. maltensis*	Manoel Island, Malta Island	14.507	35.902
19	*P. f. maltensis*	Zejtun, Malta Island	14.532	35.853
20	*P. f. maltensis*	Siggiewi, Malta Island	14.437	35.854
21	*P. f. maltensis*	Zurrieq, Malta Island	14.469	35.825
22	*P. f. filfolensis*	Filfla Islet	14.409	35.787
23	*P. f. laurentiimuelleri*	Linosa Island	12.867	35.864
24	*P. f. laurentiimuelleri*	Lampione Islet	12.317	35.550
25	*P. f. laurentiimuelleri*	Lampedusa Island	12.595	35.509

*Islands with surface area <1 km^2 are designated as islets.

Table 2 Sample sizes, codes and frequencies of the mitochondrial and nuclear haplotypes, and estimates of haplotype diversity (*h*) and nucleotide (*π*) diversity for the studied populations of *Podarcis filfolensis*. For geographical and taxonomic details on sampling locations, see Table 1

Sample	nd4				mc1r				pod15b				pod55			
	Haplotypes (*n*)	Sample size	*h* (SD)	$\pi*10^2$ (SD)	Haplotypes (*n*)	Sample size	*h* (SD)	$\pi*10^2$ (SD)	Haplotypes (*n*)	Sample size	*h* (SD)	$\pi*10^2$ (SD)	Haplotypes (*n*)	Sample size	*h* (SD)	$\pi*10^2$ (SD)
1	h14 (2); h3 (2); h13 (1)	5	0.80 (0.16)	0.12 (0.03)	m1 (2); m3 (1); m4 (2); m5 (2); m6 (1); m7 (1); m17 (1)	5	0.89 (0.08)	0.70 (0.07)	p1 (4); p3 (2); p9 (1); p10 (1)	4	0.75 (0.14)	0.23 (0.07)	d1 (3); d2 (1); d5 (1); d7 (2); d8 (1)	4	0.64 (0.18)	0.18 (0.06)
2	h3 (3)	3	0.00 (0.00)	0.00 (0.00)	—	—	—	—	p1 (2); p3 (2)	2	0.83 (0.22)	0.48 (0.17)	d1 (2); d4 (1); d6 (1); d9 (1); d10 (1)	3	0.93 (0.12)	0.32 (0.07)
3	h13 (9); h14 (1)	10	0.20 (0.15)	0.05 (0.04)	m1 (10); m2 (1); m6 (1); m9 (1); m16 (1)	7	0.51 (0.16)	0.23 (0.09)	p1 (1); p3 (3); p5 (4); p12 (1); p23 (1)	5	0.80 (0.10)	0.38 (0.05)	d1 (2); d2 (1); d3 (5); d4 (1); d8 (1)	5	0.76 (0.13)	0.28 (0.06)
4	h1 (1); h2 (1)	2	1.00 (0.50)	0.23 (0.12)	m1 (1); m4 (1); m6 (2)	2	0.83 (0.22)	0.58 (0.19)	p6 (1); p24 (1)	1	—	—	d1 (2)	1	—	—
5	h3 (3); h14 (1); h24 (1)	5	0.70 (0.22)	0.19 (0.08)	m1 (2); m3 (1); m4 (2); m5 (2); m6 (1); m7 (1); m17 (1)	5	0.93 (0.06)	0.59 (0.07)	p1 (2); p4 (1); p13 (1); p14 (2); p25 (1); p26 (1)	4	0.93 (0.08)	0.60 (0.10)	d5 (1); d7 (2); d11 (1)	2	0.67 (0.2)	0.16 (0.05)
6	h3 (2)	2	0.00 (0.00)	0.00 (0.00)	—	—	—	—	p1 (2); p4 (1); p6 (1); p12 (1); p27 (1)	3	0.93 (0.12)	0.62 (0.15)	d1 (3); d3 (1)	2	0.50 (0.27)	0.12 (0.06)
7	h20 (1); h21 (1)	2	1.00 (0.50)	0.58 (0.29)	m9 (1)	1	—	—	p9 (1); p28 (1)	—	—	—	d1 (1); d12 (1)	1	—	—
8	h5 (1); h6 (1)	2	1.00 (0.50)	0.35 (0.17)	—	—	—	—	—	—	—	—	—	—	—	—
9	h6 (2)	2	0.00 (0.00)	0.00 (0.00)	—	—	—	—		1	—	—	—	—	—	—

Table 2 *Continued*

Sample	nd4 Haplotypes (n)	nd4 Sample size	nd4 h (SD)	nd4 π*10² (SD)	mc1r Haplotypes (n)	mc1r Sample size	mc1r h (SD)	mc1r π*10² (SD)	pod15b Haplotypes (n)	pod15b Sample size	pod15b h (SD)	pod15b π*10² (SD)	pod55 Haplotypes (n)	pod55 Sample size	pod55 h (SD)	pod55 π*10² (SD)
10	h16 (5); h3 (1); h6 (1)	7	0.52 (0.21)	0.09 (0.04)	m1 (1); m2 (1); m3 (1); m9 (2); m11 (1); m18 (1); m19 (1)	4	0.96 (0.08)	0.45 (0.07)	p1 (2); p11 (1); p13 (1); p15 (1); p16 (1)	3	0.93 (0.12)	0.53 (0.12)	d3 (2); d5 (4); d8 (1); d13 (1)	4	0.75 (0.14)	0.35 (0.08)
11	h9 (2); h10 (1)	3	0.67 (0.32)	0.16 (0.07)	m2 (1); m4 (1); m9 (1); m20 (1)	2	1.00 (0.18)	0.53 (0.13)	p7 (1); p17 (1); p30 (1); p31 (1)	2	1.00 (0.18)	0.39 (0.10)	d1 (2); d2 (2); d4 (1); d5 (1)	3	0.87 (0.13)	0.29 (0.07)
12	h3 (2); h6 (1); h8 (1); h21 (1); h25 (1); h26 (1)	7	0.95 (0.10)	0.20 (0.04)	m2 (3); m3 (2); m4 (1); m6 (3); m9 (1); m10 (1); m21 (1)	6	0.89 (0.06)	0.38 (0.06)	p1 (1); p4 (1); p7 (1); p10 (1); p16 (1); p17 (1); p18 (2); p32 (1); p33 (1); p34 (1); p35 (1)	6	0.99 (0.04)	0.63 (0.08)	d1 (3); d2 (1); d3 (1); d4 (3); d6 (1); d14 (1)	5	0.87 (0.09)	0.35 (0.08)
13	h16 (2)	2	0.00 (0.00)	0.00 (0.00)	m3 (3); m22 (1)	2	0.50 (0.27)	0.32 (0.17)	p1 (1); p36 (1)	1	—	—	d1 (1); d3 (1)	1	—	—
14	h3 (7); h23 (2); h22 (1)	10	0.51 (0.16)	0.06 (0.02)	m1 (6); m2 (1); m3 (3); m5 (3); m12 (3); m13 (1); m23 (1)	9	0.84 (0.06)	0.50 (0.05)	p1 (6); p2 (7); p6 (1); p8 (3); p37 (1)	9	0.75 (0.06)	0.39 (0.06)	d1 (2); d2 (3); d4 (3)	4	0.75 (0.1)	0.26 (0.04)
15	h18 (2); h13 (2)	3	0.67 (0.31)	0.55 (0.26)	—	—	—	—	p1 (2); p15 (1); p19 (2); p38 (1)	3	0.87 (0.13)	0.28 (0.06)	d6 (4)	2	0.00 (0.00)	0.00 (0.00)
16		3	0.67 (0.31)	0.08 (0.04)		3	0.60 (0.22)	0.16 (0.07)	p1 (1); p29 (1)	3	0.73 (0.16)	0.37 (0.10)		2	1.00 (0.18)	0.36 (0.09)

Table 2 *Continued*

Sample	nd4 Haplotypes (n)	nd4 Sample size	nd4 h (SD)	nd4 $\pi*10^2$ (SD)	mc1r Haplotypes (n)	mc1r Sample size	mc1r h (SD)	mc1r $\pi*10^2$ (SD)	pod15b Haplotypes (n)	pod15b Sample size	pod15b h (SD)	pod15b $\pi*10^2$ (SD)	pod55 Haplotypes (n)	pod55 Sample size	pod55 h (SD)	pod55 $\pi*10^2$ (SD)
	h27 (2); h15 (1)				m2 (4); m3 (1); m24 (1)				p1 (3); p4 (2); p7 (1)				d1 (1); d5 (1); d6 (1); d9 (1)			
17	h7 (2)	2	0.00 (0.00)	0.00 (0.00)	m1 (1); m5 (2); m25 (1)	2	0.83 (0.22)	0.64 (0.18)	p1 (2)	1	—	—	d1 (1); d2 (1)	1	—	—
18	h27 (2); h28 (1)	3	0.67 (0.31)	0.31 (0.15)	m2 (1); m7 (4); m26 (1)	3	0.6 (0.22)	0.11 (0.04)	p1 (2); p39 (1); p40 (1)	2	0.83 (0.22)	0.39 (0.15)	d1 (1); d15 (1)	1	—	—
19	h15 (1)	1	—	—	m1 (1); m2 (1); m6 (1); m7 (1)	2	1.00 (0.18)	0.43 (0.1)	p1 (2)	1	—	—	—	—	—	—
20	h15 (2); h19 (1)	3	0.67 (0.31)	0.08 (0.04)	m1 (1); m11 (2); m27 (1)	2	0.83 (0.22)	0.43 (0.12)	p1 (2); p11 (1); p20 (1)	2	0.83 (0.22)	0.39 (0.13)	d1 (2)	1	—	—
21	h19 (1)	1	—	—	m3 (1); m28 (1)	1	—	—	—	—	—	—	—	—	—	—
22	h11 (6); h12 (1)	7	0.29 (0.20)	0.13 (0.09)	m2 (1); m3 (2); m10 (1); m14 (2)	3	0.87 (0.13)	0.39 (0.08)	—	—	—	—	d2 (2)	1	—	—
23	h3 (3); h4 (1)	4	0.50 (0.26)	0.12 (0.06)	m4 (1); m8 (3)	2	0.50 (0.27)	0.64 (0.34)	—	—	—	—	—	—	—	—
24	h17 (2)	2	0.00 (0.00)	0.00 (0.00)	m8 (2)	2	0.50 (0.27)	0.64 (0.34)	—	—	—	—	—	—	—	—
25	h13 (3)	3	0.00 (0.00)	0.00 (0.00)	m1 (2); m4 (2); m13 (1); m29 (1)	3	0.87 (0.13)	0.68 (0.12)	—	—	—	—	—	—	—	—

Table 3 Primers used in this study

Gene	Primer	Primer sequence	Reference
nd4	ND4	5′ - CAC CTA TGA CTA CCA AAA GCT CAT GTA GAA GC - 3′	Arévalo *et al.* (1994)
	Leu	5′ - CAT TAC TTT TAC TTG GAA TTT GCA CCA - 3′	
mc1r	MC1RF	5′ - GGCNGCCATYGTCAAGAACCGGAACC - 3′	Pinho *et al.* (2010)
	MC1RR	5′ - CTC CGR AAG GCR TAG ATG ATG GGG TCC AC - 3′	
pod15b	pod15bf	5′ - AAT CCT GGC TAA ATG CAA GCC TTG G - 3′	Pereira *et al.* (2013)
	pod15br	5′ - GCC AGG AGA ATA AGC TAC TCC ATC C - 3′	
pod55	pod55f	5′ - GGA TCT TTA TAG GAG AGT GCA GGC C - 3′	Pereira *et al.* (2013)
	pod55r	5′ - TTC CAG ATT GTG TTT ATC CTG GTG G - 3′	

pod15b, and *pod55*: Pereira *et al.* (2013)]. Purification and sequencing of PCR products were carried out by a commercial company, Macrogen (www.macrogen.com).

Data analysis

Sequences were visually checked and aligned using Bioedit v 7.1 (Hall 1999) and the Clustal X (Thompson *et al.* 1994) extension implemented therein. The haplotype phase of nuclear genes was inferred using the coalescent-based Bayesian algorithm in PHASE v. 2.1 (Stephens *et al.* 2001; Stephens & Donelly 2003) as implemented in DnaSP v. 5.10.01 (Librado & Rozas 2009). The possible occurrence of recombination events was assessed using the Pairwise Homoplasy Index (PHI) test (Bruen *et al.* 2006) implemented in SPLITS-TREE v. 4 (Huson & Bryant 2006). Sequence divergence (*p*-uncorrected distance) was assessed using MEGA v. 5 (Tamura *et al.* 2011). To assess patterns of genetic diversity, we computed the number of haplotypes (*n*), haplotype diversity (*h*) and nucleotide diversity (π) with their standard deviations (SD) for each gene fragment using DnaSP.

We inferred the genealogical relationships between haplotypes using a network approach, which is the most appropriate method for intraspecific gene evolution, particularly when few characters for phylogenetic analysis are available due to shallow levels of divergence (Posada & Crandall 2001). Statistical parsimony networks were constructed under the 95% probability criterion for a parsimonious connection employing the software TCS v. 1.21 (Clement *et al.* 2000).

To define the genetic structure of *P. filfolensis* in the Maltese archipelago, we used the spatial analysis of molecular variance (SAMOVA) approach implemented in SAMOVA v. 1.0 (Dupanloup *et al.* 2002). This method allows defining groups of geographically proximate populations that are maximally differentiated from each other, through a simulated annealing procedure without the prior assumption of group composition. The program was run with 100 random initial conditions for 10,000 iterations, testing for the grouping options from a predefined number of groups (*K*) = 2–10, excluding the samples composed of single individuals (samples 19 and 21) and the Pelagian samples (samples 23–25) as they are likely introduced (see results and discussion).

In order to estimate the time to the most recent common ancestor of *P. filfolensis* and the evolutionary rate for the *nd4* fragment, we used the least-squares method (Xia & Yang 2011) as implemented in DAMBE v. 5.2 (Xia & Xie 2001). This distance-based method allows a fast and accurate estimate of divergence times, providing the data set conforms to the molecular clock hypothesis and a proper substitution model is used for estimating distances (Xia & Yang 2011). The general time reversible (GTR) model was selected from 88 models as the best model of sequence evolution under the Akaike's information criterion (AIC) using JModeltest v. 2 (Darriba *et al.* 2012). The GTR model was used to generate a distance matrix from which a phylogenetic tree was derived using the neighbour-joining (NJ) method in MEGA v. 5, including *P. raffonei*, and the sister species *P. wagleriana* as outgroups. Using the same software, the molecular clock test was performed by comparing the maximum-likelihood (ML) value for the given topology with and without the molecular clock constraints under the GTR model. Based on this test, the null hypothesis of equal evolutionary rate throughout the tree was not rejected at a 5% significance level. Given the possible time dependency of the molecular clock (Ho *et al.* 2005; but see also Bandelt 2008), we use both an old and a recent calibration point in order to minimize the bias due to rate variation across temporal scales. As calibration points, we used (i) the palaeogeographical event resulting in the isolation of the Maltese Islands group from the Sicilian landmass – and thus presumably to the vicariance between the ancestor of the Maltese lizard *P. filfolensis* from its sister species the Sicilian *P. wagleriana*, – and (ii) the age of the split between *P. wagleriana* and its allied species, the Aeolian wall lizard *P. raffonei*. The first vicariance event took place after the reopening of the Gibraltar Strait 5.33 Mya; during this time, a rapid refilling of the Mediterranean

basin occurred, after desiccation during the Messinian Salinity Crisis (Krijgsman *et al.* 1999; Pierre *et al.* 2006), likely promoting vicariance and speciation in several Mediterranean groups including *Podarcis* lizards (Poulakakis *et al.* 2005; Pinho *et al.* 2006; Brown *et al.* 2008). The split between *P. wagleriana* and *P. raffonei* is much more recent and has likely occurred during the Pleistocene – about 700 kya – when the volcanic islands of the Aeolian archipelago emerged (Maramai *et al.* 2005) and were presumably colonized by the *wagleriana*-like ancestor as inferred from genetic distance data between *P. wagleriana* and *P. raffonei* by Capula (1994b). The dating procedure was performed using the previously estimated tree topology, and the composite ML genetic distance implemented in DAMBE for the Tamura—Nei substitution model (Tamura *et al.* 2004; Xia 2009). The use of the simultaneous estimated (SE) ML composite distance is recommended over the independently estimated (IE) distances allowed by the GTR model (Xia 2009). Confidence intervals of the time estimates were assessed by 1000 bootstrap replicates.

To check the consistency of dating results, we also estimated divergence times using the BEAST v. 1.7.4 package (Drummond *et al.* 2012). Although computationally more intensive than distance-based methods, BEAST allows the incorporation of the uncertainty associated with phylogenetic estimates, calibration dates and among-lineage variation of substitution rates in a single analysis (Drummond *et al.* 2012). We used the normal distribution to incorporate the calibration priors on the root of the tree ($\mu = 5.325$, $\sigma = 0.001$) and on the split between *P. wagleriana* and *P. raffonei* ($\mu = 0.695$, $\sigma = 0.001$). We performed both strict clock and lognormal relaxed clock analyses assuming the GTR model of sequence evolution and a coalescent tree prior. All BEAST analyses were run twice, with 100 million iterations per run, sampling every 10 000 steps. Convergence diagnostic and summary of posterior samples of parameters were assessed in TRACER v. 1.5 (Rambaut & Drummond 2007), and sampled trees from independent runs were combined in LogCombiner (burn-in = 2500). As results using strict and relaxed clock models were similar, we summarized only results based on strict clock analyses in TreeAnnotator.

The historical demography of the Maltese wall lizard was assessed by a suite of statistical tests for detecting changes in population size over time and space for the main lineages recovered by the phylogenetic and SAMOVA analyses. First we performed a demographic reconstruction by means of an Extended Bayesian Skyline Plot analysis (EBSP) implemented in BEAST. The EBSP combines information from multiple loci, thus incorporating the stochastic differences between gene genealogies in the estimation of population parameters.

As a further advantage over previous skyline methods, the EBPS approach is not constrained *a priori* to a specific model of historical population size change (Heled & Drummond 2008; Ho & Shapiro 2011). This was ensured using a Poisson prior with mean $\lambda = 0.693$ for the number of population changes (ψ), which places an equal probability (50% prior weight) to a constant and to a nonconstant population size. To allow for continuous population size dynamics, a piecewise linear model was used. As the quality of sequence data is crucial for an accurate EBSP estimate of population size dynamics (Heled & Drummond 2008), we verified consistency of results between two runs using either all the nuclear sequences or only those sequences phased with a probability higher than 0.9. The EBSP analysis was run using strict clock models, substitution models and trees unlinked across all markers. The *nd4* substitution rate was set according to the estimated value in DAMBE and used as a reference to estimate relative rates for nuclear genes. Three runs each of 3×10^8 generations sampled at 10^4 intervals were performed and checked for convergence and effective sample size of parameters (ESSs) with TRACER. The EBS plots were generated using the Python scripts provided in Heled (2010). To assess the time frame at which the EBSP reconstructions have high reliability, we generated a detailed demographic function description following Heled (2010) and we inspected the number of demographic functions which had an X point at each interval of time.

Additionally, independent assessments of demographic expansion were obtained by analysing mtDNA pairwise nucleotide differences (mismatch distribution) and through Tajima's *D* (Tajima 1989), Fu's F_S (Fu 1997), and Ramos-Onsin and Roza's R_2 (Ramos-Onsins & Rozas 2002) tests. Mismatch distribution analyses were carried out in ARLEQUINN v. 3.5 (Excoffier & Lischer 2010) comparing the observed distribution of nucleotide differences between pairs of haplotypes with those expected under demographic (Rogers & Harpending 1992) and spatial (Excoffier 2004) expansion models, using a generalized least square approach. This allowed estimation of the age of the expansion as the time in generations *t*, given the parameter τ and the mutation rate per sequence and per generation *u* ($\tau = 2ut$). We used the sum of squared deviations (SSD) as goodness-of-fit statistics for the observed and expected mismatch distributions. We tested the significance of the fit of the expansion model and the confidence intervals for the associated parameters estimates using 10 000 bootstrap replicates. The statistics *D*, F_S and R_2 were used to test for an excess of singleton mutations in each clade genealogy, which is an expected consequence of population expansion. We used DnaSP to estimate the *D*, F_S, and R_2 values, and we assessed their significance through

10 000 coalescent simulations under the hypothesis of population equilibrium and selective neutrality.

Results

The mitochondrial DNA sequences (855 bp) from 94 individuals from 25 populations comprised 40 variable positions. The nuclear DNA data sets consisted of 130 sequences for *mc1r* (628 bp), 108 for *pod15b* (522 bp) and 86 for *pod55* (416 bp) with 21, 34 and 11 polymorphic positions, respectively (see Table S1, Supporting information for GenBank accession numbers). An overview of genetic data including estimates of haplotype (*h*) and nucleotide (π) diversity per locus for each population is given in Table 2. When comparing the mitochondrial diversity of samples from the three main islands (Gozo, Comino and Malta) with their neighbouring islets, higher values were observed in the former, whereas low diversity was found on islets with the exception of Tac-Cawl and Selmunett islets (Table 2). Pelagian samples showed either low values (Linosa Island) or a complete lack (Lampione and Lampedusa islands) of mitochondrial diversity. Nuclear diversity was high in most of the localities of sample size >5 except for the Fungus Rock islet samples where low nucleotide diversity was observed at all loci.

The phylogenetic relationships between the mitochondrial haplotypes, as inferred by the parsimony approach, are represented by a single network (maximum connection steps at 95% probability = 12), which shows a clear phylogeographical structure (Fig. 2A). Twenty-six mtDNA haplotypes clustered in two main clades (*p*-distance between lineages = 1.29%): the Maltese clade, grouping haplotypes from most samples of Malta and Filfa (in green colours in Fig. 2A), and the Gozitan clade, grouping haplotypes from Gozo and Comino and their neighbour islets, Selmunett islet and Ta' Fra Ben (Malta) and the Pelagian islands (in blue colours in Fig. 2A). Two haplotypes, sampled in four individuals from two Maltese samples, Balzan and Ta' Fra Ben (in yellow in Fig. 2A), were differentiated from both the main clades by at least 5 mutational steps. Otherwise, haplotypes within clades are connected by a maximum of three mutational steps, while a minimum of eight steps separated the two main clades. The phylogenetic networks depicting the relationships between nuclear haplotypes (maximum connection steps at 95% probability ranging from 8 to 10) and the haplotype distribution among samples show that nuclear diversity is not geographically structured (Fig. 2B). The number of nuclear haplotypes observed in each sample (with size>2) ranged from 2 to 11; most haplotypes were restricted to a single locality (65%, 33% and 55%, of *mc1r*, *pod15b* and *pod55* haplotypes, respectively), and

the remaining ones were mostly shared by the three main islands (Table 2 and Fig. 2).

The SAMOVA analysis on the mtDNA data set (Fig. 3) identified three groups of populations ($K = 3$) as the grouping option which best explains the among-group spatial partitioning of molecular variance. Higher values of K produced genetic structures which either showed lower F_{CT} values (from $K > 6$) or similar F_{CT} values, but their population groupings were less informative (i.e. from $K = 4$–6, where one population at a time is removed from the groups structure). The three-group genetic structure identified by the SAMOVA explains 79.30% of the overall molecular variance ($F_{CT} = 0.79$; $P < 0.001$), 7.86% was explained by differences among-populations within-group ($F_{SC} = 0.38$; $P < 0.001$), and 12.84% by differences within populations ($F_{ST} = 0.87$; $P < 0.001$). The population composition of the three groups strictly reflects the geographic partition of haplotype lineages, as the first group included the samples carrying Gozitan clade haplotypes (samples 1–14), the second those carrying Maltese clade haplotypes (samples 16, 18–22) and the third the samples Ta' Fra Ben and Balzan (samples 15, 17), which carried haplotypes distinct from both the main clades. SAMOVA analyses based on nuDNA data did not allow identification of a population grouping best explaining the data (see Fig. S1, Supporting information). Indeed, F_{CT} values never exceeded 0.22, 0.24 and 0.34 for the *mc1r*, *pod15b* and *pod55* loci, respectively, whereas for the same loci, molecular variance within populations (F_{ST}) was above 0.70, 0.71 and 0.57.

The age of the most recent common ancestor of the mitochondrial lineages of *P. filfolensis* estimated in DAMBE was 476 kya (±138 ky SD). Time estimates for the most recent common ancestor for the two main clades were 183 kya (±81 ky SD) and 224 kya (±105 ky SD) for the Gozitan and Maltese clades, respectively (see Fig. S2A, Supporting information). According to the calibration used, the estimated *nd4* substitution rate was 1.15% (±0.12% SD) per million years. This value is in agreement with substitution rates previously proposed for *Podarcis* lizards for this gene fragment (Pinho *et al.* 2007). Dating results from BEAST were concordant with DAMBE estimates, placing the divergence between the main clades of *P. filfolensis* at 576 kya (95% high posterior density interval: 331–848 kya) and the divergence within the Gozitan and Maltese clades at 229 (115–351) kya and 171 (65–295) kya, respectively (Fig. S2B, Supporting information). The similarity between divergence times and associated confidence intervals estimated in BEAST and DAMBE corroborates the results from the simulation study by Xia and Yang (2011) suggesting that the latter method is not only very simple and extremely fast, but also accurate.
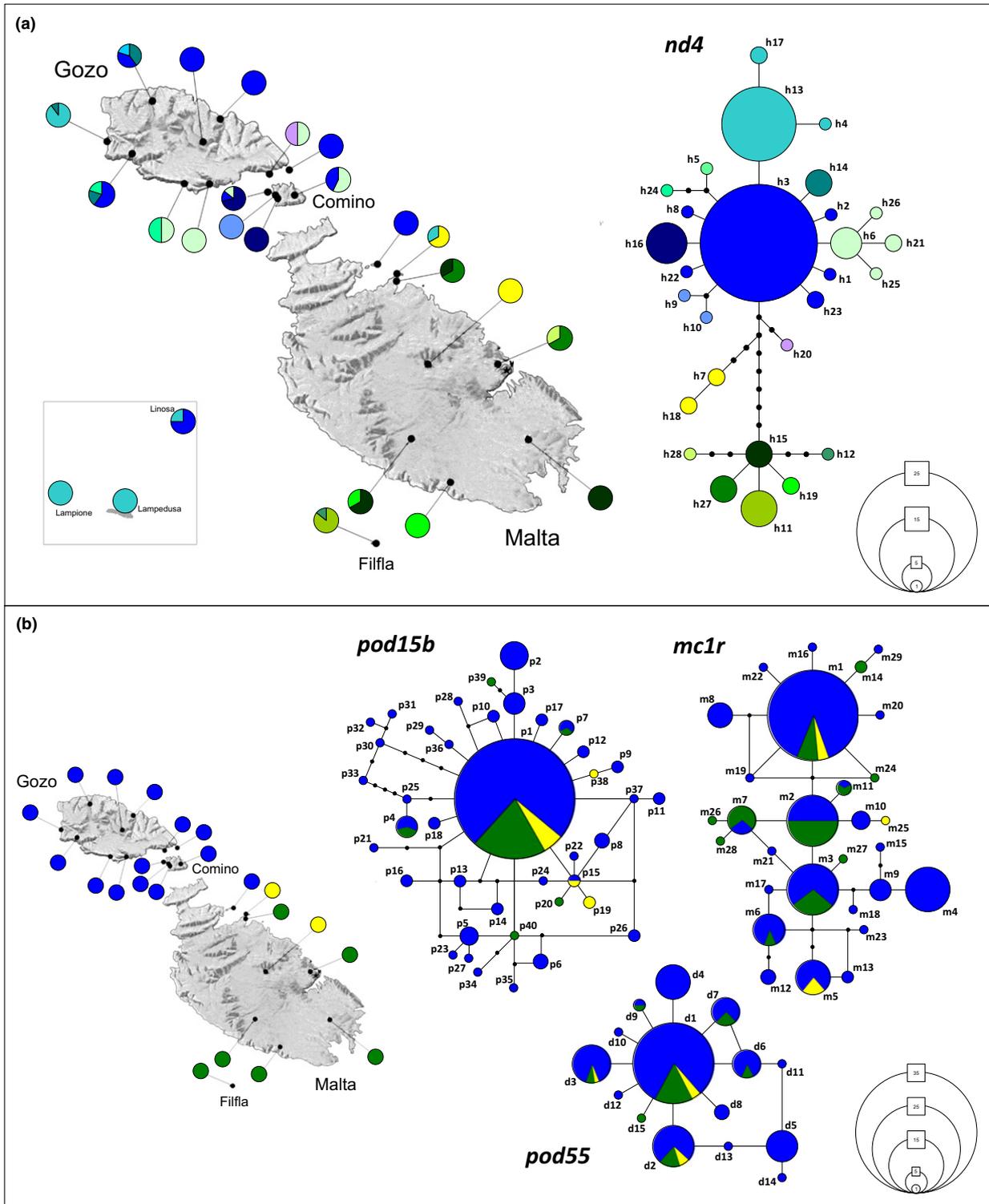
Fig. 2 Geographical distribution and genealogical relationships between the mitochondrial (*nd4*: box a) and the nuclear (*mc1r*, *pod15b* and *pod55*: box b) haplotypes of *Podarcis filfolensis*. In the map of box a, pie diagrams show the geographical distribution and frequencies of haplotypes in each population; in the map of box b, the populations are coloured according to the geographic distribution of the main mitochondrial lineages as presented in box a. Statistical parsimony networks show the phylogenetic relationships between the haplotypes, which are represented by circles with size proportional to their frequencies and with small filled circles representing 'missing' or extinct haplotypes (right). For geographical details on sampling locations, see Table 1, for sample sizes, codes and frequencies of the mitochondrial and nuclear haplotypes see Table 2.
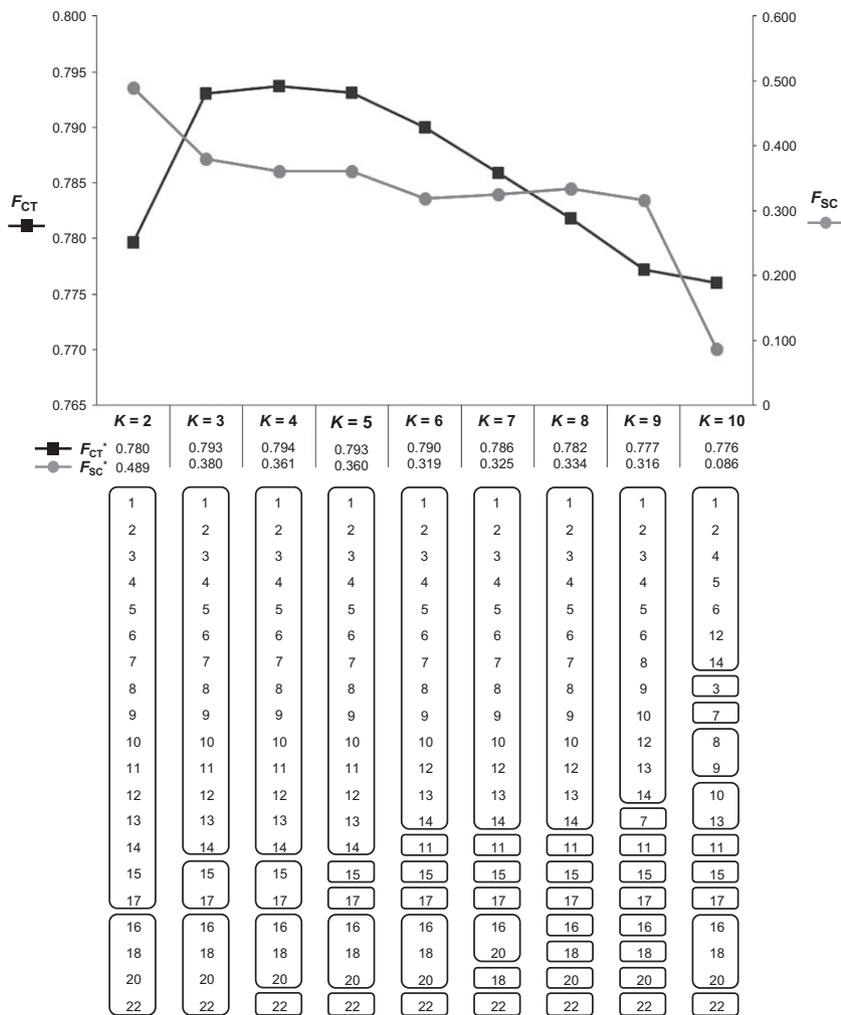
Fig. 3 Summary of results of spatial analysis of mitochondrial molecular variance (mtDNA SAMOVA) in *Podarcis filfolensis* populations. Associated with each predefined value of K from 2 to 10 is reported the group composition for the best clustering option and relative fixation indices F_{CT} and F_{SC} ($P < 0.001$).

The results from EBSP indicate that the Gozitan clade experienced a demographic expansion during the last 110 kya (see Fig. 4B). The occurrence of a historical population expansion for this clade was also supported by significant D, F_S and R_2 values (as well as their sign and magnitude) and by the mismatch analyses (see Fig. S3, Supporting information). The observed mismatch distribution was not significantly different from that expected either under a pure demographic expansion model (SSD = 0.005; $P = 0.153$), or under a sudden spatial expansion model (SSD = 0.005; $P = 0.095$). Given the value of parameter τ and the mutation rate estimated in DAMBE, considering the generation time of *Podarcis* species (estimated mean generation time = 2.09 years; Galán 1999; see also Barbault & Mou 1988; Galán 1996; Adamopoulou & Valakos 2000), the time since the spatial-demographic expansion of the Gozitan clade is estimated to have occurred during the first half of the last glacial period (83 kya; confidence interval: 59–114 kya).

For the Maltese clade, the EBSP shows a trend of recent expansion from 100 to 20 kya (see Fig. 4C). The posterior probability of the number of demographic changes was the highest when it was 1, and 95% credible intervals (HPD) excluded 0 for all runs thus rejecting the hypothesis of a constant population size. However, the wide credibility intervals suggest that the signal is not strong. Also the mismatch distribution is consistent with that expected under a model of population expansion (SSD = 0.019; $P = 0.192$). On the other hand, the nonsignificant D, F_S and R_2 values (as well as their sign and magnitude) suggested a history of demographic stability (see Fig. S4, Supporting information).

Discussion

The evolutionary history and historical demography of the Maltese wall lizard, as inferred from its pattern of genetic diversity, provide insights on how this species coped with Pleistocene palaeoecological and

palaeogeographical changes. Furthermore, these findings have direct implications in the context of the island biogeography of the Maltese archipelago, as well as for the application of the EC model in coastal settings and refugial areas. The evolutionary history of *P. filfolensis* is therefore discussed in the context of these issues.

Evolutionary history of Podarcis filfolensis

The phylogenetic network and the spatial analysis of molecular variance show the existence of two main mitochondrial phylogroups within *Podarcis filfolensis*: one occurring on the island of Malta and its satellite islet Filfla (Maltese lineage) and the other one mainly distributed in the northern islands of the Maltese archipelago (Gozo and Comino islands and their surrounding islets) and in the Pelagian islands (Gozitan lineage) (Fig. 1). Two haplotypes found in four lizards from Malta were fairly differentiated from both these lineages (in yellow, in Fig. 2). They may represent a poorly sampled mitochondrial clade endemic to NE Malta or an ancestral polymorphism perhaps from an ancient Gozitan stock (currently extinct) that expanded to Malta during an early glacial stage. The low frequency of these haplotypes and their occurrence in only two nonadjacent sites led us to consider them as the result of the retention of an ancestral polymorphism rather than as a further phylogeographical group, but more sampling is needed to address this issue.

In contrast to mitochondrial data, nuclear sequence data do not show any phylogeographical structure across the study area. Indeed, the nuclear genetic patterns of *P. filfolensis* show high diversity, shallow haplotype divergence and no significant differences in haplotype frequencies between populations, suggesting a low divergence rate and historically high effective population size of nuclear markers. Thus, difference in rates of lineage sorting due to the fourfold difference in effective population sizes between the haploid, uniparentally inherited mitochondrial genome and the nuclear genome may explain their discordant patterns (Brown *et al.* 1979; Moore 1995; Hare 2001) as already reported in several phylogeographical studies based on nuclear and mitochondrial sequence data (Toews & Brelsford 2012; Bisconti *et al.* 2013; Salvi *et al.* 2013). Alternative explanations for the observed cytonuclear discordance could be either sex-biased gene flow with stronger female phylopatry (e.g. Ujvari *et al.* 2008; reviewed in Prugnolle & Meeus 2002) or stochastic processes acting on mtDNA and generating a spurious phylogeographical break (Irwin 2002); however, both seem unlikely in this case. Indeed, the fact that the main lineages are endemic to different islands makes it highly improbable that the observed phylogeographical break arose as a consequence of a stochastic process rather than as result of allopatric divergence. Moreover, although in *Podarcis* lizards, males usually disperse more than females (e.g. Vignoli *et al.* 2012), in the case of *P. filfolensis,* it is hard to believe that while females carrying Gozitan haplotypes would have moved only a few kilometres (approximately 20 km, cf. samples 14–15 in NE Malta) during a hundred thousand years, during the same period males have been extensively carrying nuclear alleles between the Gozitan and the Maltese compartments. Therefore, although male-biased gene flow may have contributed to the observed pattern, considering the relatively recent estimated time frame for the mitochondrial divergence between the two lineages (see below), a failure of the nuclear markers to diverge and differentiate appears the most plausible hypothesis for the observed genetic patterns (Palumbi *et al.* 2001; Hudson & Turelli 2003; Toews & Brelsford 2012). Data from hypervariable nuclear markers, such as microsatellites, may help in reaching a firm conclusion on this issue.

The coalescence time of the mitochondrial lineages and their divergence dates back to the Middle Pleistocene (around 500 kya; Fig. S2, Supporting information). The most prominent driver for the palaeogeographical and palaeoenvironmental evolution of the Maltese archipelago during the entire Pleistocene was undoubtedly associated with sea-level changes due to glacial-interglacial cycles. In particular, in the Maltese archipelago interglacials were occasions for allopatric divergence for those species having limited over-sea dispersal ability such as lizards, as their populations were separated on different islands by rising sea level (see e.g. Pedley 2011). Remarkably, interglacial periods prior to 480 kya were apparently longer and colder than the typical interglacials of the latest Quaternary, as documented by palaeoclimatic records (EPICA Community Members 2004). Thus, a prolonged isolation on distinct islands of the Maltese palaeoarchipelago during the Middle Pleistocene seems a particularly plausible scenario for the allopatric divergence between the main lineages of *P. filfolensis*.

The geographical distribution of the Gozitan and the Maltese lineages and their estimated divergence time suggest that these lineages from the Middle Pleistocene survived the climatic oscillations in separate refugia in the northern and in the southern portion of the Maltese archipelago, respectively. However, while during interglacial periods, these lineages were likely confined on distinct islands, during glacial periods the wide connection between Maltese islands suggests that these lineages would have had occasion for demographic and range expansion, and admixture following secondary contact.

The historical demographic analyses based on four independent assessments consistently indicate a marked population and spatial expansion of the Gozitan lineage during the last glacial period (Fig. 4B; Fig. S3, Supporting information). According to the palaeogeographical reconstruction of the Maltese archipelago based on high-resolution seafloor geological data (Micallef *et al.* 2013; see also Pedley *et al.* 2002), there was a noticeable increase in the size of Gozo island during this period, which was merged with Comino and Malta in a single macro-island because of the glaciation-induced sea-level drop. As a consequence of such marine regression, newly exposed lowlands were readily available for colonization by terrestrial organisms from all over the palaeo-Maltese landmass and in particular a wide expanse of coastal plain extended on the eastern side (Figs 4A and 5). This area might not only have provided for larger effective population sizes of insular lineages due to an increased carrying capacity, but might also have allowed their spatial expansion. The finding of haplotypes of the Gozitan lineage in two localities in what is now northeasternmost Malta (Selmunett Islet and Ta' Fra Ben, samples 14–15) and the inferred pattern and timing of demographic and spatial expansion for this lineage closely fits in this scenario. This hypothesis is preferable over the alternative explanation of a recent introduction of Gozitan lizards in NE Maltese localities because there we observed multiple haplotypes, in Selmunett Islet also private haplotypes (h22 and h23), and because the human-mediated arrival of Gozitan lizards in this small islet off Malta would have required a two-step introduction process.

The historical demography of the Maltese lineage as inferred by mismatch and EBPS analyses also shows a trend of recent expansion during the last glacial period, but neutrality test statistics did not recover a signal of such a demographic event (Fig. 4C; Fig. S3, Supporting information). Within this group, we observed a strong population differentiation with only one fairly widespread haplotype (h15) and all others being private to one or two populations. Population fragmentation patterns such as this can hinder the detection of past demographic (and spatial) expansion leading to a signal of population stability in mismatch distribution analysis and neutrality test statistics (Wakley 1999; Hein *et al.* 2005; Chikhi *et al.* 2010); therefore, the hypothesis of demographic expansion cannot be rejected for the Maltese lineage.

On the other hand, the lack of evidence for admixture between Maltese and Gozitan lineages suggests that even if both these lineages would have undergone spatial expansions during the last glacial period, demographic exchange between them was still limited. Moreover, the occurrence of two spatially structured
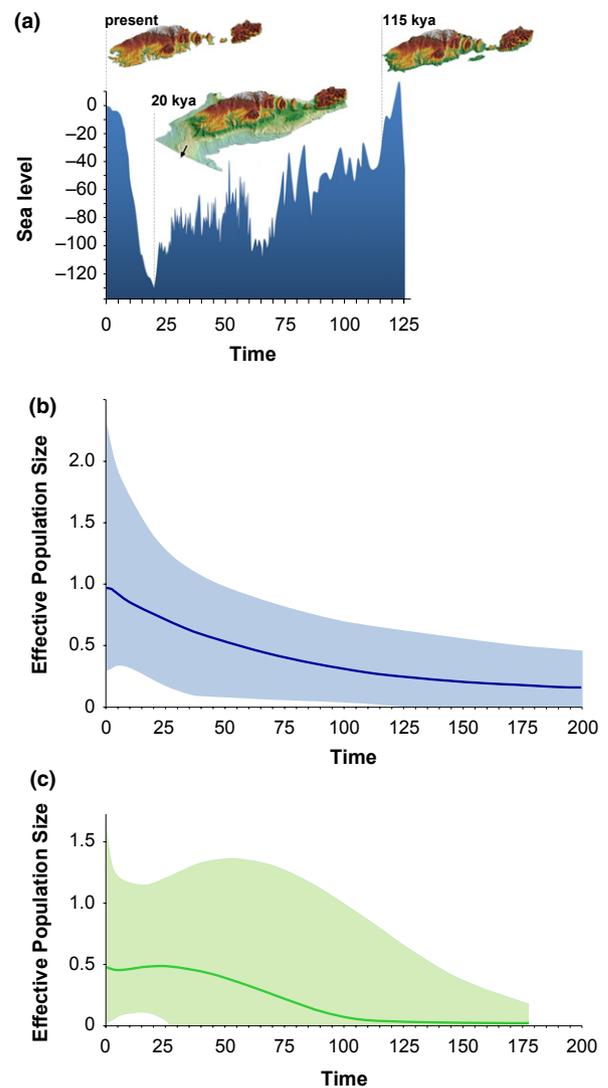

Fig. 4 Sea-level changes during the last 125 kya (redrawn from Siddall *et al.* 2003 and Lambeck *et al.* 2011) and associated 3D Maltese palaeoshorelines reconstructed based on high-resolution seafloor geological data (from Micallef *et al.* 2013) (box a). Historical demographic analysis for the Gozitan (box b) and the Maltese (box c) lineages of *Podarcis filfolensis* as inferred by Extended Bayesian Skyline Plot analyses; along the *y*-axis the effective population sizes are expressed in units of $N_e\tau$ the product of the effective population size per generation length. Solid lines are median estimates, whereas shaded areas represent confidence intervals; prior to 200 kya, the demographic function for the Gozitan lineages (box b) contains few *x*-axis points (data not shown), and so not enough power to pinpoint the exact population size value.

mitochondrial lineages suggests that admixture between these lineages can be ruled out for previous glacial periods as well. Indeed, simulations of coalescence under an island model of population subdivision show that the formation of geographically confined clusters of mitochondrial haplotypes is prevented by even very

Fig. 5 The coastline of the Maltese Islands and Sicily during the Last Glacial Maximum (redrawn from Pedley 2002 and Micallef *et al.* 2013)

low levels of dispersal between them (Papadopoulou *et al.* 2008).

Island biogeography of the Maltese archipelago

The inferred evolutionary history of *P. filfolensis* has at least three important implications for the biogeography of the Maltese archipelago. First, the estimated coalescence time for the mitochondrial haplotypes of *P. filfolensis* dates back to the Middle Pleistocene. Thus, although the genetic variability accumulated prior to that time became extinct without any direct contribution to modern genetic lineages, this species exhibits genetic signatures of long-term persistence in the Maltese palaeoarchipelago throughout a series of full glacial/interglacial cycles since at least the Middle Pleistocene. In keeping with the prediction that in Mediterranean islands, the climatic buffering of the sea may have provided suitable habitats for stable populations throughout the Quaternary (Médail & Diadema 2009), the present study shows that at least two main areas – Gozo and Malta – within the Maltese archipelago acted as 'long-term refugia'.

Second, the inferred evolutionary and demographic history of *P. filfolensis* points to a major role of Pleistocene sea-level fluctuations, rather than of direct effects of climate changes, in the formation and distribution of intraspecific genetic diversity. According to available palaeontological data, sea-level changes shaped the assembly and evolution of the entire Maltese biota

(reviewed in Hunt & Schembri 1999; Pedley *et al.* 2002, 2007; Schembri 2003). Interestingly, while interglacial high-stands primed the allopatric differentiation between populations from Gozo and Malta, on the other hand despite these islands being extensively connected during glacial low-stands, demographic and genetic interchange between their populations was limited, suggesting that the Gozitan and Maltese compartments evolved as independent biotic units during the Pleistocene.

Third, the genetic analysis of lizards from the Maltese and the Pelagian archipelagos provides useful information on biotic exchange between islands across the Sicilian Channel. Lizards from the Pelagian islands (samples 23–25) carry exclusively mitochondrial haplotypes of the Gozitan lineage and in particular those having the highest frequency on Gozo (h3 and h13, Fig. 2). The small population of Lampedusa has been introduced in the last decade from nearby Linosa (Lo Cascio *et al.* 2005), while the haplotype composition and the low genetic diversity of Lampione and Linosa (see Table 3) suggest that *P. filfolensis* colonized the Pelagian islands from the Maltese archipelago in historical times as argued by Capula (1994a) based on allozyme data. Furthermore, our data clearly indicate Gozo as the most probable source island for this colonization. Although the Pelagian and the Maltese archipelagos shared most of their early geological origin as part of the Pelagian block (Pedley *et al.* 1978; Grasso & Pedley 1985; Pedley 1990; Gatt 2007), they have had independent histories since the end of the Messinian Salinity Crisis during the Miocene (approximately 5.33 Mya; Krijgsman *et al.* 1999; Pierre *et al.* 2006), as the deep sea channel between them (up to 1000 m) prevented their connection during the Plio-Pleistocene marine regressions (Pedley *et al.* 2002). Thus, for the Pelagian *P. filfolensis* populations, an origin through long-distance rafting or human transportation is suggested (see Capula 1994a). Although it is difficult to disentangle whether this species colonized the Pelagian Islands by natural means or through human agency, the latter scenario seems more plausible considering the small size of the Pelagian islands (and thus low colonization rates) and in the light of the pattern of interisland variation observed within the Maltese archipelago, which clearly indicates very limited over-sea dispersal for *P. filfolensis*.

Perspectives on the EC model of Pleistocene biogeography

The Maltese archipelago and the endemic lizard *P. filfolensis* present a simplified experiment for testing two key predictions of the EC model: (i) temperate species experienced a maximum demographic/spatial

contraction during glacial stages and (ii) the high level of intraspecific genetic diversity harboured in glacial refugia is a direct consequence of the persistence of large and demographically stable populations.

The inferred evolutionary and demographic history of *P. filfolensis* clearly does not fit the prediction from the EC model of populations of temperate species contracting in refugia during glacials. This basic prediction is based on the scenario whereby during glacials, as a consequence of global cooling, temperate species suffered a global reduction of habitat availability. However, this model, mainly centred around the terrestrial biota of continental Europe, does not account for geographical settings such as found in coastal areas where (local) climate conditions were not too severe during glacials, and terrestrial habitat availability dramatically increased as a consequence of glacio-eustatic sea-level lowering (Thiede 1978; Rohling *et al.* 1998). In these conditions, a converse EC response for populations of temperate species may be expected throughout the Pleistocene cycles with glacial (demographic/spatial) expansions during sea-level low-stands and interglacial contractions owing to contraction of islands size. Apart from *P. filfolensis*, glacial expansion of temperate species has been recently inferred for populations occurring in coastal lowlands both in Mediterranean islands (Bisconti *et al.* 2011) and in continental setting (Canestrelli *et al.* 2007; Canestrelli & Nascetti 2008). Similarly to *P. filfolensis*, in the tree frog *Hyla sarda* endemic to (Tyrrhenian) Mediterranean islands, the estimated time since the expansion was placed in the first half of the glacial period (Bisconti *et al.* 2011) during a period well known for rapid and extensive glacio-eustatic sea-level lowering (of nearly −50 m between 80 and 70 kya; Ruddiman *et al.* 1980; Shackleton 1987; Larsen & Sejrup 1990). In the mainland species *Hyla intermedia* and *Pelophylax lessonae*, genetic data suggest that demographic expansions might have occurred in coastal refugial areas of the NE Italian Peninsula where the glaciation-induced increases in the extent of coastal plains overcame the negative demographic effects of glacial climate (Canestrelli *et al.* 2007; Canestrelli & Nascetti 2008). In all these cases, the scenario of glacial expansion of coastal populations is supported by independent evidence from palaeogeographical, sedimentological, palaeontological (fossil and pollen) and palaeodistribution model data (see references in Canestrelli *et al.* 2007; Canestrelli & Nascetti 2008; Bisconti *et al.* 2011). Moreover, spatial expansions of temperate species during glacial marine regressions have recurrently been invoked to explain dispersal of temperate species between islands of Mediterranean archipelagos and/or with the continent (e.g. Fattorini 2002; Salvi *et al.* 2011; Garnatje *et al.* 2012; Rodriguez *et al.* 2013; see also Thompson 2005; Blondel *et al.* 2010).

Thus, notwithstanding that the ranges and populations of temperate species have had an expansion–contraction response to Pleistocene climatic change, to predict the timing of these EC dynamics it is necessary to extend the simple EC model for continental species by including the relative effect of climate and sea-level variations on habitat availability. Although a considerably smaller number of studies have been made on coastal/insular populations compared with continental populations, data available up to now strongly suggest that the balance between these effects seems to have had an opposite EC outcome during glacial periods for Western Palaearctic temperate species in coastal as compared to continental ecosystems.

Archipelagos such as the ones studied here offer various insights for understanding what processes were triggered by these EC dynamics and what consequences those processes had on the genetic diversity pattern of temperate species within Pleistocene refugia. The genetic variation observed within *P. filfolensis* is mostly accounted for by the differentiation between two main lineages (79%, Fig. 3) primed by the allopatric fragmentation of the Maltese archipelago during Pleistocene cycles. The evolutionary independence of these lineages was maintained also during glacial stages, despite the islands of this archipelago being merged together in a macro-island of increased surface area and the sea buffering considerably the Maltese climate during periods of cooling (Schembri 1997; Hunt & Schembri 1999). Thus, the evolutionary history of *P. filfolensis* supports a major role for allopatric fragmentation in the *formation* of the observed genetic diversity, while the habitat availability and the long-term stability of Maltese climate during glacials would account for the *maintenance* of such diversity and for the narrow secondary admixture following parapatric contact between lineages. When considering the relevance of the processes of allopatric divergence in the context of the formation of intraspecific diversity, the processes inferred for *P. filfolensis* or other insular systems (e.g. Jordan *et al.* 2005) provide interesting analogues to the processes taking place in Pleistocene glacial refugia. Studies carried out in well-established glacial refugia such as the three Mediterranean peninsulas – Iberia, Italy and the Balkan – suggest that in various species, including amphibians, reptiles and mammals, the high intraspecific diversity observed arose because of allopatric divergence of populations from multiple subrefugia occurring *within* the main refugial areas, rather than as a direct outcome of prolonged stability of large populations within these areas (e.g. Martínez-Solano *et al.* 2006; Sotiropoulos *et al.* 2007; Ursenbacher *et al.* 2008; Vega *et al.* 2010; Miraldo *et al.* 2011; Canestrelli *et al.* 2012; for an overview see Tzedaki 2009; and Nieto

Feliner 2011). In the specific case of southern Italy, EC dynamics until the Middle Pleistocene were indeed associated with the palaeoinsularization of the current mountain massifs of this main refugial area (Canestrelli *et al.* 2010; Vega *et al.* 2010). Here, genetic signatures of allopatric isolation and differentiation have been documented also across small geographical scales, in some of which demographic and spatial expansion of allopatric lineages promoted their secondary admixture generating a melting pot of diversity (Canestrelli *et al.* 2010). Building on this evidence and on the well-established paradigm of refugia-within-refugia (Gómez & Lunt 2007), the cross-archipelago pattern shown in this study illustrates a general model in which genetic diversity is formed and shaped by cycles of allopatric divergence and repeated contact driven by population EC dynamics acting *within* highly compartmentalized refugia.

Conclusion

Since Darwin's first formulation, islands have been used as natural laboratories for developing and testing ecological and evolutionary theories (Darwin 1859; Whittaker & Fernández-Palacios 2007). Indeed, being discrete and isolated spatial entities surrounded by an inhospitable matrix, islands permit a number of simplifying assumptions to be made in empirical and theoretical models, which are relevant also to mainland geographical analogues such as patchy terrestrial and freshwater habitats (MacArthur & Wilson 1967; Whittaker & Fernández-Palacios 2007; but see also Walter 2004).

However, although Mediterranean islands have long been identified as key hotspots and glacial refugia for biodiversity (e.g. Médail & Quézel 1999; Médail & Diadema 2009), they have never been considered in empirical and theoretic models of Pleistocene phylogeography (Dapporto *et al.* 2009). This study corroborates the huge significance of Mediterranean archipelagos for testing, refining and extending these models and also as a simplified setting for studying the evolutionary and demographic processes responsible for the high diversity patterns observed within glacial refugia.

When combined with recent literature on island and coastal populations, the case study of *P. filfolensis* shows how the incorporation of Pleistocene sea-level variations in the EC model allows prediction of a reverse demographic and range response of insular and coastal temperate biotas relative to those of the continent. What is more, the Mediterranean archipelago here studied represents a simplified model of glacial refugia in which allopatric diversity is formed and shaped by cyclic contraction-expansion of habitats during the Pleistocene. A similar scenario has been invoked by a growing number of phylogeographical studies to explain the high genetic diversity of populations from mainland refugia, suggesting a key role of the Pleistocene EC dynamics in shaping the pattern of genetic diversity of temperate species not only into and out-of refugia but also *within* these areas.

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D.S., D.J.H. and P.J.S. designed the research; A.S. collected the samples; D.S. generated the sequence data, analysed the data, and drafted the article; D.J.H. and P.J.S. contributed reagents/materials and critically revised the manuscript; all authors read and approved the final manuscript.

Data accessibility

DNA sequences: GenBank accession numbers KJ027723—KJ027980; for each individual, details on sampling and the GenBank accession number for its sequence data uploaded as online supplemental material (Table S1, Supporting information). DNA sequence alignments: Dryad doi:10.5061/dryad.6hs29. DAMBE and BEAST dating analyses input files and output trees: Dryad doi:10.5061/dryad.6hs29.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Geographical location and codes for the studied specimens of *Podarcis filfolensis* and the outgroup species *Podarcis wagleriana* (*) and *Podarcis raffonei* (**).

Fig. S1 Results of Spatial Analysis of Molecular Variance (SAMOVA) analyses based on nuclear DNA data (loci *mc1r*, *pod15b*, and *pod55*).

Fig. S2 Chronogram showing the estimated times to the most recent common ancestor of the mitochondrial lineages of *P. filfolensis*.

Fig. S3 Historical demographic analysis for the two main mitochondrial lineages of *Podarcis filfolensis*.