BIODIVERSITY RESEARCH

Foundations for conservation of intraspecific genetic diversity revealed by analysis of phylogeographical structure in the endangered endemic lizard *Podarcis lilfordi*

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ABSTRACT

Aim To describe and analyse phylogeographical patterns in the endangered endemic lizard *Podarcis lilfordi* from across its remaining range and thereby establish baseline information on genetic diversity that will help determine conservation priorities and assist future reintroduction programs.

Location Balearic Islands, Spain.

Methods We analysed mitochondrial DNA (2382 bp sequence from eight genes) from 118 individuals and characterized the relationships among haplotypes using parsimony networks, as well as phylogenetic inference. Analyses of historical gene flow and population growth were used to provide further insights into population histories.

Results Four unconnected parsimony networks were obtained that mirrored the main clades in the phylogenetic tree: (I) all Menorcan populations, (II) Dragonera, Malgrats and Toro islands (Western Mallorca) (III and IV) and the remaining populations from Cabrera and Mallorca. Two major haplotype groups were detected in Menorca (I) and these provided signatures of a demographic expansion and asymmetrical historical gene flow, respectively, concordant with the expected direction of colonization from south to north of the island. Populations from western Mallorca (II) showed evidence of historical allopatric fragmentation events following isolation around the start of the Pleistocene. In networks III and IV, Cabreran populations appear to have become isolated from north and south Mallorca quite recently, with asymmetric gene flow indicating a northwards dispersal direction.

Main conclusions *P. lilfordi* is a genetically diverse species that shows substantial mtDNA structuring both between regions and, at a finer scale, between some islet populations within regions. The precarious state of some islet populations shown here to be quite divergent (e.g. Toro island in western Mallorca) means that conservation of this intraspecific biodiversity requires urgent action.

Keywords
Balearic archipelago, conservation biogeography, mtDNA, *Podarcis lilfordi*, phylogeography, migration.

INTRODUCTION

Geographical isolation is one of the most important processes underlying genetic differentiation. Islands constitute perfect choices to study this differentiation because current and historical gene flow is virtually non-existent, and so each island may be regarded as a discrete evolutionary unit. However, this assumption does not always hold, particularly for islands found in shallower seas which may have undergone repeated connections in tandem with falling sea levels. Phylogeographical patterns are likely to reflect this periodic contact across the land bridges (Glor *et al.*, 2004; Gifford *et al.*, 2005). The Mediterranean provides an ideal

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arena for such studies because it contains many small islands with genetically differentiated populations that have spent long periods in isolation. This has been punctuated by occasional, well-documented falls in sea level. It also harbours rich endemic fauna and flora that have evolved through a complex interplay of geological and palaeoclimatic dispersal and vicariance events (Stace, 1989; Blondel & Aronson, 1999).

The genus *Podarcis* is one of the most diverse and abundant reptile groups in southern Europe, with 17 currently recognized species (Harris & Arnold, 1999). The taxonomy of the genus is complex and has been subject to several recent revisions, in part due to the discovery of new phylogenetic relationships through the use of molecular markers (e.g. Poulakakis *et al*., 2003, 2005). It also provides important insights into the historical biogeography of the Mediterranean, due to the substantial intra- and interspecific genetic diversity that it shows (e.g. Capula, 1997; Harris & Arnold, 1999; Harris & Sá-Sousa, 2002; Podnar *et al*., 2005; Pinho *et al*., 2006, 2007). Phylogeographical relationships among insular populations have received particular attention because of the often substantial morphological variation found between island conspecifics (Harris *et al*., 1998; Pérez-Mellado, 1998a,b; Sá-Sousa *et al*., 2002; Arnold *et al*., 2007). To date these analyses have demonstrated biogeographical effects associated with sea level changes and northern hemisphere glaciations, as well as older physical events that predate the desiccation of the Mediterranean during the Messinian Salinity Crisis (MSC). For example, a phylogeographical survey of *Podarcis sicula* populations (representing 52 subspecies) from the Adriatic region has revealed the likely influence of climatic effects, i.e. retreats into refugia during post-Messinian glacial maxima and subsequent postglacial expansions (Podnar *et al*., 2005). In contrast, divergences between some Greek populations of *Podarcis erhardii* are indicative of much older physical events during the Miocene (Poulakakis *et al*., 2003). Many of these species show wide distributions and are associated with continental and island areas leading to quite complex patterns, some of which are further complicated by recent introductions (e.g. Podnar *et al*., 2005).

The Balearic islands should present a simpler system because of their isolation from any major land mass during recent glacials. Two endemic species of *Podarcis* inhabit the archipelago: *Podarcis lilfordi* in the Eastern Gymnesic island group (Mallorca, Menorca, Cabrera and associated islets) and *Podarcis pityusensis* in the Western Pityusic group (Ibiza, Formentera and coastal islets). Current evidence suggests they originated within the *Podarcis* clade that includes *P. tiliguerta* from Sardinia and Corsica rather than from within the Iberian/North African *Podarcis* clade that includes *P. hispanica*, *P. vaucheri* and *P. carbonelli* (Arnold *et al*., 2007). They constitute reciprocally monophyletic species (among others Harris & Arnold, 1999; Arnold *et al*., 2007; Brown *et al*., 2008) that originated during the reflooding of the Mediterranean at the end of the MSC, 5.33 Ma (Krijgsman *et al*., 1999; Duggen *et al*., 2003; Brown *et al*., 2008).

Post-Messinian isolation of the Balearics from continental areas, and their separation into Eastern and Western groups, also appear to have led to independent evolutionary lineages within several other taxa, e.g. within the extinct bovid *Myotragus balearicus* (Lalueza-Fox *et al*., 2000), the plant *Hippocrepis balearica* (Rossello *et al*., 2002) and the midwife toad *Alytes* (Arntzen & García–Paris, 1995, 1997). However, there have been very few detailed analyses of evolution within the island groups to date (but see López de Heredia *et al*., 2005). This could provide new insights into how recent population connections mediated by sea level changes may have moulded fine-scale diversity within Mediterranean islands. The distributions of Balearic *Podarcis* make them ideal subjects for this type of analysis.

When and how might land connections have been established and broken between *P. lilfordi* populations? Unlike the situation between *P. lilfordi* and *P. pityusensis*, where the depth of the intermediate channel will have precluded any contact between since the end of the MSC, repeated connections must have occurred between island populations of *P. lilfordi*. For example, the last major ice ages, namely the Riss (200,000 years ago) and the Würm (25,000 years ago), led to decreases in sea level (relative to present levels) of 130 and 110 m, respectively. During these sea level minima, Mallorca, Menorca and Cabrera became one large island (the 'Gran Balear') (Cuerda, 1989; Gracia & Vicens, 1998). In contrast, the warmer interglacial periods produced marine transgressions that caused sea levels to rise by up to 11 m above present-day levels, causing the subaerial land mass to be reduced by a half and thus fragmenting populations (Cuerda, 1989; Ginés & Ginés, 1993; Goy *et al*., 1997). These events are expected to have modified the genetic content and structure of the populations, and are likely to have left signatures through the accumulation of mutations, the extent of which will be associated with time of separation (Hewitt, 2004).

P. lilfordi is classified as endangered (IUCN), and comprises 43 known extant insular populations: 11 from islets around Mallorca, 16 from Menorcan islets and 16 from the Cabrera archipelago. The species became extinct on the main islands of Mallorca and Menorca during the past few thousand years after the introduction of foreign predators and/or competitors (Kotsakis, 1981; Alcover, 2000). Offshore islands have provided refuges for the lizards in a similar way to that seen in Tuatara species, from New Zealand (Daugherty *et al*., 1990). The latter provides a clear illustration of how a lack of baseline knowledge on intraspecific biodiversity and taxonomy can influence conservation of endangered species: the belief that the Tuatara represented a single monotypic species is thought to have contributed to extinctions of 25% of the remaining populations during the last century (Daugherty *et al*., 1990).

Previous attempts to establish the biodiversity and intraspecific taxonomy of *P. lilfordi* have been based on the considerable morphological variation found between islets. Twenty-eight subspecies have been described, although a smaller number (23) probably better describe the observed morphological diversity (Pérez-Mellado *et al*., 2008). While these taxonomic designations have contributed to the formal protection of many specific islet populations of *P. lilfordi*, knowledge of the historical component of the intraspecific biodiversity is still lacking. This is of vital importance when formulating conservation plans because it provides a relatively objective criterion on which priorities can be assessed. One of the main aims of this study was to examine the species history of *P. lilfordi*, primarily through analysis of mtDNA markers.

Figure 1 Geographical locations of *Podarcis lilfordi* populations under study. Populations are represented by name or by a two or three letters code with the first letter representing the name of the island group (M, Mallorca; m, Menorca; C, Cabrera Archipelago). The subsequent letter relates to the site from which the population was captured.

A preliminary analysis of six *P. lilfordi* populations (Terrasa *et al.*, 2004) was found to have inadvertently included nuclear mitochondrial DNA (numts) sequences. We have now corrected this and additionally obtained samples covering nearly all extant islet/island populations of the species. This allows us to undertake a detailed analysis of *P. lilfordi* to achieve our two objectives of: (1) defining the distributions of the main evolutionary lineages to help guide future conservation strategies, and (2) an analysis of intraspecific diversity in relation to the historical connections between islands.

METHODS

Samples

A total of 118 individual *P. lilfordi* from 43 localities covering all living subspecies and 41 of the 43 populations known to date were included in the analysis (Fig. 1, Table 1). One individual of *P. pityusensis* (from Pityusic Islands) was sequenced for use as an outgroup. One to five individuals were analysed for each locality. The tail tip of each individual was clipped off and stored in 100% ethanol. Live animals were released at the site of capture.

DNA isolation and sequencing

Total genomic DNA was extracted using the same protocol as that used for tail-tips from other lizards (Gonzalez *et al.*, 1996) with minor modifications. The following partial mitochondrial genes were amplified using polymerase chain reaction and sequenced: 12S rRNA, cytochrome *b* (two regions obtained separately), control region and an 800 bp (ND) fragment that included part of the ND1 gene, three tRNA genes, $tRNA_{Ile}$, $tRNA_{Gln}$, and $tRNA_{Met}$ and part of the ND2 gene. The total length of mitochondrial sequence analysed for each animal was 2382 bp. A

Table 1 Localities sampled, population identifier (ID), haplotype number in bold and number of individuals in bracket.

Locality	ID	Haplotypes
Menorca Island (m)		
Bledes	mBM	**2**(2)
Sanitja	mS	**10**(3), **16**(1)
Tosqueta	mT	**13**(5)
Rovells	mRo	**1**(2), **13**(1),
Porros de Fornells	mPr	**13**(2)
Sargantana	mSt	**1**(1), **4**(1), **6**(1)
Addaia Gran	mAg	**1**(3), **5**(1), **11**(1)
Addaia Petita	mAp	**1**(1), **18**(2)
Carbó	mCb	**17**(1), **19**(1), **20**(1)
Aguiles	mSA	**3**(1)
Colom	mCm	**1**(2), **14**(1)
Mel	mMe	**12**(2), **15**(2)
Rei	mRe	**7**(1), **9**(1)
Aire	mA	**8**(2)
Binicodrell gros	mCI	**15**(2)
Binicodrell petit	mCII	**15**(3)
Mallorca Island (M)		
Dragonera	MD	**22**(1), **23**(1), **24**(1), **25**(1)
Malgrat Gran	MMg	**21**(2)
Malgrat Petit	MMp	**21**(2)
Toro	MTo	**26**(3)
Porrassa	MPs	**28**(2)
Guardia	MGu	**31**(2)
Moltona	MMo	**27**(1), **31**(1)
Pelada	MPe	**31**(2)
Caragol	MCa	**31**(2)
Colomer	MCr	**43**(1), **44**(2), **45**(1)
Cabrera Archipelago (C)		
Foradada	Cfo	**41**(2), **42**(1)
Pobre	CPo	**35**(2)
Plana	CPl	**30**(1), **32**(1), **40**(1)
Conillera	CCo	**29**(1)
Rodona	CRoad	**33**(1), **34**(1)
Esponja	CEj	**39**(3)
Cabrera Gran (port)	CCp	**36**(1), **38**(1), **50**(1), **54**(1)
Cabrera Gran (far)	CCf	**62**(1), **63**(1)
Cabrera Gran (Miranda)	CCm	**37**(1), **58**(1), **59**(1)
Bledes	CB	**49**(3), **60**(1)
Fonoll	CFl	**52**(3), **55**(1)
Imperial	CI	**49**(1), **56**(1), **57**(1)
Rates	CRt	**53**(2)
Estell Xapat	CX	**47**(1), **48**(1)
Estell de s'Esclata-sang	CEt	**46**(2), **61**(1)
Estell des Coll	CEc	**49**(1), **51**(1)
Estell de Fora	CEf	**47**(2)

fragment of the nuclear gene *c-mos* (397 bp) was also sequenced. Primers and amplification conditions are given in Appendix. Both heavy and light strands were sequenced for most regions on an automated ABI 3130 sequencer using a Taq DyeDeoxy™ Terminator Cycle sequencing kit (Applied Biosystems Inc., Palo Alto, CA, USA). Sequences (GenBank: EF694760–EF694792, EF694794–EF694817, EF990517–EF990552) were aligned within BioEdit version 7.0.5.2 (Hall, 1999).

Haplotype network and phylogenetic analyses

Haplotypes were identified for the concatenated sequences and a haplotype network was constructed using the program TCS version 1.21 (Clement *et al.*, 2000). TCS creates a network using statistical parsimony (Templeton *et al.*, 1992; Templeton & Sing, 1993) which outputs the 95% plausible set of most parsimonious linkages among sequences.

We considered the robustness of the clades detected by the parsimony network by comparison with a phylogenetic tree obtained using Bayesian inference on the haplotypes (MrBayes version 3.0; Huelsenbeck & Ronquist, 2001). Intraspecific sequence divergence was relatively low, so applying a single evolutionary model to the concatenated sequence was preferable to applying distinct models to different partitions because some of the latter would have been phylogenetically uninformative (although preliminary analyses revealed similar results between the two approaches). Model selection was based on comparison of likelihoods of neighbour-joining trees under different models of DNA evolution (MrModeltest: Nylander *et al.*, 2004). An individual *P. pityusensis* from Ibiza island was used as the outgroup. Bayesian MCMC analyses were conducted in parallel with random starting trees, run 1.5×10^6 generations, and sampled every 100 generations using a general-time-reversible model of evolution. In both sampling chains, stationarity of the Markov Chain was determined by stable split-standard deviations (between the two runs) and stable sampled log likelihood values. 'Burn-in' data sampled from generations preceding this point were discarded. All data collected at stationarity were used to estimate posterior nodal probabilities and a majority-rule consensus tree. Two independent replicates were conducted and inspected for consistency to check for local optima (Huelsenbeck & Bollback, 2001).

Genetic diversity, AMOVA and substitution rate

Basic genetic diversity indices were calculated for each gene and also for the entire fragment. To measure genetic differentiation between the four haplotype networks obtained by TCS and between groups formed within some of them, we conducted an analysis of molecular variance (AMOVA) using an analogue of Wright's F_{ST} (1951) which accounts for pairwise distances between haplotypes. AMOVA and pairwise difference tests were performed in the program Arlequin version 3.11 (Excoffier *et al.*, 2005). Neutrality of mutations are assumed in most of the analyses we used, so we tested this using Tajima's test (Tajima, 1989) as implemented in DnaSP version 4.0 (Rozas *et al.*, 2003). The substitution rate for the concatenated sequence was estimated from the divergence between *P. lilfordi* and *P. pityusensis* based on vicariance between them 5.33 million years ago. There is strong evidence to support divergence of these taxa around this time: (1) the Gymnesic and Pityusic Islands became isolated at this time, (2) very close correspondence with the expected degree of divergence in well-characterized molecular markers (Brown

et al., 2008) and (3) independent node estimates from other studies (Arnold *et al.*, 2007). Although this provided only simple estimates, it was done for comparison with recent phylogenetic dating of major clade divergence times (Brown *et al.*, 2008).

Demographic and migration events

Fu's F_S test of neutrality uses estimates of the parameter θ to detect excesses of younger or older mutations. An excess of young mutations, assuming neutrality and an infinite sites model, can provide evidence of population expansions and produce large negative values of F_S (Fu, 1997). Mismatch distributions were calculated for each network to examine changes in population size. Under the infinite-sites model, mismatch distributions are relatively smooth and unimodal under population expansion but ragged and generally multimodal for stationary populations (Harpending *et al.*, 1998). Fu's F_S and parameters for the mismatch distributions were calculated using Arlequin version 3.11 (Excoffier *et al.*, 2005).

A maximum-likelihood (ML) method based on the coalescent (as implemented in MIGRATE version 2.0.6; Beerli & Felsenstein, 1999; Beerli & Felsenstein, 2001) was used to assess migration rates between different regions within the archipelago. This has advantages over equilibrium approaches because it estimates long-term (i.e. historical) rates for a given direction, and so allows assessment of historically asymmetrical gene flow. Analyses required pooling of populations from the same geographical areas (e.g. Menorcan islets) which violates certain assumptions of MIGRATE. However, this only impedes accurate estimation of population size, with robust assessments of gene flow remaining feasible (Beerli & Felsenstein, 2001; Pfenninger & Posada, 2002; Pfenninger *et al.*, 2003). An ML estimate of transition: tranversion ratio was obtained for a neighbour-joining tree, using PAUP (Swofford, 2002), and used as input. Starting values for other parameter estimates were obtained using F_{ST} on the first run, with ML estimates being input into subsequent runs. These were carried out with differing input trees and random number seeds and checked for consistency. Ten short chains (50,000 steps, sampling interval 500, first 100 samples discarded as burn-in) and two long chains (500,000 steps, sampling interval 100, first 100 samples discarded) were run, with adaptive heating of chains used in all runs. Ten replicates were performed.

RESULTS

Haplotypes and sequences

A total of 63 different haplotypes defined by 190 polymorphic sites were characterized from the studied mitochondrial fragment. We observed 153 transitions and 42 transversions from a total of 195 substitutions. A unique within-species indel in $tRNA_{Ile}$ was detected. A neutral pattern of variation was observed when we applied both Tajima's test ($D = 0.899$, $P > 0.05$) and Fu's test ($F_S = -1.038$, $P > 0.05$). The most variable gene studied in terms of the proportion of variable positions per site was cytochrome *b*, with the next being ND2 (Table 2). As generally expected at these

Table 2 Summary statistics for studied genes. Note that 'All mtDNA' refers to the concatenated mtDNA sequences.

Gene	Base pairs	Indels	Variable positions total	First	Second	Third	Ratio ti/tv	A + T (%)	No. of different haplotypes	Nucleotide diversity	Mean pairwise differences	Haplotype diversity	Fu's F_S statistic	*D* Tajima
Cyt *b*	834	no	92	17	4	71	74/23	60.75	42	0.025 ± 0.012	21.032 ± 9.348	0.911 ± 0.021	-0.533^{ns}	0.710^{ns}
12SRNA	373	no	13	5	5	3	12/0	56.50	8	0.010 ± 0.005	3.631 ± 1.846	0.756 ± 0.022	3.979^{ns}	1.342^{ns}
RCIII	481	no	27	11	10	6	15712	69.99	22	0.014 ± 0.003	6.833 ± 3.240	0.910 ± 0.011	-0.572^{ns}	1.034^{ns}
ND1	68	no	8	0	5	3	6/2	64.75	10	0.017 ± 0.012	1.556 ± 0.752	0.712 ± 0.019	-3.007^{ns}	-0.543^{ns}
$tRNA^{Ile}$	73	yes	7	2	1	4	5/2	50.30	7	0.009 ± 0.008	0.638 ± 0.501	0.478 ± 0.050	-2.654^{*}	-1.176^{*}
$tRNA^{Gln}$	71	no	1	0	1	0	1/0	56.31	2	0.000 ± 0.001	0.034 ± 0.098	0.034 ± 0.023	$-1.624^{\dagger}$	$-0.910^{\dagger}$
$tRNA^{Met}$	68	no	0	0	0	0	0	57.36	1	0	0	0	0	0
ND2	415	no	42	10	4	28	40/3	59.71	29	0.028 ± 0.014	11.716 ± 5.344	0.884 ± 0.021	-0.013^{ns}	1.506^{ns}
All mtDNA	2382	yes	190	46	30	115	153/42	61.33	62	0.019 ± 0.009	45.552 ± 19.881	0.979 ± 0.005	-1.038^{ns}	0.899^{ns}
c.mos	397	no	2	0	0	2	0/2	55.42	3	0.0004 ± 0.0006	0.550 ± 0.231	0.157 ± 0.065	-1.599^{ns}	-1.050^{ns}

$^{*}P < 0.10$, $^{\dagger}P < 0.05$, ^{ns}not significant.

intraspecific levels, only negligible variation was detected in the studied nuclear sequence (*c-mos*: 387 bp): single, unique substitutions were detected in two Mallorcan populations with an A-T transition in the Toro population (position 237) and a G-T transversion (in heterozygotic form) in the Colomer population at position 24. All remaining populations were identical. Note that the *c-mos* analyses were based on only 57 individuals, due to the lack of variation in this marker. We excluded *c-mos* from subsequent analyses due to the lack of phylogenetic information it provided.

Phylogeny and sequence divergence

The Bayesian tree (Fig. 2), indicated several well-supported major clades, the first cladogenesis event (A) led to the separation of Menorcan individuals from the remaining lineages. The second (B) separated the populations from the western Mallorcan islands from the remaining Mallorcan and Cabreran islands. The subsequent node (C) represents cladogenesis of populations from Mallorcan islets to the north east and south of the island and those from the northern Cabrera islets from the remaining Cabreran populations. Finally, D is subdivided into two additional clades, D1 corresponding to Cabrera and coastal islets, and D2 to the three Estells islets (southern coast of Cabrera island). This phylogenetic pattern appears very robust, as it provides the same topology as that found by a broader phylogenetic analysis that included both Balearic species of *Podarcis* (but contained fewer *P. lilfordi*) (Brown *et al.*, 2008). The main clades also mirrored the distinct networks and subgroups within these networks detected under the probability of parsimony criterion (see later). Two recent introductions were detected. One of these was detected in the Porrassa population, an island located in western Mallorca, near to Toro and Malgrats islands. Lizards from this population were genetically similar to those from Cabrera archipelago (although they showed a unique change with respect to the islets of Pobre, Moltona and Conillera). Translocation of individuals to this islet was subsequently confirmed by the Natural Environment Council of the Balearic Islands Government. Similarly, an individual from Plana (Cabrera Archipelago) shared a haplotype with lizards from Dragonera island (Mallorca), and was excluded from the analyses.

The average (total) net sequence divergence (Da) at the *P. lilfordi*/*P. pityusensis* root was 0.04815 ± 0.00830, providing an estimated substitution rate under a molecular clock of 0.0045 ± 0.0008 changes per site per million years, calculated following Nei (1987)(formula 10.22). This suggests an isolation time for the first clade (A, Menorca) from remaining populations of around 2.88–2.66 Ma. The western Mallorcan islands (clade B) would similarly correspond to an isolation time of 2.30–2.20 Ma. The remaining divergence times (between and within clades C and D) appear more recent, estimated at < 600 Ka in all cases.

Haplotype network

The 63 haplotypes formed four unconnected networks under the 95% probability of parsimony criterion (see Fig. 3). The distributions of these networks are given in Fig. 4. Network I corresponds to the most basal phylogenetic lineage (clade A, Menorca) and Network II to clade B (west Mallorca, i.e. Dragonera, two Malgrats islets, and Toro islet; the latter being separated by 11 mutational steps). Network III represents clade C, northeast and southern Mallorcan populations together with populations from islands located in the channel between Mallorca and Cabrera (i.e. Conillera, Plana, Pobre and Rodona) as well as some individuals from the main island of Cabrera. Network IV (South Cabrera, clade D) contains most individuals from the main island of Cabrera as well as associated islets, such as Bledes, Imperial, Fonoll and Rates. This contains a clade separated by 16 mutational steps and represents haplotypes from three of the most southern insular populations, Estell Xapat, Estell de Fora and two samples from Estell de s'Esclata-sang, situated to the south of Cabrera (Fig. 2). The genetic structuring of *P. lilfordi* among these four geographical groups was confirmed using tests of differentiation between pairs and an AMOVA among the four networks (within and between population variation being 92.46% and 7.54%, respectively) and calculation of a fixation index ($F_{ST} = 0.075$, $P < 0.001$).

Network I (Menorcan populations) contains two genetically well differentiated ($F_{ST} = 0.111$, $P = 0.002$) subsets of haplotypes: one with haplotype 1 and its derivatives and another with haplotype 15 and derivatives. The two subsets occupy partially overlapping geographical distributions (Fig. 4). Population expansion within the first subset is supported by: (1) significantly large negative values of F_S, and (2) the parameters of the mismatch distributions that do not differ significantly from a sudden-expansion model (see Table 3). However, no evidence of an expansion was detected in the second subset. Analyses of gene flow in this network indicated an asymmetrical pattern, with northward gene flow between the two subsets (Table 4).

Within network II, the two haplotype groups corresponded to: (1) Dragonera, and (2) Malgrats and Toro islands. Toro and Malgrats are separated by 11 and six mutational steps, respectively, from the Dragonera haplotypes, with the level of differentiation being significant ($F_{ST} = 0.725$, $P < 0.001$).

Haplotypes from Colomer island (northeast Mallorca, network III) show an odd pattern in that the three haplotypes from Colomer are separated from each other by a minimum of 10 mutational steps. However, Colomer haplotypes are all well differentiated from south Mallorca ($F_{ST} = 0.374$, $P < 0.001$), but are not significantly differentiated from Cabrera samples ($F_{ST} = 0.086$, $P = 0.063$), despite being geographically more distant.

Network IV haplotypes from three small islets known as the Estells (Estell de Fora, Estell Esclata-sang and Estell Xapat) are statistically differentiated ($F_{ST} = 0.143$, $P < 0.001$) from the remaining haplotypes (South Cabrera) by 16 mutational steps, with approximately symmetrical gene flow being detected between them (Table 3, Fig. 4). The only case of substantial asymmetrical gene flow between Networks was detected between this network and network III (Table 4), with the inferred direction being from South (South Cabrera, network IV) to North (Cabrera and channel islets, network III).

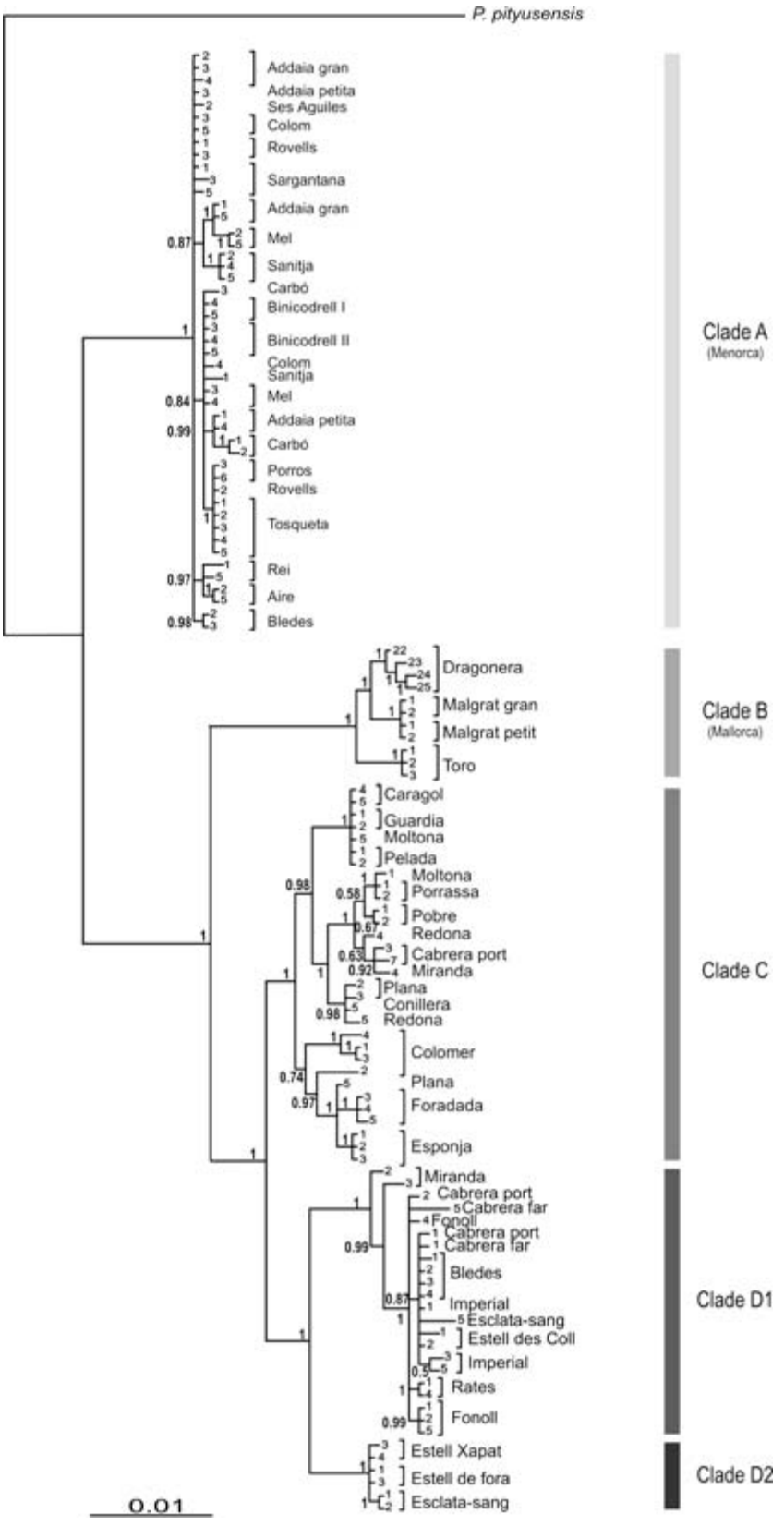

Figure 2 Phylogenetic tree constructed using Bayesian inference. The main clades (A-D2) are indicated. Numbers above branches represent clade credibility values obtained from the posterior distribution of topologies.

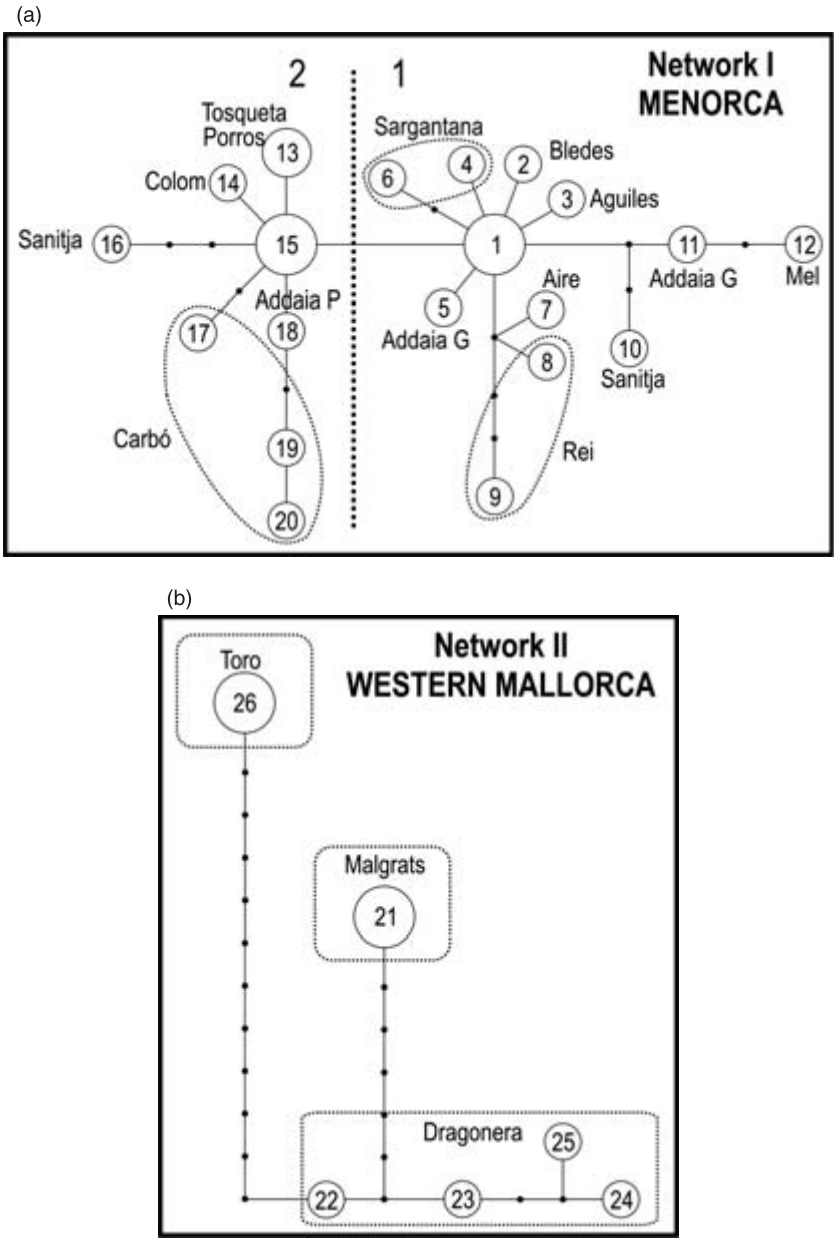

Figure 3 Statistical parsimony networks (TCS program). Haplotypes are designated by numbers as defined in Table 1.

DISCUSSION

Both Bayesian phylogenetic and parsimony networks support the structuring of *P. lilfordi* populations into four main lineages. Two of them, Menorca (16 islands) and western Mallorca (four islands), are geographically isolated both from one another and from the remaining populations while the remaining two clades show some distributional overlap. Here and elsewhere (Brown *et al.*, 2008) it has been shown that the first cladogenesis event (involving Menorca) is quite old (around 2.8 Ma) with the subsequent event involving Dragonera, Malgrats and Toro populations from western Mallorca being only slightly more recent (~2.3 Ma). It is interesting to note this latter period broadly coincides with the first Donau glaciation, some 2.35 Ma (Gracia & Vicens, 1998). The absence of detectable migration between these networks indicates a lack of subsequent introgression between clades, even during the Riss (200 Ka) and Würm (25 Ka) glacials when sea levels decreased by more than 100 m uniting all Menorcan, Cabreran and Mallorcan islands into one large land mass (the 'Gran Balear'). Two possible explanations for the lack of introgression are: (1) limited dispersal due to inhospitable environmental conditions on newly formed land bridges, specifically, high salinity levels are likely in deeper channels, such as between Mallorca and Menorca (Ryan, 1976), and (2) populations came into contact but with negligible introgression, due to selection against hybrids as found in tension zones (e.g. Phyllips *et al.*, 2004). This pattern appears likely for other populations of island lizards that are currently observed in secondary contact (e.g. Brown *et al.*, 2000; Gubitz *et al.*, 2005).

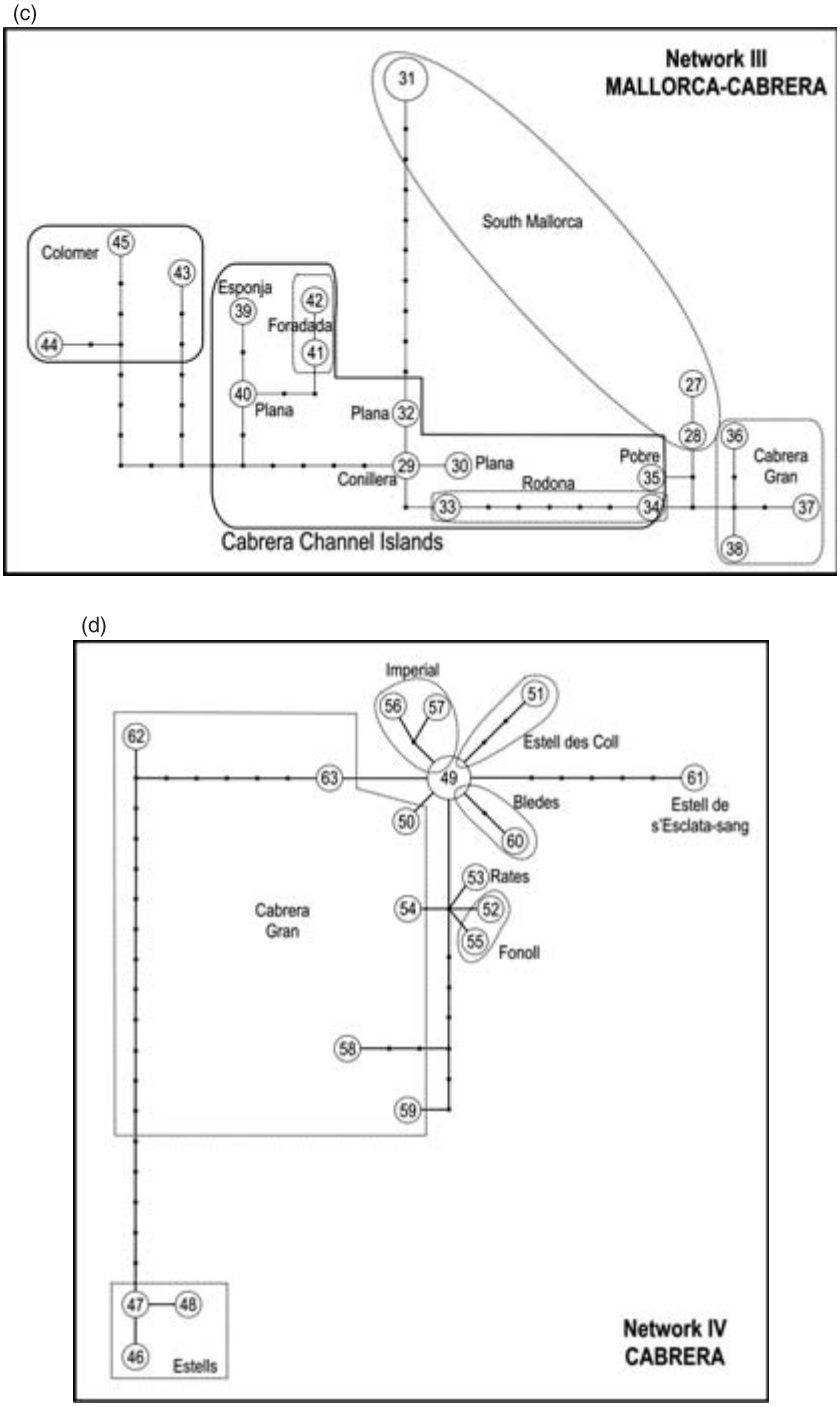

Figure 3 *Continued*

The genetic structuring across Menorcan islets (16 insular populations: network I) does not show a clear spatial pattern as might be expected if, for example, population vicariance had occurred within this group. In fact, several factors also indicate that Menorcan populations were largely panmictic prior to recent extinction on the main island: (1) low levels of genetic diversity, as reflected by the reduced number of mutational steps between haplotypes (maximum 5) and the fact that the mean of the pairwise differences between haplotypes (Pi) is five times smaller than that in other networks, (2) the widespread distribution of shared haplotypes 1 and 15, and (3) a low number of missing haplotypes. The star-shaped topologies of the two clades within this network are suggestive of demographic expansion (Mulcahy *et al.*, 2006). We found statistical evidence for such an expansion as well as a south-north direction of colonization across Menorca, which suggests that historical dispersal occurred across Menorca towards an extreme of the species range, followed by isolation of islet populations through recent rises in sea level.

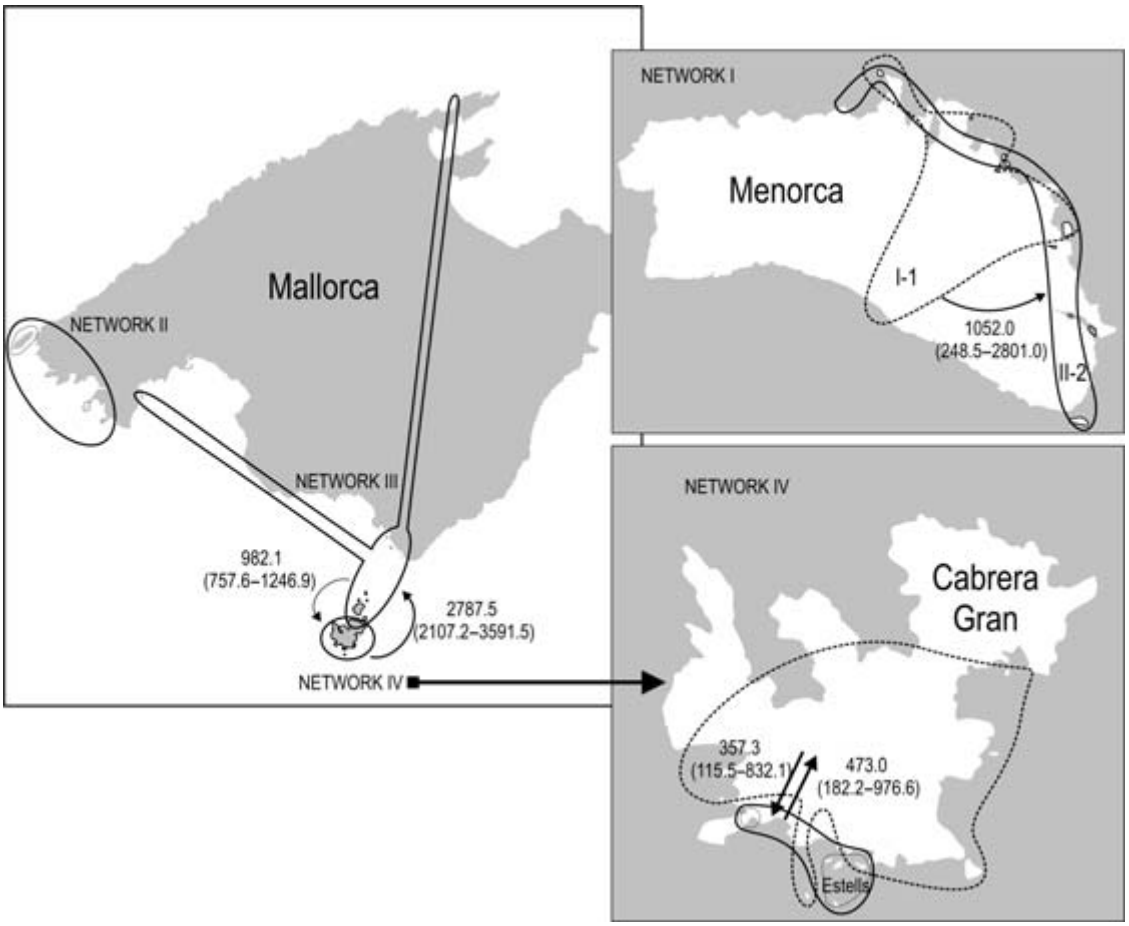

Figure 4 Geographical distribution of four networks and the significant clades. Migration events are indicated.

Table 3 Pi is the mean of pairwise differences. F_S (Fu, 1997) tests population growth with associated *P*-value denoted by P_F (the probability that the simulated F_S is less or equal to the observed F_S). Parameters of the mismatch distribution are denoted by τ, θ_0, θ_t and the associated significance, *P* (the probability that random mismatch distributions (1000 bootstrap replicates) have a larger sum or squared deviation than the model distributions). Raggedness is Harpending *et al.* (1998) raggedness index and measures the smoothness of the mismatch distribution.

Group	Pi	F_S	P_F	τ	θ_0	θ_t	*P*	Raggedness
Network I								
Menorca	2.903	−9.380	0.000	2.945	0.304	18.984	0.600	0.020
Subset 1	2.200	−4.200*	0.019	1.672	0.594	67.734	0.200	0.044
Subset 15	4.182	−0.790	0.310	5.234	0	9999.0	< 0.001	0.190
*Network II**								
W. Mallorca	9.091	2.362	0.856	15.604	0.002	33.057	< 0.001	0.310
Dragonera/Malgrats	5.429	0.969	0.650	10.143	0.004	12.319	0.100	0.403
Network III								
Mallorca/N. Cabrera	12.267	−1.750	0.289	15.603	0.002	33.057	0.200	0.020
Colomer	8.833	5.921	0.997	0	0	9999.0	< 0.001	0.345
S. Mallorca	6.422	2.417	0.836	16.021	0	33.459	0.100	0.417
N. Cabrera	12.948	1.188	0.283	7.551	8.029	24.743	0.600	0.013
Network IV								
Estells/S. Cabrera	11.370	−2.119	0.208	0.642	0	99999.0	< 0.001	0.078
Estells	0.867	−0.426	0.183	1.113	0	99999.0	0.400	0.347
S. Cabrera	7.342	−3.390	0.090	1.303	0	99999.0	< 0.001	0.216

*No statistics were computed for Toro islet within this network, as only one haplotype was detected.

Table 4 Matrix of estimated migration rates between regions occupied by distinct networks.

Migration events within networks

	M			
	To			
From	I	II	III	IV
I Menorca	–	0	0	62.34 (62.34–227.37)
II Western Mallorca	0	–	0	874.60 (7.89–272.08)
III Mallorca-N. Cabrera	0	0	–	2787.46 (2107.18–3591.49)
IV South Cabrera	0	0	982.09 757.85–1246.88	–

Migration events within networks

		M	
	From	To	
Network I		Subset 15	Subset 1
	Subset 15	–	1052.04 (248.50–2801.90)
	Subset 1	0	–
Network IV		1. Estells	2. South Cabrera
	1. Estells	–	473.05 (182.17–976.62)
	2. South Cabrera	357.34 (115.49–832.07)	–

M, absolute number of migrants exchanged per generation.

In contrast, the phylogeographical pattern in western Mallorca (network II) appears more likely to be explained by allopatric fragmentation between the Dragonera population and those from Malgrat Gran and Petit and Toro islands. Haplotype distributions are spatially non-overlapping while levels of genetic divergence are two-three times higher than the mean divergence detected within other networks. Studies in other species from the neighbouring Iberian peninsula, such as the amphibian *Lissotriton boscai* and the lizard *Lacerta schreiberi*, have postulated similar processes of fragmentation in the Upper Pliocene or Lower Pleistocene (Paulo *et al.*, 2001; Martinez-Solano *et al.*, 2006). The distribution of western Mallorca haplotypes comprises the western extreme of the northern mountain chain that dominates the island (Serra de Tramuntana). Here, the islets on which *P. lilfordi* are found are close to the coast but their topography is abrupt and steep. Interestingly, while two of them (Malgrat Gran and Malgrat Petit) contain the same haplotypes, there is a clear genetic differentiation between these islets and Toro (despite a separation of less than 2 km).

Populations from the Cabrera archipelago and the north and south of Mallorca were grouped into two independent large clades (networks III and IV). Considerable asymmetry in historical gene flow exists between them, with northward migration exceeding that in the opposite direction. This is surprising as migration from the centre of the range (Mallorca) to the periphery would intuitively appear more likely. It is therefore possible that Cabrera has acted as a refugial area, a hypothesis that is compatible with the extremely high levels of diversity found within this tiny archipelago (see later).

The lack of genetic differentiation between Colomer and North of Cabrera and South of Mallorca is surprising given their current geographical isolation. The finding of one confirmed introduction involving a network III haplotype (from Cabrera to Porrassa islet) shows that some recent anthropogenic dispersal has occurred and so this cannot be completely ruled out. However, Colomer is a single isolated population in northern Mallorca into which introductions are extremely unlikely due to its steep-sided, almost inaccessible nature. Instead, its close relationship with islets to the south of Mallorca (and Cabrera) seems more likely to be due to the recent extinction of populations occupying the centre and east of the main island Mallorca.

Network IV comprises two clades with almost symmetrical gene flow between their respective areas. The differing geographical areas and large number of mutational steps between these clades is consistent with allopatric fragmentation. It is interesting to note that one of these groups is largely restricted to a southern site on the main island of Cabrera Gran itself, together with three very steep adjacent islets Estell de s'Esclata-sang, Estell Xapat and Estell de Fora. Considerable sea-depths are found in this area (> 50 m) suggesting infrequent connections between these islets.

Haplotypes detected in Cabrera Gran correspond to both networks III and IV. This fact, together with the lack of clear geographical associations, suggests possible colonizations and recolonizations at different time periods between Cabrera Gran and the other small islands, such as Rates, Fonoll, Bledes and Imperial, between which sea depths are relatively shallow. Thus, the phylogeographical distribution in this region lends support to the theory that Cabrera Gran has harboured refugia populations

during extreme climatic periods, particularly during times of increased sea levels when many neighbouring islets would have been submerged.

The phylogeographical pattern in *P. lilfordi* can be attributed to several different processes. However, two general scenarios could explain the overall pattern of genetic diversity within this species after the early cladogenesis of the Menorcan populations. First, the Balearic lizard originally had a wide distribution across Mallorca and Cabrera, of which the north Mallorcan Colomer population represents a unique relict. Recent extinction in the south Mallorcan islands of Caragol, Guardia, Moltona and Pelada was followed by recolonization from the Cabrera archipelago (likely to be Conillera and/or Plana islets). An alternative to this is that it subsequently became restricted (by climatic and marine conditions) to the most southern part of the Balearic archipelago (i.e. Cabrera) with subsequent expansion leading to colonization of the northern part of the range. In support of the latter, Colomer samples clustered with Conillera and Plana from the Cabrera archipelago.

Our study also allows us to determine which evolutionary significant units (ESU) might require more protection, as well as guiding potential reintroduction programs. The existence of four major lineages provides a starting point for the recognition of populations with unique maternal histories, and indicates that at least four genetic groups should be recognized for conservation purposes. However, other components of the intraspecific diversity are also significant. There are three well-supported lineages within clade C alone. At a finer-scale, several geographically restricted populations with unique mtDNA histories are evident, which also qualifies them as ESUs. These include haplotypes found exclusively on the islet of Toro. This divergent islet population likely consists of only a small number of individuals and its unique maternal history therefore justifies the highest conservation priority. A similar case can be made for the population from the islet of Colomer.

It has been argued that the subspecies rank in taxonomy needs substantial reorganization, as it has a major impact on conservation (Zink, 2004). However, constructing taxonomies based on phylogenetic species concepts removes the need for subspecies (Haig *et al*., 2006) so our study cannot be used in isolation when assessing the validity of subspecies designations within *P. lilfordi*. Despite this, there was often considerable overlap between subspecies and mtDNA lineage. For example, the divergent network IV islets populations from Estell de s'Esclata-sang, Estell Xapat, and Estell de Fora, together are recognized as a single subspecies. It could be argued that the original subspecies designations may be of greater use than knowing the phylogenetic history in some cases (see Haig *et al*., 2006). For example, we did not detect unique mtDNA histories among several populations from Menorcan islets that are morphologically differentiated from one another and therefore classified as distinct subspecies. One of these, the type subspecies *P. lilfordi lilfordi* from the islet of Aire, is morphologically highly distinctive but shares haplotypes with other islets. The complexity of these patterns of morphological and mtDNA differentiation makes *P. lilfordi* a model organism for investigating the relationships between biodiversity, subspecific taxonomy and conservation priorities.

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Appendix Primer sequences used in polymerase chain reaction (PCR) amplification and sequencing. Primers numbers refer to the 3′ end of the human mitochondrial genome (Anderson *et al.*, 1981), where L and H correspond to light and heavy strands, respectively.

Primer	Sequence	Reference
Cytochrome *b*		
L14724	5′-TGACTTGAARAACAYCGTTG	Palumbi (1996)
H15175	5′-CCCTCAGAATGATATTTGTCCTCA	
L14841	5′-AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA	Kocher *et al.* (1989)
H15149	5′-AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA	
L15347	5′-CATGAAACTGGATCAAACAACCC	Fu (2000)
H15915	5′-GTCTTCAGTTTTTGGTTTACAAGAC	
RNA 12s		
L1091	5′-AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT	Kocher *et al.* (1989)
H1478	5′-TGACTGCAGAGGGTGACGGGCGGTGTGT	
ND2 and RNAt		
L4178	5′-CARCTWATACACYTACTATGAAA	Macey *et al.* (1998)
H4980	5′-ATTTTTCGTAGTTGGGTTTGRTT	
Control region		
L15022	5’-TACCCTTGCTCATAGCATAACTG	Modified from Brehm *et al.* (2003)
H00292	5’-GTCTTGTTGACTGTAATTAACCGATA	
c-mos		
G73	5’-GCGGTAAAGCAGGTGAAGAAA	Saint *et al.* (1998)
G74	5’-TGAGCATCCAAAGTCTCCAATC	

	Initial denaturing		Denaturing		Annealing		Extension			Final extension	
PCR conditions	T(°C)	t (min)	T(°C)	t (s)	T(°C)	t (s)	T(°C)	t (min)	Cycles	T(°C)	t (min)
Cytochrome *b*	96	5	94	60	50	60	72	1	35	72	5
RNA 12s	96	5	94	60	55	60	72	1	35	72	5
Control region	96	5	94	60	55	60	72	1	35	72	5
ND2 and RNAt	94	5	94	35	50	35	70	2	30	70	5
c-mos	94	3	94	45	50	45	72	1	35	72	6