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Use of NCPA to understanding genetic sub-structuring of *Podarcis lilfordi* from the Balearic archipelago

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Abstract. *Podarcis lilfordi* is an endemic lizard found on islands and islets off the coast of Mallorca and Menorca (including the Cabrera archipelago) in the Balearic Islands, which is extinct on the two main islands themselves. Analyses of mtDNA revealed four unconnected parsimony networks. These represented (I) all Menorcan populations, (II) Dragonera, Malgrats and Toro islands (Western Mallorca), (III and IV) the remaining populations from Cabrera and Mallorca. We applied NCPA to evaluate its utility in understanding the genetic sub-structure. Within network I, contiguous range expansion was inferred for both main clades. Populations from western Mallorca (II) have undergone historical allopatric fragmentation events following isolation around the start of the Pleistocene. Clades within networks III and IV showed some allopatric fragmentation and restricted gene flow (isolation by distance) among islands from Cabrera, as well as long distance dispersal between the Cabrera archipelago and southern Mallorca. The NCPA strongly supports the importance of allopatric fragmentation, with some additional support for range expansion. The primary advantage of the NCPA approach was to provide statistical support for these inferences.

Keywords: Balearic archipelago, mtDNA, NCPA, *Podarcis lilfordi*.

Introduction

The lizard genus *Podarcis* has been the subject of a large number of biogeographical studies, in part due to the substantial genetic diversity that it shows around the Mediterranean (Capula, 1997; Harris and Arnold, 1999; Harris and Sá-Sousa, 2002; Podnar et al., 2005; Poulakakis et al., 2005a, 2005b; Pinho et al., 2006, 2007). Phylogeographical relationships among insular populations have received particular attention because of interest in assessing previous taxonomic designations based on the often substantial morphological variation found within many species (Harris et al., 1998; Pérez-Mellado, 1998a, 1998b; Sá-Sousa et al., 2002; Arnold et al., 2007). In addition, these studies can provide insights into historical biogeography in relation to sea-level changes and northern hemisphere glaciations, although the action of many different effects can obfuscate the current pattern. For example, evolution within *P. sicula* in the Adriatic region (52 subspecies) (Podnar et al., 2005), and Greek populations of the Balkan wall lizard *Podarcis taurica* (Poulakakis et al., 2005a) have revealed histories that are not dominated by one simple overriding scenario. Instead, they show complex patterns resulting from several natural events, which probably include range contraction into glacial refuges and postglacial areas expansions, in addition to multiple introductions by man.

The Balearic Islands should present a simpler system because of their isolation from any major land mass during recent glaciations. Two endemic species of *Podarcis* inhabit the archipelago: *Podarcis lilfordi* in the Gymnesic island group (Mallorca, Menorca, Cabrera and associated islets) and *Podarcis pityusensis* in the Pityusic group (Ibiza, Formentera and coastal islets). They constitute reciprocally monophyletic sister species, which originated during the re-flooding of the Mediterranean at the end of the Messinian Salinity Crisis (MSC),

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5.33 Ma ago (Krijgsman et al., 1999; Duggen et al., 2003; Brown et al., 2008).

There are currently 43 different insular populations of *P. lilfordi*, 11 from islets around Mallorca, 16 from Menorcan islets and 16 in the Cabrera archipelago. It became extinct on the main islands of Mallorca and Menorca during the past few thousand years as a result of the introduction of foreign predators and potential competitors (Kotsakis, 1981; Alcover, 2000).

Two recent studies have addressed the genetic variability that may underpin this variation (Brown et al., 2008; Terrasa et al., 2009). The first of these established the overall phylogeny within the Balearic *Podarcis* and divergence times for major nodes (Brown et al., 2008). A second paper examined the fine-scale genetic diversity within *P. lilfordi* from a conservation perspective. These studies used Bayesian phylogenies and parsimony networks, and showed that *P. lilfordi* populations were structured into four main lineages. Two of them, Menorca (16 islands) and Western Mallorca (4 islands), are geographically isolated both from one another and the remaining populations while the remaining two clades show some distributional overlap. Brown et al. (2008) have indicated that the first cladogenesis event (involving Menorca) is quite old (around 2.6 Ma), with the subsequent event involving Dragonera, Malgrats and Toro populations from western Mallorca being only slightly more recent (∼2.0 Ma). It is interesting to note this latter period broadly coincides with the final Donau glaciation, some 2.0 Ma ago (Gracia and Vicens, 1998). The absence of detectable migration between these networks indicates a lack of subsequent introgression between clades, even during the Riss (200 Ka ago) and Würm (25 Ka ago) glaciations when sea levels decreased by more than 100 metres uniting all Menorcan, Cabreran and Mallorcan islands into one large land mass (the "Gran Balear").

Nested clade phylogeographic analysis (NCPA) is a popular method for reconstructing the demographic history of spatially distributed populations from genetic data, although the power of this method to detect the correct process has been questioned. Several criticisms have been levelled at this approach. In particular, it has been suggested that methods based on other analytical models better incorporate the stochasticity inherent in the evolutionary process (Knowles and Maddison, 2002; Knowles, 2004). Despite this, the approach can provide valuable insights when used in conjunction with other methodologies for investigating phylogeographic patterns (Templeton, 2008). Here, we apply NCPA to the *P. lilfordi* mtDNA data to test its utility in revealing the causes of geographical substructuring in *Podarcis lilfordi* from the Balearic Islands.

Materials and methods

Samples

A total of 118 individual *Podarcis lilfordi* from 43 localities covering all extant subspecies and 41 of the 43 insular populations known to date were included in the analysis. The samples, genes and the methodology used for DNA isolation and mtDNA sequencing are indicated in Terrasa et al. (2009).

Nested clade analyses

We analyzed the spatial component of the genetic diversity using Nested Clade Phylogeographic Analysis (NCPA) (Templeton, 1998, 2004). A statistical parsimony haplotype network (Templeton et al., 1992; Templeton and Sing, 1993) was constructed using the program TCS version 1.21 (Clement et al., 2000). Haplotypes were grouped following a hierarchical nested design (Templeton et al., 1995) and the statistical significance of the relationships between the geographical location of individuals and their position in the nested haplotype network, tested using the software GeoDis version 2.5 (Posada et al., 2000). The statistics calculated for clades were: (i) the clade distance (D_C), a measure of the average distance of all clade members from the geographical centre of distribution, (ii) the nested clade distance (D_N), a measure of how widespread a particular clade is relative to the distribution of its sister clades in the same nesting group, and (iii) interior-tip distances (I-T_C and I-T_N), to indicate how widespread evolutionary younger clades (tip clades) are, relative to their ancestral clades (interior clades). The statistical significance of the different NCPA distance measures was calculated by comparison with a null distribution generated using randomization. Interpretations of patterns for clades with significant geographical associations were obtained by means of the latest inference key (http://darwin.uvigo.es/download/geodisKey_11Nov05.pdf) (Templeton et al., 1995; Templeton, 1998, 2004).

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 (see Terrasa et al., 2009, for more information).

NCPA

The results of the NCPA are summarized in fig. 1. Sequences were collapsed into 63 haplotypes which formed four unconnected networks under the 95% probability of parsimony criterion. Network I included the Menorcan populations, network II west Mallorca, i.e., Dragonera, two Malgrats islets, and Toro islet; the latter being separated by 11 mutational steps.
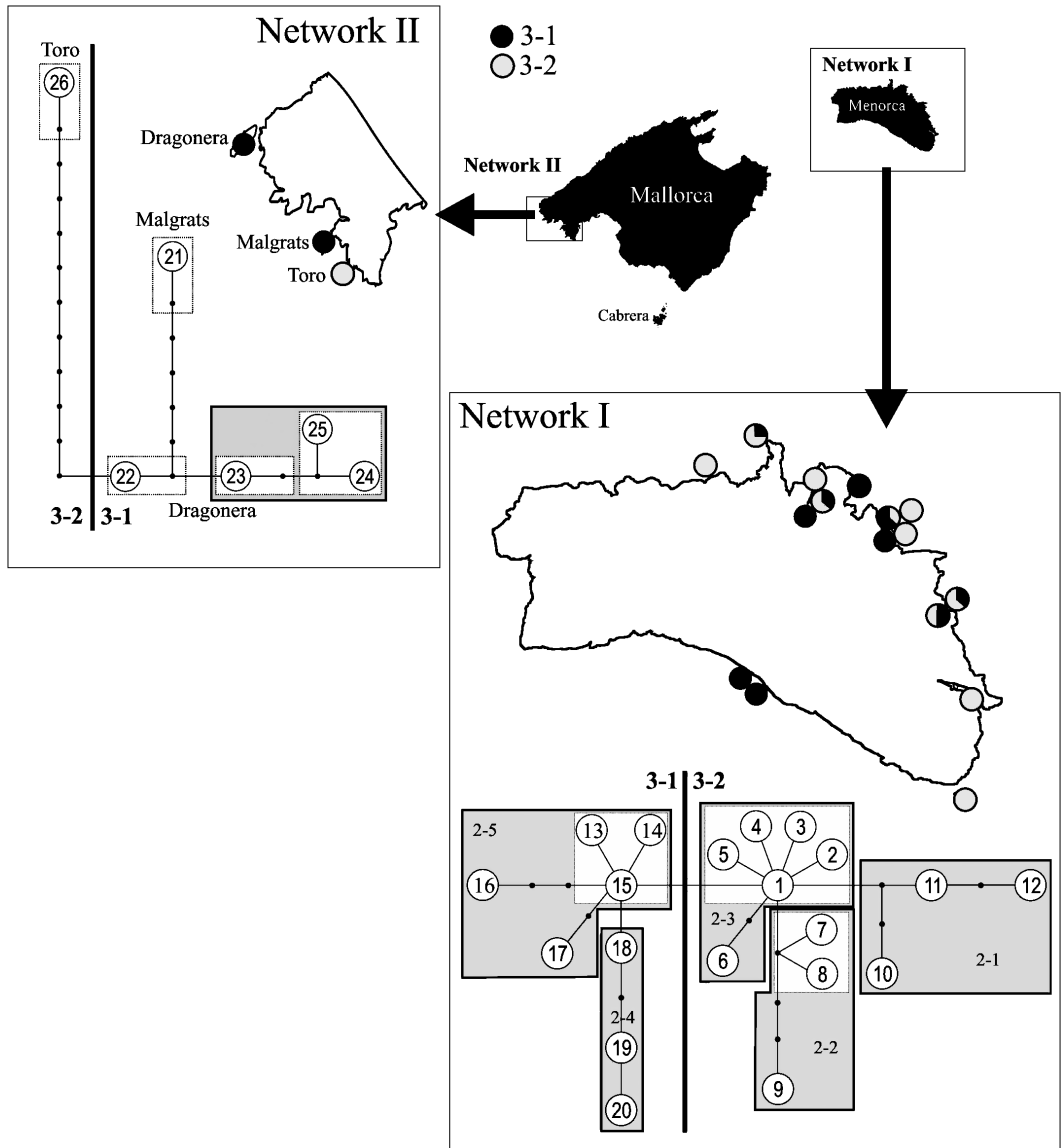
Figure 1. Statistical parsimony networks (TCS) and associated nested design. Haplotypes are designated by numbers as defined in table 1. Boxes group the haplotypes nested together into one, two, three and four step clades.

Figure 1. (Continued).
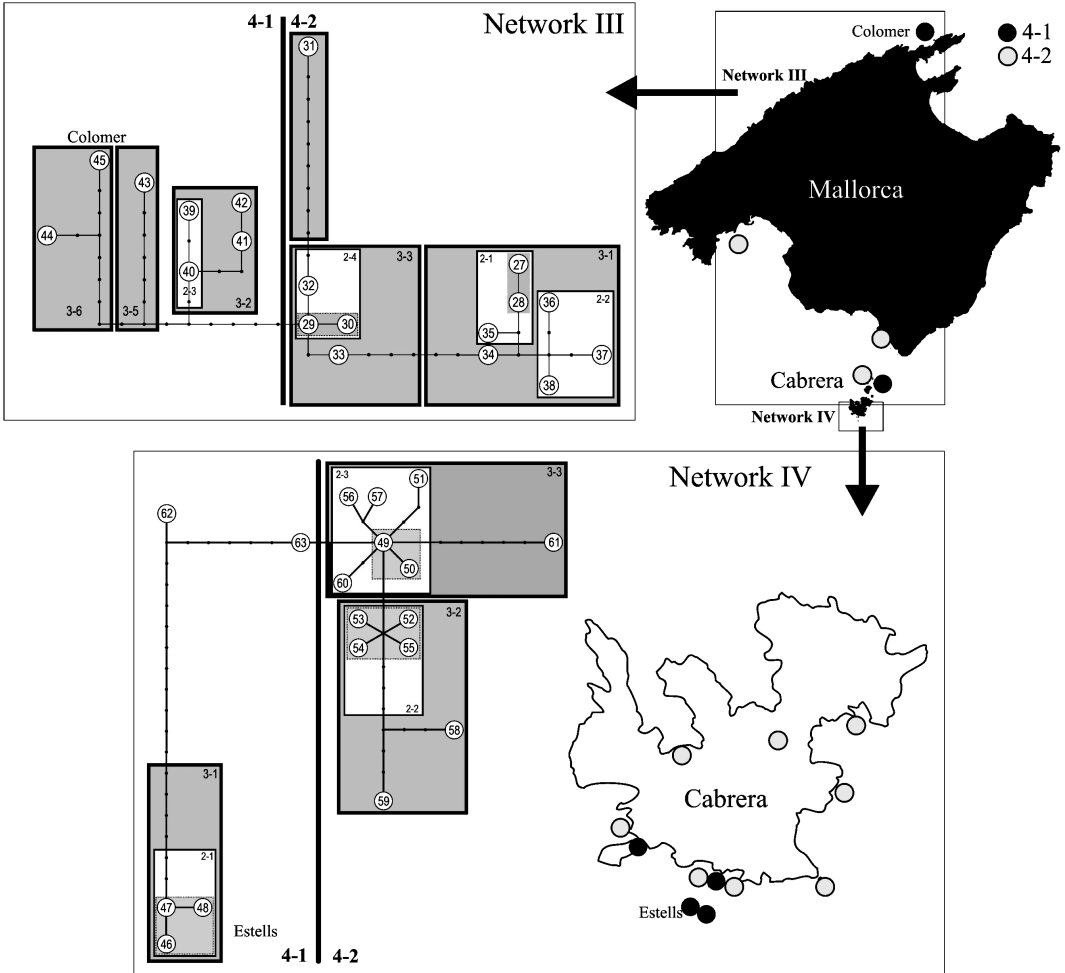
Network III represents north-east and southern Mallorcan populations together, with populations from islands located in the channel between Mallorca and Cabrera (i.e., Conillera, Plana, Pobre and Rodona), as well as some individuals from the main island of Cabrera. Network IV (Cabrera South) contains most individuals from Cabrera (main island), as well as associated islets, such as Bledes, Imperial, Fonoll and Rates. This contains a nested clade separated by 16 mutational steps (clade 1-1), and represents haplotypes from three of the most southern insular populations: Estell Xapat, Estell de Fora and two samples from Estell de s'Esclatasang.

Thirty three of the 83 clades generated in the nesting design showed significant geographical contingency, and so the significance of the magnitudes of the clade distances D_C, the nested clade distances, D_N, the within-clade interior-tip distances, I-T_C and the nested clade interior-tip distances I-T_N were tested. Results are given in fig. 2 (I, II, III and IV). In some of the lower level nested clades, the null hypothesis that gene flow is not restricted cannot be rejected although alternative processes were inferred for clades at different, higher nesting levels, possibly due to greater statistical power (table 1).

Network I (Menorca populations) provides no inference at the total cladogram level, al-

Network I

Haplotypes			1-step clades			2-step clades			3-step clades		
NUM	Dc	Dn	NUM	Dc	Dn	NUM	Dc	Dn	NUM	Dc	Dn
1	5.4	5.9									
2	0	12.2									
3	0	4.4									
4	0	3.2									
5	0	4.1									
I-T	5.4	-1.3									
			1-1	6.6	6.6						
6	0	0	1-2	0	3.0						
			I-T	6.6	3.6	2-3	6.4S	7.9L			
7	0	6.4									
8	0	3.2	1-3	4.2	4.8						
9	0	0	1-4	0	4.8						
			I-T	4.2	0	2-2	4.8	18.2L			
10	0	0	1-5	0S	10.1						
11	0	0	1-6	0	4.5S						
12	0	0	1-7	0.0	12.0						
			I-T	0.0	-6.4S						
						2-1	9.3	9.9			
						I-T	-1.3	-5.0S			
									3-2	10.6	10.8
13	1.6S	7.9S									
14	0.0	13.5									
15	6.1S	10.9									
I-T	4.7L	2.4L									
			1-8	9.7	9.6						
16	0.0	0.0	1-9	0.0	12.3						
17	0.0	0.0	1-10	0.0	7.4						
			I-T	9.6	-0.3						
						2-5	0.3S	6.7			
18	0	0	1-11	0.0	0.3	2-4	9.6	9.5			
19	0	0				I-T	-9.3S	-2.8			
20	0	0	1-12	0.0	0.3				3-1	8.9	8.9
			I-T	0.0	0.0						

Figure 2. Results of the nested geographycal analysis. **NUM** is the number of clade, **Dc** is the clade distance and **Dn** the nested clade distance at each one of the levels of the analysis (haplotype, one-step, two-step, three-step and four step levels). The row I-T indicates the average difference between interior and tip clades. S means that the statistic was significantly small and L that the statistic was significantly large (both at he 5% level). The significant clades are indicated by shading.

though it contains two 3-step clades (3-1 and 3-2) which all have the same outcome, i.e., contiguous range expansion. The latter have overlapping distributions but, 3-2 is found in southeast Menorca and not found near Binicodrell islets.

Evidence for allopatric fragmentation was detected in several clades. First, in network IV, haplotypes from three small islets known as the Estells (clade 4-1 [see fig. 1]: Estell de Fora, Estell Esclatasang and Estell Xapat), are separated from the remaining haplotypes by 16 mutational

Network II

Haplotypes			1-step clades			2-step clades		
NUM	Dc	Dn	NUM	Dc	Dn	NUM	Dc	Dn
21	-	-	2-1	0.3S	4.9S			
22	-	-	2-2	0.0	9.7			
23	-	-						
24	-	-						
25	-	-	2-3	0.0	9.7			
			I-T	-0.2L	2.8			
						3-1	6.5	5.8
26	-	-	2-4	-	-	3-2	0S	6.8

Network III

Haplotypes			1-step clades			2-step clades			3-step clades			4-step clades		
NUM	Dc	Dn	NUM	Dc	Dn	NUM	Dc	Dn	NUM	Dc	Dn	NUM	Dc	Dn
27	0.0	30.4												
28	0.0	15.2												
I-T	0.0	-15.2	1-1	20.2	25.2									
35	-	-	1-7	0.0	21.3									
			I-T	-20.2	-3.9	2-1	23.6	22.8						
36	-	-	1-3	0.0	0.9									
37	-	-	1-4	0.0	0.9	2-2	0.9S	13.5						
38	-	-	1-5	0.0	0.9									
			I-T	0.0	0.0									
34	-	-	1-10	-	-	2-5	0.0	12.5						
						I-T	15.1	-6.8						
									3-1	18.4	17.6L			
29	0.0	0.4												
30	0.0	1.2												
I-T	0.0	-0.8	1-2	0.6	0.7									
32	-	-	1-6	0.0	1.0	2-4	0.8	0.8	3-3	0.8S	7.8			
33	-	-	1-11	-	-	2-7	0.0	2.2						
31	-	-	1-12	-	-	2-8	-	-	3-4	1.5S	8.1			
									I-T	10.2	-5.7			
												4-2	12.6	13.6S
39	-	-	1-8	0.0	0.1									
40	-	-	1-9	0.0	0.5									
			I-T	0.0	0.3	2-3	0.2S	0.7S						
41	-	-												
42	-	-	1-13	-	-	2-6	0S	0.9L						
						I-T	0.2	0.2S						
									3-2	0.8S	25.1S			
43	-	-	1-14	-	-	2-9	-	-	3-5	0.0	58.6	4-1	35.2L	29.6L
44	-	-	1-15	-	-									
45	-	-	1-16	-	-	2-10	-	-	3-6	0S	58.6L			
									I-T	0.7	-29.3S			

Figure 2. (Continued).

Network IV

Haplotypes			1-step clades			2-step clades			3-step clades			4-step clades		
NUM	Dc	Dn	NUM	Dc	Dn	NUM	Dc	Dn	NUM	Dc	Dn	NUM	Dc	Dn
46	0.0	0.2												
47	0.3	0.3												
48	0.0	0.3												
I-T	0.3	0.1L	1-1	0.3	0.3	2-1	0.3	0.3	3-1	0.3S	0.5S			
62	-	-	1-12	-	-	2-7	-	-						
63	-	-	1-13	-	-	2-8	-	-	3-5	0.0	1.3L			
									I-T	-0.3	0.0	4-1	0.7S	1.1
49	1.3	1.4												
50	0.0	1.7												
I-T	1.3	-0.3	1-2	1.5	1.5									
56	-	-												
57	-	-	1-6	0.0	1.0									
51	-	-	1-7	0.0	1.5									
60	-	-	1-8	0.0	1.3									
			I-T	1.5	0.2	2-3	1.4	1.4						
61			1-11			2-6	0.0	0.7						
						I-T	1.4	0.7	3-3	1.3	1.5	4-2	1.5	1.5L
52	0.0	2.0												
53	0.0	1.8												
54	0.0	0.7												
55	0.0	2.0	1-3	1.7	1.7	2-2	1.7	1.8L						
59	-	-	1-9	-	-									
58	-	-	1-10	-	-	2-5	0.0	0.8S	3-2	1.5	1.5			
						I-T	1.7L	0.9L	I-T	-0.1	0.0			

Figure 2. (Continued).

Table 1. Results of paths taken for clades with significant associations with geography through the latest NPCA inference key (see text). X^2 is the statistic used to measure the contingency of haplotype frequency on geography (Templeton et al., 1995), with probability values (P) obtained via randomization.

Network	Clade	X^2	P	Chain of inference	Inference
I	3-1	16.917	0.047	1-2-11-12-No	Contiguous range expansion
I	3-2	43.571	0.000	1-2-11-12-No	Contiguous range expansion
I	Entire clagogram	27.250	0.005		Non significant D_C and D_N values at nesting level
II	3-1	8.000	0.020	1-19-No	Allopatric fragmentation
II	3-2				Not significant
II	Entire clagogram	1.000	0.004	1-19-No	Allopatric fragmentation
III	4-1	11.000	0.118	1-2-3-5-15-21-No	Past fragmentation and/or long distance colonization
III	4-2	35.587	0.002	1-2-3-5-6-7-Yes	Restricted gene flow/dispersal but some long distance dispersal
III	Entire clagogram	27.129	0.000	1-2	Inconclusive outcome
IV	4-1	0.000	0.000	1-2-3-4-9-No	Allopatric fragmentation
IV	4-2	17.980	0.001	1-2-3-4-No	Restricted gene flow with isolation by distance
IV	Entire clagogram	24.733	0.001	1-2-3-5-6-7-Yes	Restricted gene flow/dispersal but some long distance dispersal

steps. Second, in network II, this is inferred for clade 3-1 (Dragonera and Malgrats islands) and clade 3-2 (Toro island). Toro and Malgrats are isolated by 11 and 6 mutational steps, respectively, from the Dragonera haplotypes. Finally, haplotypes from Colomer island (northeast Mallorca, network III) may also have resulted from past fragmentation, although long distance colonization can not be ruled out: the three haplotypes from Colomer are found within clade 4-1 and separated from other clades by a minimum of 10 mutational steps.

Restricted gene flow/dispersal but with some long-distance dispersal was inferred in network III (clade 4-2). This included samples from Cabrera Gran (two sites: Miranda and Cabrera port) and two neighbouring islets (Fonoll and Rates). There is restricted gene flow between the two samples from Cabrera Gran. Restricted gene flow with isolation by distance was inferred for clade 4-2 (network IV), which includes populations from the south of Mallorca and the Cabrera archipelago.

Discussion

It seems clear that *P. lilfordi* populations are structured into four main clades; both Bayesian phylogenetic inference and the parsimony networks support this fact (Brown et al., 2008; Terrasa et al., 2009; present work). Two of them, the Menorca (with 16 islands) and Western Mallorca (with 4 islands) clades, are geographically isolated, while the other two clades show some distributional overlap.

The genetic structuring across Menorcan islets (16 insular populations: network I) is unclear. Several factors indicate that Menorcan populations were largely panmictic prior to recent extinction on the main island: (i) low levels of genetic diversity, (ii) the widespread distribution of shared haplotypes 1 and 15, (iii) the reduced number of mutational steps between haplotypes, (iv) the low number of missing haplotypes. Despite this, the NCPA detected clear evidence of range expansion (clades

3-1 and 3-2, contiguous range expansions), but nonsignificant values at higher nesting levels. Thus, NCPA suggests historical dispersal across Menorca to one extreme of the species range, followed by isolation of islet populations through recent rises in sea-level, rather than transmarine colonization. This result could be a false positive, as suggested by critiques of NCPA (Knowles and Maddison, 2002; Petit and Grivet, 2002; Panchal and Beaumont, 2007; Petit, 2008). However, the significance ($P < 0.001$) obtained by Fs (Fu, 1997) and the star-shaped topologies of clades 2-3 and 2-5 (Mulcahy et al., 2006) are highly suggestive that the finding of range expansion is robust.

In contrast, the isolation of the islands/islets of Dragonera, Toro, Malgrats Gran and Malgrats Petit, (network II) can be attributed to allopatric fragmentation. These results are supported by the geological origin of these islands (fracture of the western edge of the Sierra de Tramuntana) as detailed in Terrasa et al. (2009). Only a reduced number of individuals could be analysed across these latter three islets. Possibly for this reason, there was no significant contingency of genetic diversity on geography (NCPA) so statistically-supported inferences cannot be made.

Two independent large clades (networks III and IV) grouped the populations from the Cabrera archipelago and the north and south of Mallorca. Although no inference could be made for network III at the total cladogram level, lower level clades did provide some insights into historical processes. The evidence of long-distance colonization/dispersal in both level 4 clades may suggest transmarine colonization. However, the finding of one confirmed "network III" introduction (from Cabrera to Porrassa islet) shows some recent anthropogenic dispersal has occurred and so this cannot be completely ruled out for other islets within this network. These events could lead to the same inference, i.e., long-distance dispersal. A similar inference for the other level 4 clade arises due to affinities with Colomer island, a single isolated

population in northern Mallorca. Here, introductions are extremely unlikely due to the steep-sided, almost inaccessible nature of Colomer. Instead, its close relationship with islets to the south of Mallorca (and Cabrera) is almost certainly a result of the recent extinction of populations on Mallorca and this leads to a less robust inference. In summary, the possibility that network III inferences reflect natural phenomena such as transmarine colonization events would appear very tenuous.

Allopatric fragmentation was detected in one (4-1) of the two clades within network IV, suggesting flooding of landbridges among the three very steep islets located near to the coast, i.e., Cabrera Gran (Estell de s'Esclatasang, Estell Xapat, Estell de Fora) and a neighbouring site on Cabrera Gran itself. Restricted gene flow with isolation by distance is tentatively inferred for clade 4-2, although additional observations that would strengthen this inference are lacking, and so an inconclusive outcome is obtained. Different haplotypes from Cabrera Gran are also included within this clade. These results would be expected due to the processes of colonization and recolonization between Cabrera Gran and other smaller islands, as hypothesized by Terrasa et al. (2009). Thus, there is further support for Cabrera Gran harbouring refugia populations during extreme climatic periods, particularly when increased sea levels lead to submergence of many neighbouring islets.

In summary, application of NCPA has lent considerable weight to the importance of allopatric fragmentation as well as weaker evidence for short distance range expansions. In conjunction with previous studies, this approach does appear to reveal new phylogeographic insights for this endangered species, with the advantage of providing statistical support for the inferred processes.

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