Deep differentiation between and within Mediterranean glacial refugia in a flying mammal, the *Myotis nattereri* bat complex

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ABSTRACT

Aim The role of glacial refugia in the biogeographical patterns in the Western Palaearctic region has been widely discussed, but many questions remain unresolved. We examined the biogeography, genetic diversity, spatial distribution and evolutionary history of the *Myotis nattereri* bat species complex to investigate the presence of multiple refugia and the persistence of Quaternary differentiation between and within Mediterranean refugia in a flying mammal.

Location Western Palaearctic region (central and southern Europe and north-western Maghreb).

Methods We analysed three mitochondrial fragments (cytochrome *b*, NADH dehydrogenase subunit 1 and the control region; 1570 bp) from 136 individuals of the *M. nattereri* complex sampled from 87 different localities using a range of phylogenetic techniques. Divergences among clades were also dated using a Bayesian coalescence approach.

Results Phylogenetic analyses identified four main lineages, coincident with the four cryptic species recently described. Each species is further subdivided into well-supported lineages with evident geographical structure. Estimates of genetic diversity and polymorphism were very high for the majority of subclades, with the exception of *M. nattereri* s.s.

Main conclusions The *M. nattereri* bat complex comprises four species whose distributions in the Western Palaearctic correspond to four main glacial refugia (Iberia, Italy, Balkans and Morocco). These species are the result of long-term isolation (remarkable in a flying mammal) over several glacial cycles. The Balkan species expanded into central Europe in a rapid recolonization process. Both the Iberian and Italian peninsulas show a clear pattern of refugia-within-refugia in their genetic structuring, with a deeply differentiated southern Italian clade. Morocco shows two markedly differentiated lineages, probably separated by the Atlas Mountains. The legacy of Pleistocene cycles is evident in both the speciation and the intraspecific diversification events.

Keywords
Bats, biogeography, Chiroptera, cryptic species, *Myotis escalerai*, Pleistocene, refugia-within-refugia, species complex, Western Palaearctic.

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INTRODUCTION

Quaternary climatic changes have played a key role in shaping the distribution and genetic structure of many living taxa and habitats (reviewed in Avise, 2000; Hewitt, 2004). The cycles of climatic cooling followed by warming repeatedly transformed the continent, as the ice caps and permafrost expanded and contracted, thereby changing the distributions and dynamics of habitats and species. The timing, paths and effects of these movements, and their consequent genetic effects, have been studied for several plant and animal species, resulting in a wide variety of species-specific evolutionary histories (Stewart *et al.*, 2010), as well as a number of common phylogeographical patterns (Taberlet *et al.*, 1998;

Schmitt, 2007). In the Western Palaearctic region, the Iberian, Italian and Balkan peninsulas, together with eastern regions such as Turkey and the Caspian area, have been recognized as major glacial refugia and important sources for the post-glacial recolonization of central and northern Europe for many temperate organisms (see reviews in Hewitt, 1999, 2004; Randi, 2007; Bilgin, 2011). Repeated founder effects and bottlenecks, which occurred during rapid post-glacial expansions, are probably the main causes of the genetic paucity found in the northern populations of many European taxa, while higher demographic stability has probably prompted richer intrapopulation genetic variability in southern populations (Hewitt, 2004).

On the other hand, the traditional view of glacial refugia as single homogeneous entities has been drastically reinterpreted, and the presence of multiple isolated refugia within traditional southern areas has been proposed as an important source of genetic variability at the interpopulation level (Gómez & Lunt, 2007). Geographical isolation in allopatric shelter areas, facilitated by the complex topographies of southern European peninsulas, may have significantly contributed to the strong genetic subdivision and endemism displayed by many taxa within these areas. Evidence for the existence of multiple refugia comes primarily from the Iberian Peninsula (reviewed in Gómez & Lunt, 2007), although a similar pattern has also been demonstrated for the Italian peninsula (e.g. Canestrelli & Nascetti, 2008b; Canestrelli *et al.*, 2008a; Grill *et al.*, 2009) and other refugia (e.g. Ursenbacher *et al.*, 2008; Bilgin, 2011).

Many questions still remain unanswered concerning the different within-refugia responses to glacial cycles or the role of life-history factors (e.g. vagility) in shaping the genetic structure of species. To our knowledge, most previous studies of within-refugia structure have involved taxa with poor dispersal ability, and studies focusing on the same species complex in different shelter areas remain scarce (Hewitt, 2011; but see Bilgin *et al.*, 2008, and Flanders *et al.*, 2009).

While the role of the Quaternary climatic cycles in shaping genetic divergence at the intraspecific level has largely been described (e.g. Schmitt, 2007), its role in speciation events is still debated (Knowles, 2001; Stewart *et al.*, 2010). The main objections are based on the short-term, reversible nature of the climatic cycles and consequent selective pressures, such that ephemeral vicariant populations could have expanded and interbred during favourable periods (e.g. Bennett, 1997).

Indeed, in several species, earlier genetic signals of evolutionary history have been erased by late Pleistocene events (i.e. local extinctions or post-glacial hybridizations) (Hewitt, 2004). Nevertheless, many taxa present deep genetic differentiation indicating that ancient haplotypes survived and diverged in allopatric regions over several glacial cycles. In such cases, extreme Pleistocene conditions and demographic events could have either driven diversification or amplified pre-existing genetic structure, thereby accelerating speciation (Knowles, 2001; Hewitt, 2004).

Although the number of biogeographical studies in the Western Palaearctic has increased tremendously in recent years, there are few comprehensive phylogeographical studies available for the mammalian clades widespread over this region (e.g. Hewitt, 2011). Focusing on true sibling species or complexes of closely related species is crucial for a greater understanding of the origin of the rich European biodiversity and the role of the Quaternary period both in shaping intraspecific biodiversity and in promoting speciation (Joger *et al.*, 2007; Hewitt, 2011). The ability to fly provides bats with a potentially good dispersal capacity and the possibility to overcome geographical barriers. However, significant polymorphism and genetic structure have been found in many bat species, and have yielded valuable insights into evolutionary processes (reviewed in Burland & Worthington Wilmer, 2001) at both species and population levels (e.g. Kerth *et al.*, 2002; Ruedi & Castella, 2003; Juste *et al.*, 2004).

According to traditional taxonomy, the Natterer's bat, *Myotis nattereri* (Kuhl, 1817) (Chiroptera: Vespertilionidae), is a non-migratory mouse-eared bat widespread from Europe to East Asia. The presence of independent lineages both in mitochondrial and nuclear DNA has been recently documented, showing that the Western Palaearctic *Myotis nattereri* s.l. is actually a complex of four cryptic species (Salicini *et al.*, 2011). In this study we examine the genetic structure and geographical distribution of the *M. nattereri* complex using three mitochondrial DNA (mtDNA) fragments and a continent-wide sampling including four potential glacial refugia: Iberia, Italy, the Balkans and Northwest Africa.

We aimed to test three main hypotheses: (1) the effect of allopatric evolution in different glacial refugia is still evident in the geographical distribution of the genetic lineages; (2) major divergence/speciation events occurred during the Quaternary period; and (3) the late Pleistocene events shaped the genetic structure of populations within the *M. nattereri* complex (i.e. poor genetic diversity and evidence of population expansion in northern populations; highly variable and geographically structured southern populations).

MATERIALS AND METHODS

We analysed a total of 136 samples of *Myotis nattereri* s.l. from 87 different localities in 12 countries (Fig. 1, and see Appendix S1 in Supporting Information). *Myotis capaccini* and *Myotis mystacinus* were used as outgroups, while the related *Myotis schaubi*, known from Armenia and Iran, was included because of its close phylogenetic relationship to the *nattereri* clade.

Most of the samples were ethanol-stored wing punches (Worthington Wilmer & Barratt, 1996). Tissues were digested with proteinase K and DNA was extracted using phenol/chloroform and ethanol precipitation (Sambrook *et al.*, 1989). Fragments of mtDNA from the cytochrome *b* gene (*Cytb*), NADH dehydrogenase subunit 1 gene (*ND1*), and the second hypervariable domain (HVII) of the control region were sequenced using, respectively, the pairs of

primers Molcit-F (Ibáñez *et al.*, 2006) and Molcit-R (Salicini *et al.*, 2011), ND1F2 and ND1R (Kawai *et al.*, 2002), and Ple2 + (Spitzenberger *et al.*, 2001) and H607 (Worthington Wilmer *et al.*, 1994). Polymerase chain reaction (PCR) products were purified and sequenced in both directions using an ABI 3100 automated sequencer (PE Biosystems, Warrington, UK). All of the sequences were aligned and edited visually using SEQUENCHER 4.5 (Gene Codes Corp., Ann Arbor, MI, USA).

Phylogenetic reconstructions

To test the congruence of the phylogenetic information in the three fragments, a partition-homogeneity test (incongruence length difference, ILD) was implemented in PAUP* 4.0b10 (Swofford, 2003). Sequences of *Cytb*, *ND1* and HVII were then combined to perform the phylogenetic analyses. The whole data set was collapsed to haplotypes with the software COLLAPSE 1.2 (available from http://darwin.uvigo.es/).

Phylogenetic hypotheses were obtained using different optimality criteria: maximum parsimony (MP) and minimum evolution (ME) were implemented using PAUP* 4.0b10 (Swofford, 2003), maximum likelihood (ML) was performed with the software PHYML (Guindon & Gascuel, 2003), and Bayesian inference (BI) was implemented using MRBAYES 3.1.2 (Ronquist & Huelsenbeck, 2003). The best-fitting substitution model, used with ML, ME and BI, was selected according to the Akaike information criterion using JMODELTEST (Posada, 2008). The reliability of the MP, ME and ML topologies was assessed with nonparametric bootstrap analysis (Felsenstein, 1985) using 1000 pseudoreplicates. The model parameters used in BI were estimated in the analyses. Two simultaneous runs, each with four chains running for 10,000,000 generations and sampled every 1000th generation, were implemented. After verifying that stationarity had been reached with TRACER 1.5 (Rambaut & Drummond, 2007), the initial 25% of the trees was discarded as burn-in. The robustness of internal nodes was evaluated with Bayesian posterior probabilities.

Divergence times estimates

Divergence times between the main lineages were estimated using a Bayesian coalescence approach, implemented using BEAST 1.6.1 (Drummond & Rambaut, 2007). Owing to new insights into the taxonomy and phylogeny of the group (Salicini *et al.*, 2011), we preferred not to use the available fossil record (Horáček & Hanák, 1984) to calibrate the molecular clock analysis, given the uncertainty about the phylogenetic position of the fossil specimens. Instead, we used the *Cytb* substitution rate reported for *M. nattereri* s.l. (0.054 substitutions/site/Myr) by Nabholz *et al.* (2008). The analysis was performed with a model of uncorrelated lognormal relaxed molecular clock, which allows independent rates on different branches (Drummond *et al.*, 2006). A GTR model, with gamma distribution and invariable sites, was used with a constant population size prior. Three independent runs of 20 million steps each were implemented, sampling every 2000 generations. Convergence and suitable effective sample size (ESS) were assessed by TRACER 1.5 (Rambaut & Drummond, 2007) and the results combined by LOGCOMBINER 1.6.1 (Drummond & Rambaut, 2007) after a 10% burn-in.

Genetic structure and demographic history

The *Cytb* fragment was used to calculate net-pairwise distances within and between the major clades identified by the phylogenetic analyses, using a Kimura 2-parameter model (K2P) with MEGA4 (Tamura *et al.*, 2007). This model and gene were selected to obtain distances that could be compared with previous studies (e.g. Kawai *et al.*, 2003; Ibáñez *et al.*, 2006; García-Mudarra *et al.*, 2009).

Median-joining networks of the concatenated sequences were produced with NETWORK 4.5 (Bandelt *et al.*, 1999) for the major clades shown by the phylogenetic analyses. In addition, TCS 1.21 (Clement *et al.*, 2000) was used to reconstruct a parsimony network with both 95% and 90% confidence criterion for the same data set. The 95% parsimony connection limit has been suggested as a useful threshold for identifying evolutionarily significant units (reviewed in Hart & Sunday, 2007), while the use of the more conservative 90% limit allows for the investigation of the depth and strength of the found genetic structure.

In order to evaluate genetic variability, the number of haplotypes (h), number of mutations (η), segregating sites (S), haplotype diversity (Hd) and nucleotide diversity (π) were calculated using DNASP 4.5 (Rozas *et al.*, 2003), for the whole data set and for the major clades and subclades of the completely sequenced samples.

In order to investigate the demographic history across lineages, the same software was used to calculate Fu's F_S statistic (Fu, 1997) and R_2 index (Ramos-Onsins & Rozas, 2002), which have been suggested to be the most powerful tests for detecting expansion events (Ramos-Onsins & Rozas, 2002), as well as the more conservative Tajima's D test (Tajima, 1989). The statistical significance of the indexes was evaluated with 10,000 simulations. ARLEQUIN 3.01 (Excoffier *et al.*, 2005) was used to calculate pairwise mismatch distributions (Rogers & Harpending, 1992). This analysis compares the observed distribution of the pairwise nucleotide differences between haplotypes with those generated by 1000 bootstrap replicates according to an expansion model (Schneider & Excoffier, 1999); the sum of squared deviations (SSD) between observed and expected nucleotide differences is used to calculate the proportion of the simulated sum of squared deviations larger than the observed. Demographic analyses were performed initially for the main clades. Given their deep genetic structure, an independent demographic history is likely to exist for each of the subclades, and thus demographic analyses were repeated for subclades that were sufficiently represented ($n > 5$). For those clades that presented signals of population expansion, we calculated the

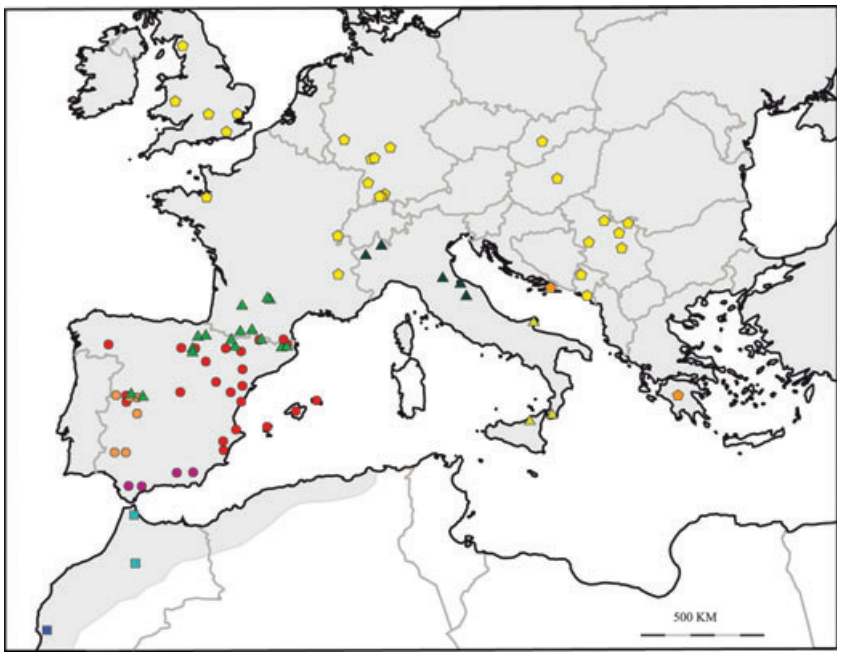

Figure 1 Map showing the sampling locations of bats in the *Myotis nattereri* complex. Symbols represent genetic clades at the species level: ⬠ *Myotis nattereri* s.s.; ○ *Myotis escalerai*; □ *Myotis* sp. B; △ *Myotis* sp. A. Different colours correspond to distinct subclades. The grey area indicates the distribution of the *M. nattereri* complex in the region.

time since the expansion (t) using the parameter tau (τ) (Rogers & Harpending, 1992), which was estimated in the mismatch distribution analysis as $t = \tau/2u$, where u is the mutation rate per sequence per generation. We used the mutation rate of 0.054 substitution/site/Myr for the *Cytb* sequence (Nabholz *et al.*, 2008), and a generation time equal to 1 year.

RESULTS

Sequences were trimmed to 700 bp for *Cytb*, 650 bp for *ND1* and 220 bp for HVII. Homology tests demonstrated congruence among the phylogenetic information of the three fragments ($P = 0.35$), allowing concatenation for phylogenetic analyses. The complete data set of the *M. nattereri* complex consisted of 136 sequences, each 1570 bp long, with 854 invariable sites, 105 singletons and 364 parsimony-informative sites. Only the Greek sample, obtained from GenBank (Ruedi & Mayer, 2001), lacked the D-loop sequence. A total of 84 different haplotypes were found.

Phylogenetic reconstructions

The best fitting model of substitution was a GTR with gamma shape (G = 0.29). The different phylogenetic approaches (BI, MP, ML and ME) produced very similar and well-supported topologies, with two major clades at the base of the tree, each of which was further subdivided into well-supported clades (Fig. 2). The main clades correspond to the four cryptic species retrieved recently by mitochondrial and nuclear phylogenetic analyses on a smaller number of samples (Salicini *et al.*, 2011).

The first major clade includes two monophyletic groups: *Myotis escalerai* includes samples from all over Spain (mainland and Balearic Islands) and is well structured with three differentiated haplogroups corresponding to different geographical areas (see Fig. 1 and discussion for their distributions); the second lineage (*Myotis* sp. B) includes all samples from Morocco. This lineage splits again into two haplogroups: one from central and northern Morocco (sharing haplotypes) and the other from southern Morocco (unique haplotype) (Fig. 2).

The second basal clade also splits into two sister groups (Fig. 2). Samples from a large part of Europe cluster together (*Myotis nattereri* s.s.) with only the differentiation of samples from Greece and Croatia. The second clade (*Myotis* sp. A) encompasses specimens from northern and central Italy, southern France (Pyrenees) and northern Spain, as well as a deeply differentiated haplogroup from southern Italy (Fig. 1).

The two main clades of the *M. nattereri* complex were found not to be monophyletic; in fact *M. escalerai* and *Myotis* sp. B cluster together with *M. schaubi* (Fig. 2).

Genetic structure

The main K2P distances for *Cytb* between the haplogroups are shown in Fig. 3. The largest between-group distance was found between the Maghrebian *Myotis* sp. B and the European *M. nattereri* (16.6% in *Cytb*). The Iberian *M. escalerai*

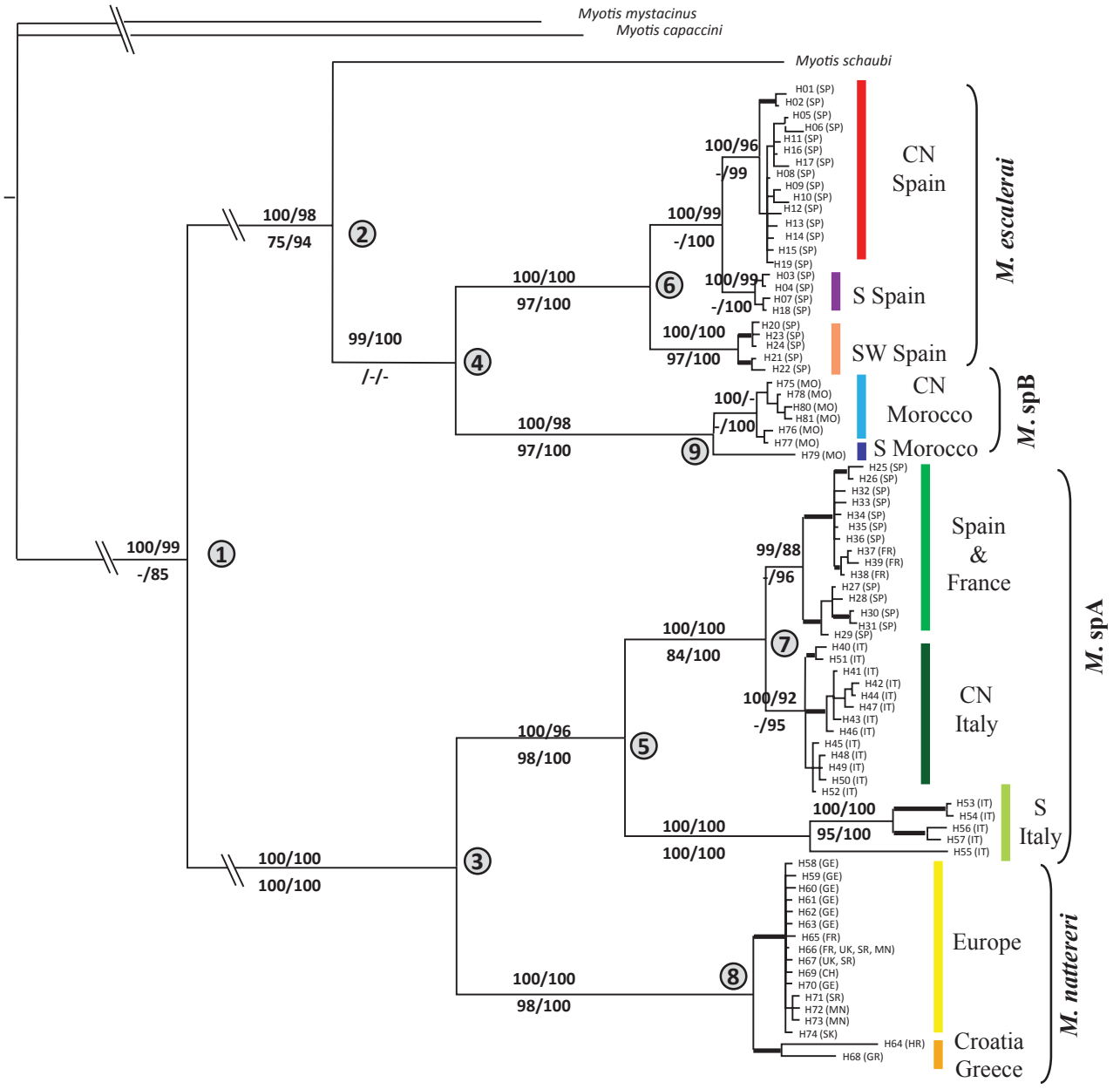

Figure 2 Phylogenetic reconstructions within the *Myotis nattereri* complex obtained by concatenating mtDNA fragments of *Cytb, ND1* and HVII in Bayesian analyses. Numbers on branches indicate, respectively, posterior probability in Bayesian analyses (> 95%) and bootstrap supports for maximum likelihood, minimum evolution and maximum parsimony (> 75%) for the node. Bold on terminal branches indicates that they are supported by at least two of the four reconstructions. The name of each haplotype is followed by the abbreviations for the countries in which it is present: Croatia (HR), France (FR), Germany (GE), Greece (GR), Italy (IT), Montenegro (MN), Morocco (MO), Serbia (SR), Slovakia (SK), Spain (SP), Switzerland (CH) and the United Kingdom (UK). The main clades and their geographical origins are also indicated. Colours correspond to those in Fig. 1. For more information on the geographical origins of haplotypes, see Appendix S1.

is more closely related to the Moroccan clade (7.5%) than to the rest of the European clades (12.6%). On the other hand, *M. nattereri* s.s. diverges from its sister lineage *Myotis* sp. A by 8.5%, and up to 15.3% from *M. escalerai*. Within-group distances vary from a low of 0.2% for *M. nattereri* s.s. (excluding the Croatian and Greek samples) and the central–northern Iberian *M. escalerai* to a high of 2.7% for *Myotis* sp. A (Table 1).

The main groups and subgroups revealed by the phylogenetic reconstructions are also clearly delimited by the network approaches (Fig. 3). A statistical parsimony network, using the 95% connection limit (equal to 17 maximum connection steps), gave rise to 12 unconnected networks in strong concordance with the phylogenetic groupings and their geographical origins. The 90% limit, corresponding to 25 maximum connection steps, still resulted in nine

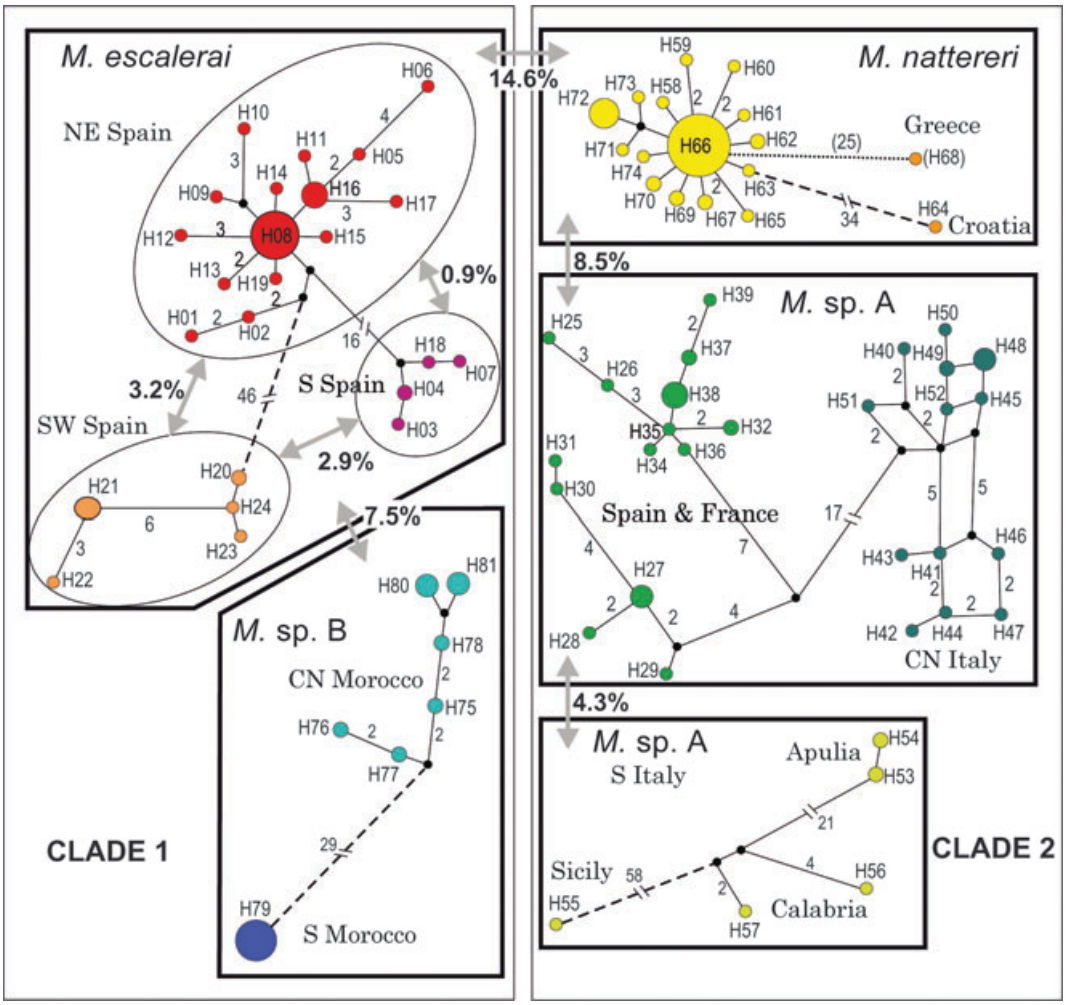

Figure 3 Haplotype network for the *Myotis nattereri* complex. Circles represent haplotypes, with circle size proportional to the number of individuals in each haplotype. Solid lines represent connections inferred by both parsimony and median-joining networks, dotted lines represent connections not inferred by the parsimony method (90% limit). Missing haplotypes are indicated by black circles. Numbers near a branch indicate the number of mutations (when more than one). The colours of the circles are the same as in Fig. 1. The analysis for the Greek sample (H68) is based on *Cytb* and *ND1* only. Haplotype names, distribution areas, names of the clades and mean values of the corrected K2P genetic distance are also indicated.

unconnected networks. Sequences from Sicily, Croatia and Greece remained completely isolated.

The estimates of genetic diversity and polymorphism are very high not only for the whole data set (Hd = 0.971; π = 0.086) and the four species, but also for the majority of subclades (Table 1). The lowest levels of variability are found in *M. nattereri*: as many as 81% of the individuals from the United Kingdom to Montenegro share the same *Cytb* haplotype, and 36% share the same haplotype of the three studied fragments (see Appendix S1). Only the Greek and Croatian samples differ from the clear star-shape network that has this common haplotype at its centre (Fig. 3). The Croatian sample alone accounts for 33 out of the 45 singleton variable sites. For this reason the analysis was repeated without this sample and the Greek one which lacks the HVII sequence. Conversely, *Myotis* sp. A shows remarkable geographical and genetic structure, possessing a monophyletic central–northern Italian clade which differs around 2% from the Franco-Iberian lineage; both groups show further genetic structure. The southern Italian clade diverges 5% from its sister group and it shows the highest variability; in this case 46 out of the 54 singletons correspond to the unique sequence from Sicily.

F_S, R_2 and D indexes test the null hypothesis of demographic stability. All three indexes are highly significant for *M. nattereri* s.s. (R_2 is significant only when the Croatian sample is excluded) and central–northern Iberian *M. escalerai* (Table 2). The concordance of the significance test results for the three indexes and the evident unimodal profile of mismatch distributions (Appendix S2) indicate that demographic expansion has occurred in these lineages. F_S suggests population expansion for the Italian lineage of *Myotis* sp. A, but in this case the three indexes show very different values, suggesting a more complicated history (Table 2).

We have subsequently estimated the time of expansion for both *M. nattereri* s.s. and the central–northern *M. escalerai* clade. Population expansion could be dated to approximately 39,682 years ago (95% confidence interval, CI: 5370–46,296) for *M. nattereri* s.s. and to around 16,917 years ago (95% CI: 7724–28,650) for the central–northern Iberian clade.

Divergence times estimates

According to our estimations, the deep split between the two main groups of species occurred around the end of the Pliocene epoch, with successive divergences taking place in the Pleistocene (Table 3).

The speciation events (i.e. the divergence between *M. escalerai* and *Myotis* sp. B and between *M. nattereri* s.s. and *Myotis* sp. A) occurred during the second half of the early Pleistocene, while the main intraspecific divergences date back to the middle Pleistocene, with the split of the southern Italian clade within *Myotis* sp. A representing the oldest internal isolation event (0.43 Ma), and the split within *Myotis* sp. B, the most recent one (0.16 Ma) (Table 3).

Table 1 Genetic variability for the clades and main subclades within the *Myotis nattereri* complex: sample sizes (n), number of haplotypes (h), number of mutations (η), singleton mutations (S), haplotype diversity (Hd), nucleotide diversity (π) and within-group diversity (d).

	n	h	η	S	Hd	π	d
Myotis escalerai	41	24	90	20	0.938	0.015	1.4%
Central–northern Iberia	27	15	29	19	0.877	0.002	0.2%
South-western Iberia	9	5	11	4	0.806	0.003	0.3%
Myotis sp. B	14	7	37	0	0.813	0.012	0.9%
Central–northern Morocco	8	6	8	0	0.929	0.002	0.2%
Myotis sp. A	37	27	60	10	0.977	0.012	2.7%
France and Spain	21	14	31	7	0.948	0.006	0.7%
Central–northern Italy	16	13	17	3	0.967	0.004	0.5%
Southern Italy	5	5	77	54	1.000	0.023	2.6%
Myotis nattereri	37	16	52	45	0.842	0.002	0.3%
excluding Croatia	36	15	18	12	0.833	0.001	0.2%
Total	134	75	445	36	0.971	0.086	

DISCUSSION

Our study confirms the extensive genetic diversification and geographical structure within the *M. nattereri* complex in the Western Palaearctic. Our results retrieve the different mtDNA lineages identified in previous studies (Ibáñez *et al.*, 2006; Mayer *et al.*, 2007; Galimberti *et al.*, 2010) and recently confirmed by nuclear markers (Salicini *et al.*, 2011). More importantly, our more intensive sampling over a wider geographical scale, as well as our more exhaustive statistical analyses, allowed us to probe phylogenetic relationships between lineages and their geographical distributions, and to formulate hypotheses concerning the evolution of the species complex.

Biogeography of the *Myotis nattereri* complex

We found four main mitochondrial lineages throughout the European and North African distribution. These clades are consistent with the lineages found by previous studies and recently identified as cryptic species (Salicini *et al.*, 2011): *Myotis escalerai* (Ibáñez *et al.*, 2006), *Myotis* sp. B (García-Mudarra *et al.*, 2009), *Myotis* sp. A (Ibáñez *et al.*, 2006), and *Myotis nattereri* s.s. (hereafter, *M. nattereri* only).

The deep basal split reveals a profound differentiation in mtDNA within the *M. nattereri* complex between central–eastern European regions and western Mediterranean areas, which were found to be more closely related to far eastern species (*M. schaubi*). This pattern suggests a former connection between the Iberian Peninsula and far eastern lineages, probably through the Mediterranean Africa and across the Strait of Gibraltar. The colonization of the European continent via Gibraltar has also been suggested for several other animal species (e.g. Cosson *et al.*, 2005; Fritz *et al.*, 2006).

Myotis nattereri is widely distributed across Europe, but presents an extremely shallow differentiation. To date,

Table 2 Demographic indexes for the clades and main subclades of the *Myotis nattereri* complex: Fu's (F_S), R_2, Tajima's (D) neutrality indexes, and the sum of squared deviations (SSD) of mismatch distributions, with their respective P value. Asterisks (*) indicate significance at the 95% confidence interval.

	F_S (P)	R_2 (P)	D (P)	SSD (P)
Myotis escalerai	0.092 (0.568)	0.121 (0.682)	0.248 (0.675)	0.036 (0.619)
Central–northern Iberia	−7.196 (0.001)*	0.050 (0.000)*	−2.154 (0.003)*	0.189 (0.006)*
South-western Iberia	1.016 (0.691)	0.190 (0.646)	−0.655 (0.773)	0.094 (0.138)
Myotis sp. B	5.308 (0.980)	0.252 (0.999)	2.609 (1.00)	0.118 (0.139)
Central–northern Morocco	−1.209 (0.177)	0.223 (0.783)	0.762 (0.797)	0.125 (0.060)
Myotis sp. A	−4.057 (0.098)	0.149 (0.891)	1.019 (0.889)	0.010 (0.409)
France and Spain	−1.736 (0.229)	0.140 (0.658)	0.293 (0.672)	0.017 (0.590)
Central–northern Italy	−4.717 (0.015)*	0.172 (0.824)	0.769 (0.815)	0.012 (0.771)
Myotis nattereri	−5.459 (0.013)*	0.106 (0.447)	−2.593 (0.000)*	0.007 (0.433)
excluding Croatia	−10.544 (0.000)*	0.044 (0.000)*	−2.050 (0.003)*	0.003 (0.791)

Table 3 Bayesian coalescent estimates of divergence times between and within species of the *Myotis nattereri* complex. Mean node ages and 95% highest posterior density interval (HPD) are given in million years ago (Ma) for a substitution rate of 0.054 substitution/site/Myr. The numbers in the first column refer to the node number in Fig. 2.

		Mean	95% HPD (Ma)
1	Clade 1/Clade 2	2.60	(1.93–3.28)
2	*M. escalerai* + *Myotis* sp. B/*M. schaubi*	1.44	(1.02–1.93)
3	*M. nattereri*/*Myotis* sp. A	1.22	(0.89–1.56)
4	*M.escalerai*/*Myotis* sp. B	0.99	(0.66–1.33)
5	*Myotis* sp. A (southern Italy)	0.43	(0.27–0.59)
6	*M. escalerai* (south-western Spain)	0.36	(0.22–0.52)
7	*Myotis* sp. A (central–northern Italy/France + Spain)	0.31	(0.20–0.44)
8	*M. nattereri*	0.29	(0.17–0.41)
9	*Myotis* sp. B	0.16	(0.07–0.25)

M. nattereri has not been found in either Italy or Iberia and our study notably reduces its distribution as previously estimated (Simmons, 2005).

The results of the present study extend the geographical distribution of a second European species (*Myotis* sp. A) from the northern mountains of Spain, throughout the Pyrenees, in the mountains of the northern Sistema Ibérico and western Sistema Central. This clade in Spain is sympatric with *M. escalerai*, although possibly with elevational segregation, as *Myotis* sp. A was found at higher elevations than *M. escalerai* (Ibáñez *et al.*, 2006). *Myotis* sp. A was the only lineage found in Italy, widespread throughout the peninsula at various elevations. The deep and ancient differentiation of the southern Italian clade and its geographical isolation suggest that this lineage could represent a subspecies of *Myotis* sp. A.

In addition to Spain and Italy, we also found this lineage in the south-western French regions of Midi-Pyrénées and Aquitaine. Both *M. nattereri* and *Myotis* sp. A are present in France, although their distributions do not appear to overlap (see also Puechmaille *et al.*, 2012).

Myotis escalerai is widely distributed across the Iberian Peninsula including the Balearic archipelago. This species shows high polymorphism and strong genetic structure, with three different haplogroups. The most differentiated group is found in south-western Iberia, while the samples from southern Spain also cluster together in a well-supported group. Finally, the most widely distributed clade occupies the northern and the eastern portions of the Iberian Peninsula, including the Balearic Islands. Recently, *M. escalerai* has also been found on the northern slopes of the eastern French Pyrenees, close to the Spanish border (Evin *et al.*, 2009; Puechmaille *et al.*, 2012).

The fourth cluster is composed exclusively of the Moroccan lineage (*Myotis* sp. B), with one branch that extends from the Rif to the Middle Atlas mountains, diverging 1.5% in the *Cytb* from another lineage distributed along the southern slopes of the Grand Atlas ridge. Due to its differentiation from the European lineages, the North African cluster represents an undescribed species (Salicini *et al.*, 2011), the distribution, morphological characterization and conservation status of which await further study.

Glacial refugia

Our results clearly demonstrate a non-random pattern of genetic structure and geographical distribution for the *Myotis nattereri* complex, a pattern that agrees with the predictions for allopatric persistence in southern glacial refugia and post-glacial recolonization of northern areas. The identified species correspond well to the three major European refugia plus the Maghreb region (Fig. 1).

The genetic isolation of the Italian lineage and the basal position of the southern Italian clade suggest, for *Myotis* sp. A, a long persistence and allopatric origin in the Italian peninsula during the second half of the early Pleistocene. During an interglacial period the species probably had a wider European distribution, which was interrupted by a later expansion of the ice cover and changes in the environment that forced its populations to retreat to southern areas, thereby splitting them into two vicariant groups. The two groups remained isolated for the subsequent late Pleistocene glaciations. The entry of the Italian clade into the Iberian Peninsula led to secondary contact with *Myotis escalerai*, already present in the peninsula. Conversely, the Alps would then have acted as a barrier and hindered the later and eastern expansion of the Italian clade.

Simultaneously, *M. nattereri* expanded rapidly from the Balkans (or even further east) to central and western Europe, as indicated by the significant indexes for population expansion, the star-shaped network and the low genetic variability within this clade.

The presence of *Myotis* sp. A in southern France may have impeded the advance of *M. nattereri* westwards towards the Pyrenees, although a more complete sampling of southern and central France is necessary to confirm this scenario and to identify past and current relationships between the Italian and Franco-Iberian *Myotis* sp. A groups.

Post-glacial recolonization of Europe by the *M. nattereri* complex appears to follow the 'grasshopper paradigm' (Hewitt, 1999), with the Balkan peninsula as the major source for the recolonization of central and northern Europe, and the Pyrenees and the Alps presenting serious barriers (Taberlet *et al.*, 1998). However, this taxon differs from the paradigmatic model in the closer relationship of the Iberian clade to the North African one, and the presence of the same clade (*Myotis* sp. A) in disconnected areas of Italy and south-western Europe. Our scenario, therefore, indicates a connection between different refugia that represents a novel contribution to the general theory of refugia (see also Valdiosera *et al.*, 2008).

Structure within refugia

Strong genetic subdivision, with deep geographically grounded patterns, is shown by both the Italian and the

Iberian lineages. Genetic structure points to a scenario of survival throughout the Pleistocene glacial cycles in separate refugia within the southern peninsulas and provides evidence for the existence of a refugia-within-refugia pattern. In both regions high variability is recognizable at two levels: (1) high intrapopulation polymorphism most likely due to its long persistence in the area; and (2) deep differentiation between populations, corroborating the hypothesis of allopatric multiple refugia (Gómez & Lunt, 2007).

In Iberia at least three subclades with non-random geographical distributions are distinguishable within *M. escalerai*, thereby suggesting the existence of at least as many refugia. Two of the clades are currently found in sympatry in the central–eastern area of Spain, in the north of the Extremadura region.

Although an exact match is virtually impossible due to the great variability in ecological and behavioural characteristics and stochastic events, the study of phylogeographical concordance is a robust tool for estimating the role of vicariance in speciation processes (Avise, 2000; Gómez & Lunt, 2007). The three identified refugia for *M. escalerai* within Iberia correspond to important putative allopatric refugia identified by previous studies of non-volant organisms; the Baetic region (south-eastern Spain) seems to have been particularly important (see Centeno-Cuadros *et al.*, 2009, and examples in Gómez & Lunt, 2007).

The genetic conformity of the Balearic samples with the Iberian sequences suggests a recent dispersal from the mainland to the archipelago, a pattern also present in the greater mouse-eared bat, *Myotis myotis* (Ruedi & Castella, 2003), and the grey long-eared bat, *Plecotus austriacus* (Juste *et al.*, 2004).

Myotis sp. A shows evident genetic structure within both the Franco-Iberian region and the Italian peninsula. Within western *Myotis* sp. A, two genetic–geographical groups are differentiated: a small haplogroup limited to the eastern Pyrenees and a widely distributed haplogroup that connects the central area of the Iberian Peninsula to France through the western Pyrenees. Long-term differentiation in allopatric refugia is evident also in the Italian peninsula, with a deep split between the southern and central–northern clades, and strong genetic structure within the southern lineage. The three lineages found in southern Italy probably correspond to three different refugia. The sample from Sicily appears especially differentiated, despite its geographical proximity to the mainland. Southern Italy and Sicily are particularly rich in lineages for many other taxa, such as the red squirrel (Grill *et al.*, 2009) and edible dormouse (Hürner *et al.*, 2010), among others (e.g. Joger *et al.*, 2007). Recently, the presence of multiple refugia in the Italian peninsula has been described for amphibians (Canestrelli & Nascetti, 2008b; Canestrelli *et al.*, 2008a) and for the European whip snake, *Hierophis viridiflavus* (Rato *et al.*, 2009). Our limited sampling in southern Italy makes it difficult to recognize putative refugial areas or identify dispersal barriers. Calabria has been identified as an area of discontinuity for many taxa, probably due to the repeated isolation and fragmentation of the Calabrian Apennine massifs during Pleistocene sea-level oscillations (Canestrelli *et al.*, 2008a; Grill *et al.*, 2009), although it is unclear how this could have had such a strong effect on a flying mammal.

In the Balkans, only two samples from Croatia and Greece diverge significantly from an otherwise genetically uniform group. Although we did not identify any geographical structure in haplotypes from this region, the area should be further investigated. A clear pattern of multiple refugia in the Balkans has been described for the nose-horned viper, *Vipera ammodytes* (Ursenbacher *et al.*, 2008), while a phylogeographical study of the edible dormouse, *Glis glis*, suggested that the actual Balkan diversity could be masked by a lack of sampling in the southern part of this region (Hürner *et al.*, 2010). Something similar could be happening in our analyses.

Morocco also shows two markedly differentiated lineages that correspond with geography. The Atlas Mountains acted as an important barrier for many other taxa (e.g. Brown *et al.*, 2002; Fritz *et al.*, 2006) and may also explain the genetic discontinuity within *Myotis* sp. B.

The strong within-lineage genetic structure found in this study confirms for the first time the predictions of the refugia-within-refugia pattern for organisms with a high dispersal capacity. Despite the subsequent disappearance of the geographical barriers that caused this structure, maintenance of deep diversification within clades may be related to ecological or behavioural characteristic of the taxa (i.e. philopatry; Rivers *et al.*, 2005).

Evolutionary timing

The wide differentiation within the *M. nattereri* complex is the result of various evolutionary processes corresponding to different periods. Caution is necessary when drawing conclusions from molecular dating (e.g. Pulquério & Nichols, 2007), even if relaxed molecular clock methods avoid assumptions of rate constancy. Hence, divergence dates should be considered as time windows.

The legacy of the Pleistocene glacial and interglacial periods is evident in the genetic structure and geographical distribution of our phylogenetic reconstructions. Speciation events occurred towards the end of the early Pleistocene and the within-species subdivisions occurred throughout the last 500,000 years, during the intense glacial–interglacial cycles of the middle and late Pleistocene (Klotz *et al.*, 2006). This timing demonstrates that allopatric populations survived in their isolated refugia for multiple climatic cycles, where they had time to accumulate genetic differences that were not lost during subsequent glacial and interglacial periods. Interestingly, the estimated divergence time of the southern Italian clade within *Myotis* sp. A seems to correspond to the estimate of isolation proposed for the southern Italian clade of the edible dormouse (Hürner *et al.*, 2010).

The results of this study are also particularly relevant for the conservation of the *M. nattereri* complex. What was

considered a single species a few years ago has been identified as a complex of four cryptic species with restricted distributions, the conservation status of which is unknown. Many other well-differentiated lineages are recognized at the intraspecific level in southern Mediterranean regions. Their long-term isolation and independent evolutionary histories, resulting from survival in a refugia-within-refugia scenario, indicate that these lineages should be considered as evolutionary significant units and that conservation efforts should be taken to preserve this outstanding and newly discovered diversity.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 List of the samples used in this study, with taxonomic, genetic and geographical data and GenBank accession numbers.

Appendix S2 Mismatch distribution graphics for clades and main subclades within the *Myotis nattereri* complex.

BIOSKETCHES

Irene Salicini is a PhD student at the Estación Biológica de Doñana with a strong interest in evolutionary ecology and phylogeography, especially of Palaearctic bats. Her PhD thesis focuses on the phylogeography and phylogeny of the *Myotis nattereri* complex.

Carlos Ibáñez is a staff professor at the Estación Biológica de Doñana, CSIC, and is interested in various aspects of evolution, biogeography and ecology of mainly Neotropical and Palaearctic bats.

Javier Juste works as an assistant professor at the Estación Biológica de Doñana, CSIC. His scientific interests cover a variety of topics in evolutionary biology from morphological evolution to evolutionary relationships at different levels, mainly among bats.