Cryptic diversity of Italian bats and the role of the Apennine refugium in the phylogeography of the western Palaearctic

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The Mediterranean Basin is typified by a high degree of species rarity and endemicity that reflects its position, geomorphology, and history. Although the composition and cryptic variation of the bat faunas from the Iberian and Balkan Peninsulas are relatively well studied, data from the Apennine Peninsula are still incomplete. This is a significant shortfall, given the presumed refugial role of this region in the context of Europe's Pleistocene phylogeography. It was thus our aim to supplement the phylogeographical information from the region, generating mitochondrial sequences and reviewing published data, with a focus on the dispersal and diversification patterns characterizing taxa with different life strategies. Site-specific lineages were ascertained, especially in the genera *Myotis* and *Plecotus* and amongst the pipistrelloid bats, representing speciose radiations. It was possible to observe disjunct ranges with patches isolated south of the Alps in several species, corresponding with evolution of elevated genetic distance. The genetic subdivision within the continuous Italian range into northern and southern lineages in several taxa indicated the possible past substructure of the refugium. Several shared lineages between the Apennine and Ibero-Maghrebian regions were observed, indicating connectivity between the Adriatic and Atlantic–Mediterranean refuges, and raising questions as to which region these clades originated from and what was the direction of faunal exchange between them. In contrast to Europe's other two main refugia, the Apennine Peninsula is a smaller region with simpler phylogeographical patterns. Nevertheless, our results support the idea that the region generated novel lineages. Whereas diversification in sedentary bats may have been driven through the generation of *in situ* adaptations, specialization, and niche differentiation, the emergence of species with a tramp strategy could have entailed the utilization of faunal drift and the taxon cycle.

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INTRODUCTION

Distribution patterns of contemporary biodiversity are valuable sources of information with which to infer past biogeographical processes (Cox & Moore, 2010). In recent decades, our capacity to reveal the fine-scale geographical structure of particular species has been boosted by the appearance of molecular tools within the field of phylogeography (Avise, 2000). This trend has led to the formulation of many novel hypotheses regarding microevolution, including the mechanisms underpinning speciation, reinforcement, the evolution of intraspecific variation, and other phenomena. Moreover, molecular markers offer a more realistic description of diversity than that obtained with alpha taxonomy, providing a process-based approach to the species problem (Hart, 2010).

The patterns of mammalian diversity in Europe are traditionally used as model data for the biogeography of temperate zones, on account of the relatively fine-scale resolution that is available. Amongst terrestrial mammals, species-richness gradients display peaks in the mountainous parts of the temperate and Mediterranean regions, whereas maxima for species rarity and endemicity tend to be located in the mountains of south-eastern Europe, and on the Iberian and Apennine Peninsulas (Baquero & Tellería, 2001). The status of the Mediterranean Basin as one of the world's biodiversity hotspots is in turn related to the position of the region at the intersection of the Palaearctic, Afrotropical, and Oriental ecozones, as well as to the elevation of the speciation rate connected with a complicated geomorphology that includes the presence of islands (Blondel *et al.*, 2010).

Considering the geomorphology of southern Europe, the classical phylogeographical paradigm interprets these patterns especially in connection with the role of the Iberian, Apennine, and Balkan Peninsulas (alternatively the Atlantic, Adriatic, and Pontic-Mediterranean dispersal centres *sensu* de Lattin, 1967). These regions acted as refugia in which some temperate-zone species were able to persist even during the glacial peaks of Pleistocene climate oscillations, and in some cases to generate new lineages via mechanisms of allopatric speciation, as well as serving as centres for potential postglacial recolonization of continental Europe (Hewitt, 2000, 2004; Schmitt, 2007; Stewart *et al*., 2010). The phylogeographical structure of individual taxa may have been complicated by various contributions made by different refugia, different glaciation peaks, and different climate envelopes of the respective taxa, and the possible roles of refugia fragmentation (Schmitt, 2007), microrefugia (Rull, 2009), northern refugia (Rowe *et al*., 2004; Stewart *et al*., 2010), cryptic refugia (Stewart & Lister, 2001), and other phenomena (Bennet & Provan, 2008). By contrast, species richness in the Mediter-

ranean may have been altered as a result of far-reaching land-use change owing to protracted periods of human settlement and the recent rise in tourism.

Most recent mammalian surveys have tended to exclude bats (Chiroptera) because of the fragmentary knowledge of distributions and the taxonomic structure of particular lineages (Baquero & Tellería, 2001). As bat circadian rhythms, spatial niches, and sensory abilities are almost totally distinct from those characterizing humans, these mammals are notoriously difficult to study, and there is often a lack of data in particular fields as compared with other vertebrates. This is especially true for the Mediterranean Basin, notwithstanding the fact that this region boasts apparently high levels of bat species richness and endemicity. This is in turn related to the position of the region within the gradient of disparity and diversity of the order Chiroptera that increases towards the Tropics, as well as to increased speciation levels. However, more complete distributional data are now available in the light of the increasing interest being shown in the region, and the application of genetic markers has allowed complex phylogeographical patterns connected with complicated local geomorphology and palaeoclimatic history to be disentangled. By way of this effort, many cryptic taxa have come to be recognized during the last decade (Mayer, Dietz & Kiefer, 2007).

Amongst the three refugia of Mediterranean Europe, the Apennine Peninsula remains relatively undersampled where bat biodiversity is concerned. It was thus with the aim of filling this gap that the work described in the present paper was carried out. To assess the contribution of the Apennine Peninsula to cryptic diversity in European bats, we performed screening for mitochondrial markers in 212 specimens from 21 bat species resident in the region. As mitochondrial DNA is a fast-evolving haploid molecule with lower effective population size compared with nuclear DNA, it will probably carry traces of allopatric processes in the course of potentially separate refugial histories. The respective genealogies may help to reveal cryptic species and serve as useful proxies for the phylogeography of particular lineages (Avise, 2000).

The aims of this study were to analyse phylogeographical patterns characterizing particular species, to summarize the presence of potentially endemic lineages in Italy, to infer the ability of the Apennine Peninsula to act as a refugium and generate novel lineages, to study its potential substructure, to identify the relationship with the remaining European refugia areas, and to discuss the role of the Apennine Peninsula in the postglacial recolonization of particular territories of Europe. A further aim on the basis of the data acquired involved the study of the impact of biological attributes of particular taxa on modes of range shifts and differentiation.

MATERIAL AND METHODS

SAMPLING

We performed field work in June and August of 2008–2013 inclusive. Bats were captured by placing 2.5–6-m and 2.5–12-m mist-nets (50 denier, mesh size 38 mm) across rivers or streams, near cattle troughs used by bats to drink, or in forest sites. In the latter case we generally employed the AUTOBAT acoustic lure devised by Sussex University (Hill & Greenaway, 2005), with a view to increasing capture success. We erected nets soon after dusk and kept them in place for 4–6 h. Captured bats were removed from nets promptly and were identified to species level in the field, using published keys (Dietz & von Helversen, 2004).

Using a biopsy punch, one or two 3-mm diameter samples were taken from each wing membrane for genetic analysis. Within this study, populations from continental Italy and Sicily were sampled. Sardinia was excluded. Twenty-one of the 34 species recorded in the region (Agnelli *et al.*, 2004) were obtained, including *Myotis punicus*, newly recorded from Sicily. Several species with rather occidental occurrence in the region are not included, as evolution of separate lineages from these species is deemed to be less likely.

SEQUENCING

DNA extraction from wing biopsy punches was performed using a DNeasy Blood & Tissue Kit (Qiagen, Germany). DNA concentration (in ng μL^{-1}) was measured using a Qubit 2.0 Fluorometer with a Qubit dsDNA HS Assay kit (Life Technologies, USA). Primers for NADH dehydrogenase subunit 1 (ND1) amplification were ER66 (5′-GTATGGGCCCGATAGCTT-3′) and ER70 (5′-CAGACCGGAGTAATCCAGGT-3′) (Mayer & von Helversen, 2001). Twenty-five μL of REDTaq Ready Mix (Sigma-Aldrich, Germany), 0.2 μM of each primer, and 1.5 mg mL^{-1} of bovine serum albumin in a total volume of 50 μL made up the PCR mix. The PCR was performed in a Veriti Thermal Cycler (Life Technologies, USA) as follows: 10 min of initial denaturation at 95 °C, followed by 40 cycles of 45 s at 95 °C, 45 s at 52 °C, and 60 s at 72 °C. The programme was completed by a final extension step at 72 °C for 10 min and a final hold at 4 °C. To assess the quality of the amplified PCR products, electrophoresis in 2% agarose gel with Midori Green DNA Stain was performed. Only specific PCR products were subjected to purification using Clean Up (A&A Biotechnology, Poland). Elution of purified products was carried out in 50 μl of distilled H_2O. Both forward and reverse sequencing PCRs were carried out using a BigDye Terminator v. 3.1 Cycle Sequencing Kit (Life Technologies, USA). In the reaction, PCR primers were used as sequencing primers. Electrophoresis of sequencing reaction products was performed on a 3500xL Genetic Analyzer (Life Technologies, USA). Data were analysed using SEQUENCING ANALYSIS SOFTWARE v. 5.4 (Life Technologies, USA) and edited with BioEdit 7.1.3 software (Hall, 1999). Sequences were submitted to GenBank (accession numbers KJ948319–KJ948240 and KJ957194; see also Appendix S1).

PHYLOGEOGRAPHICAL ANALYSIS

Comparative ND1 sequence data relevant to our study were downloaded from GenBank. The list of specimens, localities and accession numbers are given in Appendix S1. Sequences for each species were aligned in BioEdit (Hall, 1999), and edited manually. Phylogenetic analyses were performed in MEGA X (Tamura *et al.*, 2007). Genetic distances were computed as uncorrected p-values. Minimum (p_{min}) and maximum (p_{max}) genetic distances between bat populations from Italy and the remaining western Palaearctic ranges were displayed for particular species. The distance p_{max} was included to illustrate the status of Italian lineages within particular species, to show those with the highest variability within their distributional range and provide a view of those taxa, which may represent cryptic species. Further details concerning the phylogenetic relationships of Italian samples were assessed using the neighbour-joining (NJ) algorithm (Saitou & Nei, 1987), with either 1000 (Fig. 2) or 500 (Appendix S2) bootstrap replicates. Distant lineages were treated as separate integrated operational taxonomic units (Galimberti *et al.*, 2012) in further analyses in the case of the *Pipistrellus pipistrellus* and *Myotis nattereri* species complexes. However, the classification of particular species complexes depends to a certain degree on the species definition used (Hey, 2001). The distance p_{min} was therefore used to order taxa and indicate the presence of site-specific mitochondrial lineages within the Apennine Peninsula. In addition, we had no data to distinguish taxa sharing mtDNA and differing in nuclear DNA for *Myotis myotis* and *Myotis blythii* (or *Myotis oxygnathus*), for which only a mitochondrial marker was used. Therefore, we treated this species group as one unit (marked *M. myotis/blythii*). Phylogeographical context in particular species was displayed by plotting sampling localities and haplotype information into maps of the western Palaearctic with the species range indicated.

RESULTS

The range of genetic distances for particular species between samples from Italy and from the remainder of the range is displayed in Table 1 and Figure 1. The highest values for p_{min} between Italian and non-Italian conspecifics were present within several members

Table 1. Descriptive characteristics of mitochondrial data: list of species, number of sequences analysed (N), number of Italian samples (N_I, total/present study), length of the alignment (l), range of detected genetic distances between Italian and non-Italian conspecifics (p_{min} – p_{max}). Clades I and II in *Myotis nattereri* and *Pipistrellus pipistrellus* (although using 650 and 894 bp, respectively) are shown in Figure 2

Species	N	N_I	l	p_{min}	p_{max}
Family Rhinolophidae					
Rhinolophus euryale	23	16/16	926	0.000 (Turkey)	0.005 (Turkey)
Rhinolophus ferrumequinum	11	1/1	957	0.001 (Turkey)	0.011 (Greece, Turkey)
Family Vespertilionidae					
Barbastella barbastellus	6	1/1	900	0.006 (Greece)	0.010 (Turkey)
Eptesicus serotinus	16	5/5	801	0.005 (Germany, Hungary, Turkey)	0.074 (Syria)
Hypsugo savii	37	22/22	900	0.006 (Spain)	0.099 (Spain)
Myotis alcathoe	39	8/8	885	0 (Croatia, Poland, France, Hungary, UK)	0.015 (Greece)
Myotis bechsteinii	27	2/2	919	0.021 (Germany, Portugal, Greece, Bulgaria, UK)	0.060 (Caucasus, Anatolia)
Myotis brandtii	49	4/4	914	0.008 (Greece, Poland)	0.015 (Germany)
Myotis capaccinii	11	2/1	856	0.010 (Greece)	0.037 (Turkey)
Myotis daubentonii	27	18/18	956	0.006 (Germany, Scotland)	0.032 (Spain)
Myotis emarginatus	19	7/7	899	0.001 (Germany, Belgium, Greece)	0.010 (Greece, Israel)
Myotis myotis/blythii	37	26/26	799	0.010 (Germany, Poland)	0.029 (Germany, Poland)
Myotis mystacinus	104	35/35	906	0.002 (Ireland, UK, Poland)	0.045 (Morocco)
Myotis nattereri	45	15/8	798	Clade I: 0.012 (Spain); clade II: 0.063 (Spain)	Clade I: 0.148 (Spain); clade II: 0.148 (Spain)
Myotis punicus	5	2/1	899	0.008 (Morocco)	0.011 (Morocco)
Nyctalus leisleri	22	7/7	800	0 (Ireland, Italy, Turkey, Greece, Switzerland)	0.008 (Morocco)
Pipistrellus kuhlii	27	6/3	799	0 (Italy, Morocco, Switzerland. Greece)	0.058 (Spain, Turkey)
Pipistrellus pipistrellus	43	18/15	899	Clade I: 0.006 (Morocco, Turkey); clade II: 0.015 (Morocco)	Clade I: 0.030 (Morocco); clade II: 0.034 (Turkey)
Plecotus auritus	32	16/14	858	0.002 (Spain, Ireland, Switzerland)	0.058 (Spain)
Family Miniopteridae					
Miniopterus schreibersii	25	17/17	799	0.00 (Spain)	0.037 (Azerbaijan)
Family Molossidae					
Tadarida teniotis	4	1/1	1148	0.001 (Libya)	0.003 (Turkey)
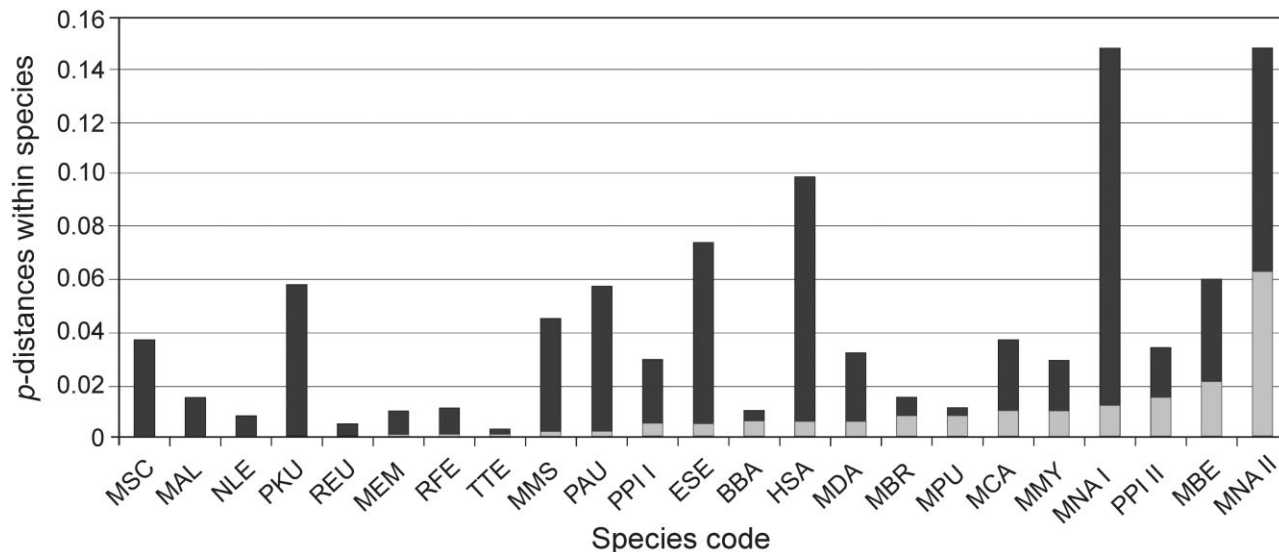
Figure 1. Minimum (p_{min}, light) and maximum (p_{max}, dark) genetic distances of mitochondrial sequence marker detected between bat populations from Italy and the rest of their western Palaearctic ranges measured as uncorrected p-distances. Species codes are as follows: MSC, *Miniopterus schreibersii*; MAL, *Myotis alcathoe*; NLE, *Nyctalus leisleri*; PKU, *Pipistrellus kuhlii*; REU, *Rhinolophus euryale*; MEM, *Myotis emarginatus*; RFE, *Rhinolophus ferrumequinum*; TTE, *Tadarida teniotis*; MMS, *Myotis mystacinus*; PAU, *Plecotus auritus*; PPI I, *Pipistrellus pipistrellus* clade I; ESE, *Eptesicus serotinus*; BBA, *Barbastella barbastellus*; HSA, *Hypsugo savii*; MDA, *Myotis daubentonii*; MBR, *Myotis brandtii*; MPU, *Myotis punicus*; MCA, *Myotis capaccinii*; MMY, *Myotis myotis/blythii*; MNA I, *Myotis nattereri* clade I; PPI II, *Pipistrellus pipistrellus* clade II; MBE, *Myotis bechsteinii*; MNA II, *Myotis nattereri* clade II (see also Fig. 2).

of the genus *Myotis*, and in pipistrelloid bats (one lineage of the *P. pipistrellus* species complex and *Hypsugo savii*). In the case of *Plecotus auritus*, an endemic lineage in sympatry with two other haplogroups occurring in Italy was detected. The phylogeographical situation is further illustrated in species with $p_{min} > 0.01$ and in species with $p_{max} > 0.05$ (Fig. 2).

In *M. nattereri*, southern Italy has an endemic haplogroup whose position is distant from those of the rest of the European haplotypes ($p_{min} = 0.063$), whereas central and northern Italy are inhabited by a further lineage with a sister branch in southern France and Spain separated by a distance of $p_{min} = 0.012$. Relatively deep divergence between Italian and non-Italian conspecifics was also observed in *Myotis bechsteinii* ($p_{min} = 0.021$). The basal branch of the *M. bechsteinii* tree is occupied by Anatolian haplotypes, followed by the Italian branch and then haplotypes from the rest of Europe, including the Balkan Peninsula (Appendix S2). Decreasing divergence was detected in *Myotis capaccinii*, *M. myotis/blythii*, *Myotis brandtii*, *Myotis punicus*, *Myotis daubentonii*, *Myotis mystacinus*, *Myotis emarginatus*, and *Myotis alcathoe*.

In *Plecotus auritus*, three sympatric haplogroups were identified in the Apennine Peninsula, one of them

endemic for the region, separated by $p_{min} = 0.027$ and $p_{min} = 0.034$ from the eastern and western continental lineages, respectively.

Within *P. pipistrellus*, the southern and central parts of the Apennine refugium bear endemic haplotypes manifesting relationships with the Maghrebian ones, as separated by a genetic distance of $p_{min} = 0.015$. The northern part is inhabited by populations similar to those across the rest of the European range (Appendix S2). In *H. savii*, the Italian haplogroup clusters along with haplotypes from the Maghreb and Iberia, separated by a genetic distance of $p_{min} = 0.012$. Some differences could also be observed between insular (Sicily) and mainland populations of *Eptesicus serotinus*.

Additional bat taxa of the region exhibited small differences from non-Italian conspecifics (Appendix S2). The base of the *Pipistrellus kuhlii* species tree is occupied by the Iberian and Anatolian branches, whereas the Italian haplotypes are part of a shallow clade that is widespread from the Ibero-Maghrebian region across to the Balkans and Anatolia. No exclusively Apennine lineages were detected in *Nyctalus leisleri* either, reflecting the shallow genetic structure within Europe. A similar situation was found for *Barbastella barbastellus* – the species from the tribe Plecotini. In
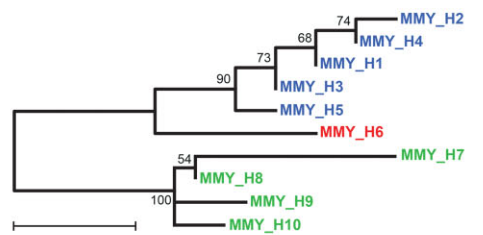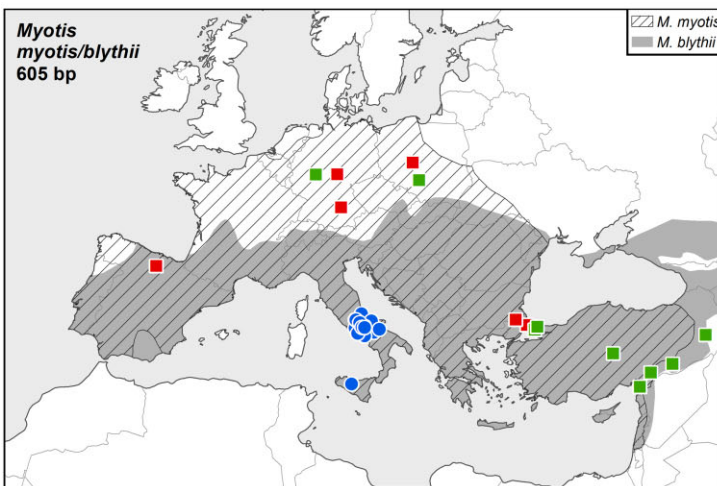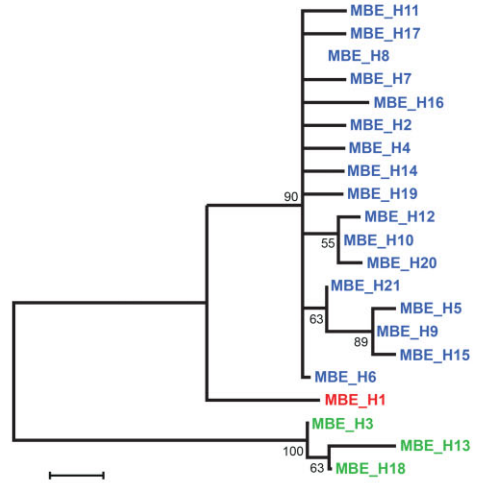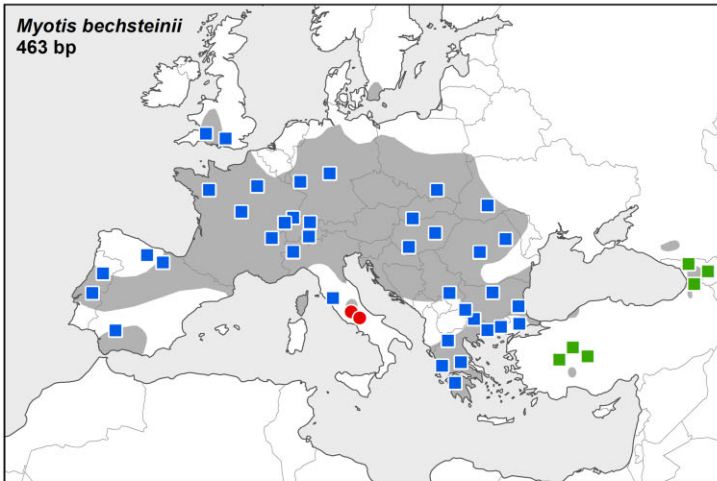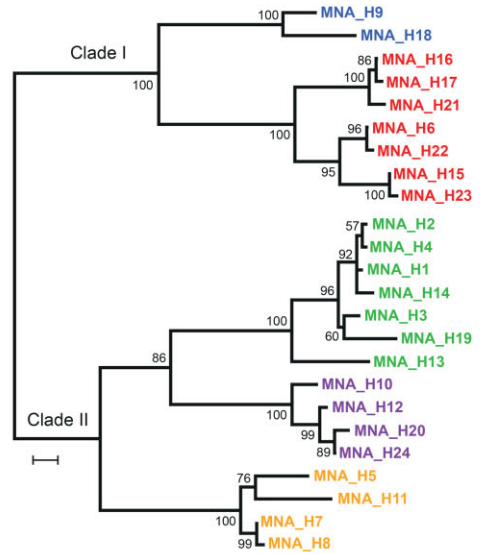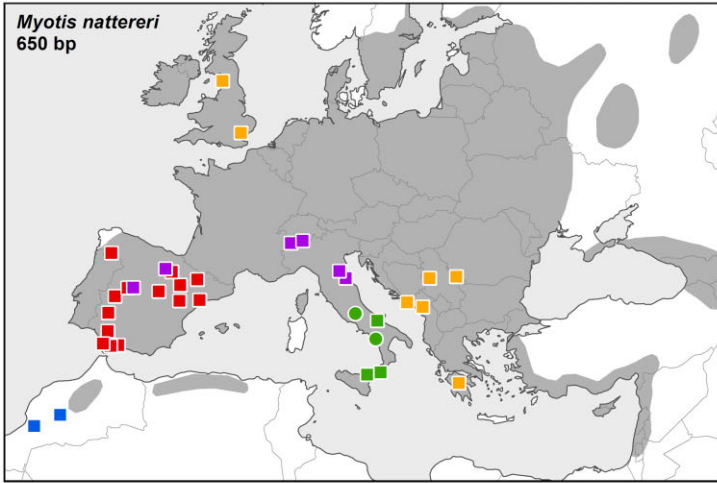
Figure 2. Phylogeographical structure of nine taxa with the most divergent lineages [with minimum genetic distance (p_{min}) > 0.01 and/or p_{max} > 0.05] ascertained in Italy. Shaded areas represent species ranges and were taken from the IUCN Red List of Threatened Species website (http://www.iucnredlist.org). Samples from known localities are marked as circles; the origin of samples marked as squares is limited to either province or country range. The number of base pairs used in the generation of particular haplotype trees is also shown (cf. Table 1). Symbols and terminal taxa are coloured according to the appearance order of clades on the haplotype tree (1, blue; 2, red; 3, green; 4, violet; 5, orange). Scale bars correspond to 0.005 substitutions/site.

◄——

Miniopterus schreibersii, a member of the newly recognized family Miniopteridae, a shallow genetic structure stretching from Turkey to Spain was ascertained. In *Tadarida teniotis*, the only European member of the family Molossidae, almost no geographical structure was found amongst the samples taken from Italy, Greece, and Libya. A shallow genetic structure was also indicated for European members of the genus *Rhinolophus*.

DISCUSSION

ISOLATION IN REFUGIAL AREAS; REFUGIA WITHIN REFUGIA

Italy is located in the centre of the Mediterranean Basin and is mainly formed by the Apennine Peninsula and the island of Sicily. Relatively recently (Pliocene to Pleistocene), it has been surrounded by the Ligurian and Tyrrhenian Seas in the west and the Adriatic and Ionian Seas in the east (Vai & Martini, 2001). The region is typified by its smaller land mass and simpler geomorphology than the other two main European refugia, and is consequently found in most cases to be characterized by simpler phylogeographical patterns (Schmitt, 2007). However, the area of the region was larger during glacial peaks owing to the decrease in the sea level, similarly to all Mediterranean peninsulas. Isolation of this region enabled the evolution of endemic mammalian lineages at different depths of divergence, for example *Elephas antiquus italicus* Osborn, 1931, *Cervus elaphus rianensis* Leonardi & Petronio, 1974, and a genetically separate lineage of *Canis lupus* (Pilot *et al.*, 2010). As insectivorous mammals dependent on active insects, bat populations should presumably have been impacted upon markedly by Pleistocene climate oscillations. Indeed, the genetic variation and number of newly discovered species increases southwards (Ibáñez *et al.*, 2006). Isolation of refugial populations may persist until recent, or may evolve recurrently owing to the geography of the region. In our study, disjunct ranges with isolated patches in Italy were observed in several *Myotis* species (*M. bechsteinii*, *M. capaccinii*, *M. brandtii*, *M. daubentonii*, and probably also *M. mystacinus* and *M. alcathoe*). Such patterns illustrate (apart from the ecological characteristics

of the genus, see below) the role of the Alps as a barrier and the tendency of some populations to remain 'trapped' south of these mountains. Several lineages are characterized by relatively high values of genetic distance from their continental relatives (Fig. 1), illustrating ongoing allopatric diversification. In these isolates, genetic drift, site-specific adaptations, and other phenomena typical for insular populations may occur (Fleming & Racey, 2009).

The patterns within the Apennine populations of several species (*M. nattereri*, *P. pipistrellus*) also indicate a more complicated genetic architecture within Italy with two haplogroups present within a presumably continuous range. Such a situation may be a result of past allopatry. The separate status of lineages in the southern part of the region, Calabria and Sicily, which were presumably especially important as allopatric differentiation centres (Schmitt, 2007), was ascertained in many organisms. The phylogeographic pattern showing differentiation between lineages from Calabria and Sicily is not frequent among recent taxa, although it has, for example, been reported in the case of the West European hedgehog (Seddon *et al.*, 2001). Microrefugia probably also existed in the northern part of Italy (Ursenbacher *et al.*, 2006). A mosaic phytogeographical composition of the glacial landscape (Huntley & Birks, 1983) may further increase the heterogeneity of the environment and contribute to species diversification within the refugial area. Further research with detailed sampling and fast-evolving markers is needed to map exactly the species distribution and population characteristics of particular demes, including their size and level of gene flow.

OUT OF ITALY – THE BIOGEOGRAPHICAL RELATIONSHIP WITH OTHER REFUGIA AND CONTINENTAL EUROPE

The exchange of terrestrial fauna between particular refugia occurs in mammals with a higher dispersal capacity, such as large carnivores (Valdiosera *et al.*, 2007). The phylogeography of bats provides some evidence of similar processes, as demonstrated in *M. nattereri*, *P. pipistrellus*, and *H. savii* in our study. Moreover, as
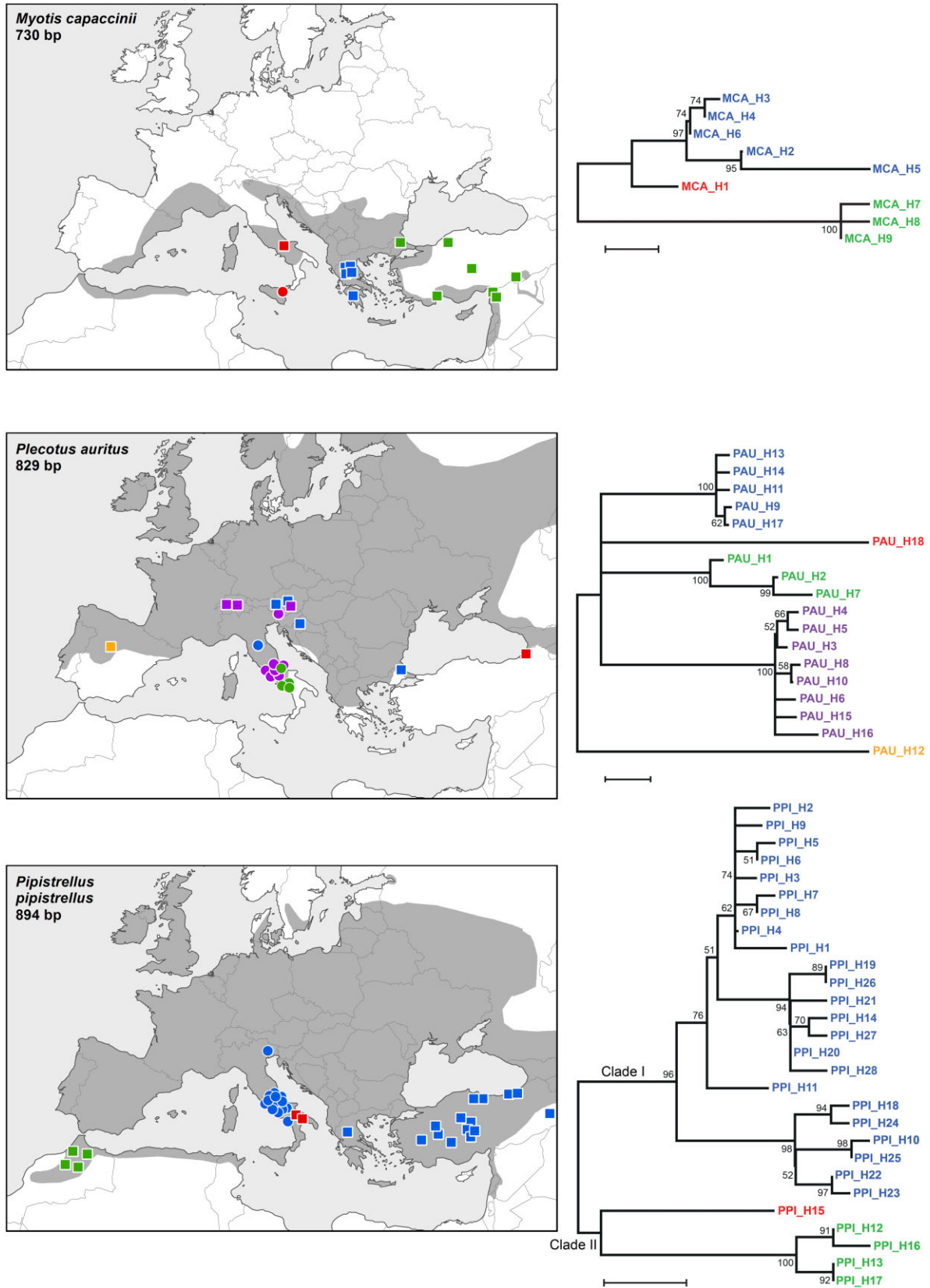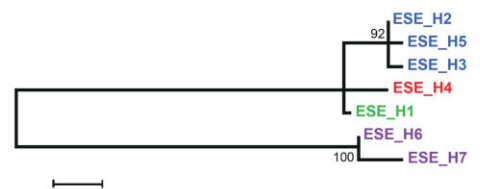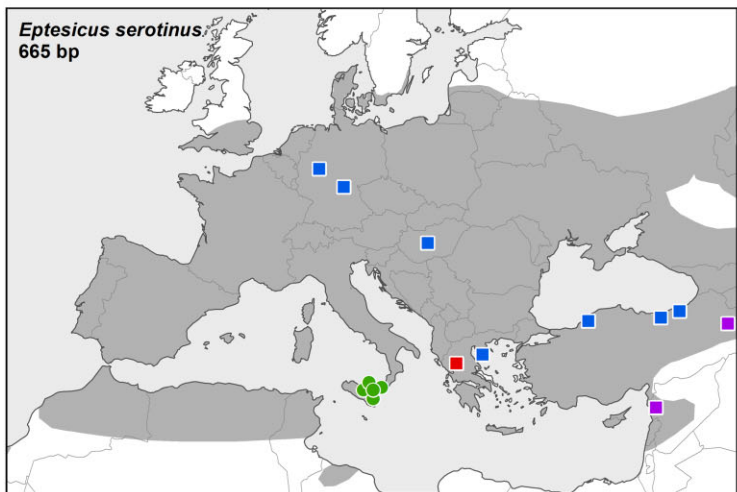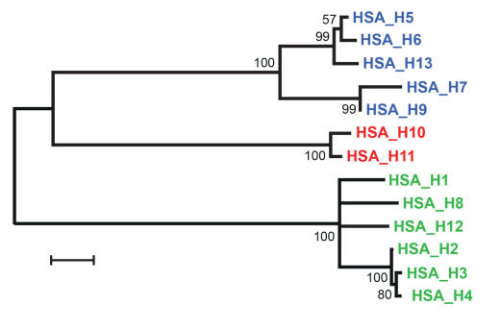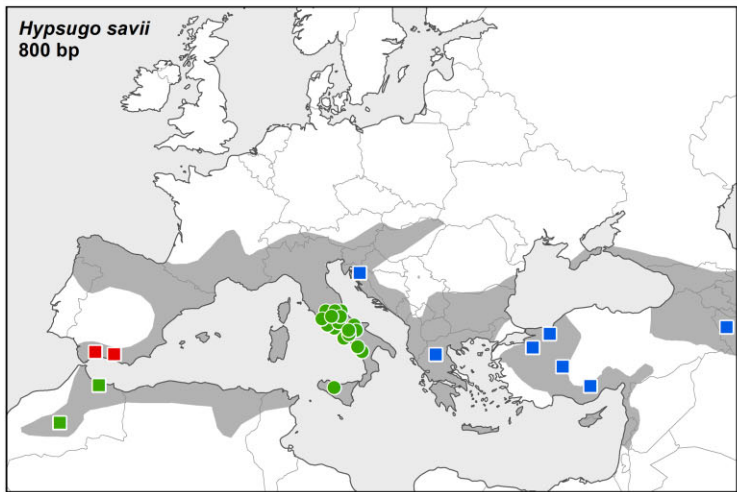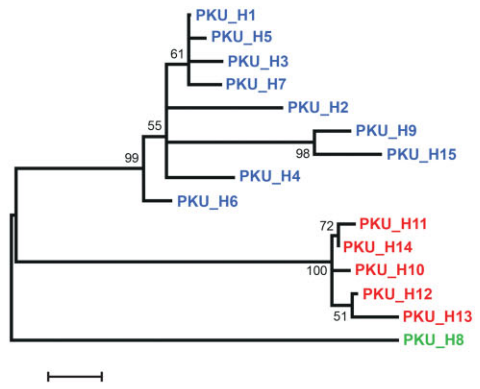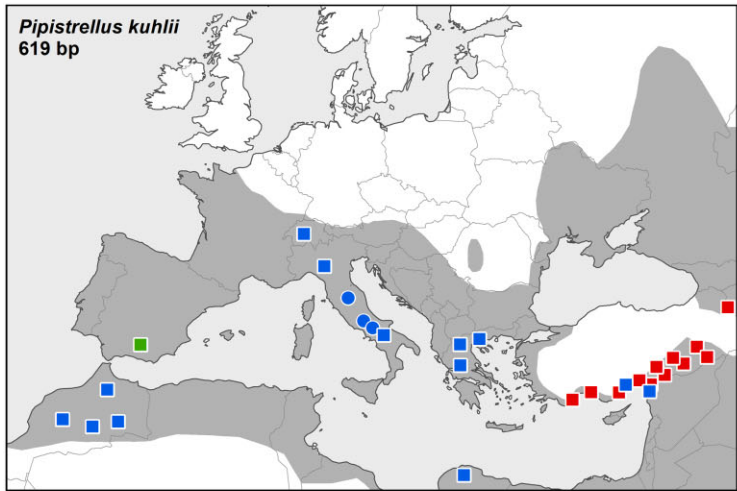
Figure 2. *Continued.*

Figure 2. *Continued.*

bats are capable of powered flight, there is the relatively easy crossing of water barriers. In line with the central position of the Apennine Peninsula in the geomorphologically complicated Mediterranean Basin, relationships with other refugia are complex. Most lineages seem to be shared between Italy and the Ibero-Maghrebian region, indicating a connection between the Adriatic and Atlantic–Mediterranean refugia. Phylogeographical relationships of the western and central parts of the Mediterranean could extend back to the Messinian salinity crisis (Duggen *et al*., 2003), indicating possible pre-Pleistocene roots of the respective divergences. The sharing of divergent lineages between these refugial areas raises questions concerning directions of faunal exchanges. The complexity of the relationships can be illustrated by reference to several bat taxa with pronounced phylogeographical substructure in the central Mediterranean. In *M. nattereri*, the most parsimonious interpretation of range history in the Italo-Iberian clade (indicated as *M*. spA in Salicini, Ibáñez & Juste, 2013) involves colonization polarity from the Apennine Peninsula to Iberia via the mainland route. In *P. pipistrellus*, the direction seems to have been from the Maghreb to Italy via Malta and Sicily, as the Maghreb hosts two mitochondrial lineages of this species, with one of them also occurring in Malta and Sicily (Hulva *et al*., 2010). A colonization route from the Maghreb and the Ibero-Maghrebian region to Italy has also been suggested for *M. punicus* (Biollaz *et al*., 2010) and *H. savii*, respectively. Relationships of Italian lineages with the Balkan refugium are not so pronounced, however (see also Çoraman *et al*., 2013).

In further bat species, the Apennine Peninsula could once have hosted endemic lineages that were later able to colonize Europe. The traceability of such a scenario depends on many factors, including, for example, the depth of the respective divergences or population biology. Reconstructing the range history may be difficult in bats, and especially in highly mobile species, as presumable genetic drift and range shifts during postglacial recolonization may erase information about refugial history (Petit, Excoffier & Mayer, 1999). For example, it was not possible to reveal a key role of the Apennine Peninsula in the colonization of Europe by large *Myotis* bats until detailed phylogeographical studies using mitochondrial and nuclear data and ancient DNA analysis could be taken advantage of (Ruedi *et al*., 2008; Bogdanowicz *et al*., 2009; Furman *et al*., 2013). Nursery colonies of *M. myotis* located in northern parts of Italy are typically of mixed population composition, indicating complex relationships with other Mediterranean refugia (especially the Adriatic region) during particular glaciation cycles. The record of *M. punicus* from Sicily expands the known range of the species, to date including Malta, Sardinia, and Corsica. Specifying the distributional status of this species in southern Italy and the possibility of permeable reproductive isolating barriers and mtDNA introgression with its sister species *M. myotis* and *M. blythii/M. oxygnathus* (Berthier, Excoffier & Ruedi, 2006; see also Furman *et al*., 2013) will require the acquisition of further data.

Colonization events may also result in secondary contacts with other allopatrically evolved lineages. In such cases, reproductive isolating mechanisms may not be fully evolved, and hybridization and introgression may occur, as demonstrated for example in Mediterranean lineages of the *P. pipistrellus* species complex (Hulva *et al*., 2010). Hybridization may be common in suture zones at crossroads of postglacial recolonization routes, e.g. in the Alps, eastern Central Europe, western Central Europe, and the Pyrenees (Schmitt, 2007; Hewitt, 2011).

DIFFERENTIATION IN ECOMORPHOLOGICAL GROUPS

Diverse taxa with differences in bauplans, life strategies, and key adaptations allow the study of various aspects of evolution, and thus a balanced view of its course. Bats, for example, can cross barriers relatively easily and serve as a model in studies of modes of dispersal. Site-specific phylogeographical patterns observed within the genera *Myotis* and *Plecotus*, and amongst pipistrelloid bats are congruent with the high level of speciation typical for these groups. The genus *Myotis* represents one of the largest mammalian radiations, with a worldwide distribution. Site-specific microevolution presumably played a crucial role in the origins of numerous species, by way of parallel occupancy of similar niches and the related occurrence of convergent ecomorphological adaptations in different biogeographical regions (Ruedi & Mayer, 2001; Ruedi *et al*., 2013). In our study, the sedentary nature of this group is illustrated by the frequent evolution of disjunct ranges with the resultant clade remaining trapped south of the Alps. The difficulty of crossing mountainous geographical barriers could have resulted in a sedentary lifestyle *sensu* Diamond (1974). For example, the structure of the *M. nattereri* species complex reflects the tendency towards speciation in allopatry, resulting in a pattern typical for temperate species, with four mitochondrial lineages evolved in the main European refugia and the Maghreb. The greatest genetic distance ($p_{max} > 0.05$) of an Apennine population corresponds to the level of species within the genetic species concept (Baker & Bradley, 2006). Isolation of an Italian population was also indicated in *M. bechsteinii* in the present study, supplementing the findings of Kerth *et al*. (2008), and in several other *Myotis* species. These patterns could serve as an example of a nascent speciation event occurring in the course of isolation within the refugial area.

The roots of the *Plecotus* radiation are probably pre-Pleistocene and are connected with the fragmentation of the Arcto-Tertiary forest (Spitzenberger *et al.*, 2006; see also Datzmann *et al.*, 2012). The basal position of the *Pl. auritus* group is occupied by the Iberian haplotype (representing *Pl. auritus begognae*, Juste *et al.*, 2004). The rest of the tree includes the Caucasian lineage, and three haplogroups occurring sympatrically in Italy – the western lineage, the eastern lineage, and a newly identified haplogroup endemic to the Appenine Peninsula (cf. Appendix S2, Spitzenberger *et al.*, 2006; Kiefer, 2007). The *Pl. auritus* group represents a further example of radiation of bats with a sedentary strategy and with differentiation in habitat selection (Ashrafi *et al.*, 2013). It may also exemplify marked changes in ecological niches, as demonstrated in the montane *Plecotus macrobullaris*, which presumably shifted from being an ancestral forest-dwelling gleaner to becoming an aerial hawker preferring open habitats (Alberdi *et al.*, 2012). The considerable diversity within the central Mediterranean region illustrates the role of geomorphological complexity in the microevolution of this taxon. Further research will be necessary to reveal if the reciprocal monophyly of Italian lineages occurs also in nuclear markers, and if the genetic differentiation is associated with ecological differentiation. In *B. barbastellus*, no exclusive Italian lineage was ascertained. However, the sharing of similar haplotypes with Great Britain (Rebelo *et al.*, 2012) points to the role that Italian populations have played in the recolonization of the northern part of Europe, being similar to that in hedgehogs, for example (Seddon *et al.*, 2001). Data from France and western Germany will be necessary if the hypothesis is to be tested more fully. Within the genus *Rhinolophus*, pre-Pleistocene colonization of Europe from west Asia is likely, with possible multiple late Pleistocene microrefugia located in the Balkans and the Ibero-Maghrebian region (Rossiter *et al.*, 2007; Dool *et al.*, 2013). In *Rhinolophus ferrumequinum*, a microrefugial role for the Apennine Peninsula (together with Iberia) cannot be precluded (Rossiter *et al.*, 2007).

Pronounced patterns within the central Mediterranean are also generated amongst pipistrelloid bats. This phenotypic group derives from several parallel radiations including the genera *Pipistrellus* (paraphyletic in relation to *Nyctalus*) and *Hypsugo* in the western Palaearctic region (Hoofer & Van Den Bussche, 2003). An elevated degree of speciation may be demonstrated by a number of species within particular lineages, in line with the phylogeographical patterns observable within particular ecozones (Hulva *et al.*, 2004; Koubínová *et al.*, 2013), or at the level of population biology in particular species (Hulva *et al.*, 2010). Pipistrelloid bats are aerial hawkers with tendencies towards migratory behaviour that entail the utiliza-tion of life strategies different from those in the above-mentioned groups with sedentary habits. Owing to their high metabolic rate, key adaptations and bauplan modifications in bats are connected with trophic strategies. Foraging modes are mirrored at the ecomorphological level (Norberg & Rayner, 1987) and may generate pre-adaptations where dispersal abilities are concerned. However, bats are representative of rather long-lived mammals displaying complex behaviour, such that evolution of genetic substructures does not necessarily relate directly to capabilities as regards to movement (García-Mudarra, Ibañez & Juste, 2009), as other factors, such as fidelity to roosting sites over generations or the cohesion of particular demes, may also play a role. The modes of gene flow may be also sex-specific and are usually shifted toward male dispersal and female philopatry in mammals, as for example demonstrated in pipistrelles across the Straits of Gibraltar, which represents a barrier for mitochondrial DNA transfer, but not for nuclear microsatellites (Hulva *et al.*, 2010). Resultant patterns will thus be influenced by many factors, including dispersal, behaviour, and ecology. In *Pipistrellus*-like species with large ranges, migratory behaviour may be pronounced only on the continental scale, whereas insular populations are rather sedentary as a result of ecological factors (Hulva *et al.*, 2010; Sztencel-Jabłonka & Bogdanowicz, 2012). Nevertheless, good flight capabilities allow for spread overseas, colonization of islands, pursuit of the tramp strategy (Diamond, 1974), and consequently of the faunal drift and taxon cycle (Wilson, 1961). The stepping-stone dispersal between the Maghreb and the central Mediterranean region and a radiation zone of *P. pipistrellus* on islands of the Mediterranean Basin could be an example of such a strategy. Nascent phenotypic diversification within this species complex, presumably connected with selection, especially related to trophic niches, was revealed using geometric morphometrics (Evin, Horáček & Hulva, 2011). Elevated gene flow may be also demonstrated through broad sympatry, which has developed between both continental lineages of presumably Mediterranean origin (*P. pipistrellus* and *Pipistrellus pygmaeus*), and is mirrored in their demographic characteristics including star-like phylogeny and other traces of population growth (Hulva *et al.*, 2010).

Low levels of geographical structure were detected in several taxa that are not closely related (*Tadarida*, *Nyctalus*, *Miniopterus*; see Table 1), typified by a fast, long-range hawking foraging strategy (Norberg & Rayner, 1987). This pattern indicates substantial gene flow across the range and/or its relatively recent colonization by recent mitochondrial lineages.

Regarding the considerable complexity of species responses to Pleistocene climate oscillations (Bennet & Provan, 2008), more research should be performed, using

dense sampling and further genetic data including nuclear markers, which may also help to reveal potential cases of reticulate evolution and sex-biased dispersal. In addition, genomic approaches and the integration of palaeofaunal, biochronological, and palaeoenvironmental data will be needed if the fine-scale phylogeographical structure of particular species is to be resolved, along with the role of the Apennine refugium in the respective species histories. Our results also draw attention to conservation issues associated with the bat fauna of Italy.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Sample and sequence details.
Appendix S2. Phylogenetic relationships based on ND1 bat samples.