

Mechanisms of radiation in a bat group from the genus *Pipistrellus* inferred by phylogeography, demography and population genetics

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Abstract

Here, we present a study of the *Pipistrellus pipistrellus* species complex, a highly diversified bat group with a radiation centre in the Mediterranean biodiversity hotspot. The study sample comprised 583 animals from 118 localities representatively covering the bats' range in the western Palearctic. We used fast-evolving markers (the mitochondrial D-loop sequence and 11 nuclear microsatellites) to describe the phylogeography, demography and population structure of this model taxon and address details of its diversification. The overall pattern within this group includes a mosaic of phylogenetically basal, often morphologically distant, relatively small and mostly allopatric demes in the Mediterranean Basin, as well as two sympatric sibling species in the large continental part of the range. The southern populations exhibit constant size, whereas northern populations show a demographic trend of growth associated with range expansion during the Pleistocene climate oscillations. There is evidence of isolation by distance and female philopatry in *P. pipistrellus sensu stricto*. Although the northern populations are reproductively isolated, we detected introgression events among several Mediterranean lineages. This pattern implies incomplete establishment of reproductive isolating mechanisms in these populations as well as the existence of a past reinforcement stage in the continental siblings. The occurrence of reticulations in the radiation centre among morphologically and ecologically derived relict demes suggests that adaptive unequal gene exchange within hybridizing populations could play a role in speciation and adaptive radiation within this group.

Keywords: hybrid speciation, introgression, Mediterranean, microsatellites, mitochondrial DNA, phylogeography, *Pipistrellus*, radiation

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Introduction

The process of species formation can be studied effectively in cases of radiations. This phenomenon is traditionally defined as the adaptive multiplication of

lineages, typically after colonizing a new environment or obtaining 'key innovations' (Schluter 2000). Adaptive radiation is usually approached from a macroevolutionary point of view; however, important insights can also be gained from a microevolutionary perspective because the great majority of important evolutionary changes are concentrated early in the phylogeny of a radiation (Gavrilets & Vose 2005). Thus, in closely

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related lineages, the crucial steps of the process are not masked by long periods of independent evolution. Such an approach is complicated by several factors. First, radiation could be viewed as an extension of the speciation process, and it is characterized by considerable complexity and influenced by many factors. Even identifying the initial stages of radiation is difficult (as the evolution of phenotype–environment correlations is not pronounced in this situation), resulting in a lack of case studies and an absence of agreement about general patterns and mechanisms of adaptive radiation (Schluter 2000). The role of phenomena studied usually in connection with speciation (e.g. allopatry, processes acting in small populations, regional selection, secondary contacts with hybridization, intergradation and reinforcement) remains unclear and may differ in particular cases.

Several attributes might make a model species group promising for studying ongoing radiation. This model taxon should exhibit sufficient diversity, indicating the initial stages of multiple species formation. The group's membership of a speciose higher-order taxon would suggest a high rate of cladogenesis within its portion of the tree of life. Large ranges provide different kinds of environments and enable isolation by distance (IBD) to occur. In addition, radiations are typically connected with geographically confined areas, and many examples originate from islands or their equivalents in a biogeographical sense. Here, we present a study of the *Pipistrellus pipistrellus* species complex, a bat group from a very species-rich genus that has radiated into a mosaic of lineages at different stages of diversification and secondary contact, in particular in the rugged environment of the Mediterranean biodiversity hotspot.

The discovery of this complex was connected with analyses of echolocation calls and mitochondrial DNA (mtDNA) in the widespread and well studied European bat species, common pipistrelle *Pipistrellus pipistrellus* (Schreber 1774). Two sympatric cryptic species within this taxon were surprisingly revealed in northern Europe (Barratt *et al.* 1997), while further lineages and the radiation centre of this group were later discovered in the Mediterranean region (Hulva *et al.* 2004, 2007). The recent view of this complex encompasses several somewhat distant populations (often also living in allopatry) inhabiting peninsulas and islands of the Mediterranean Basin, and two sibling species with secondary range overlap inhabiting most of the western Palearctic [common pipistrelle *P. pipistrellus sensu stricto* (s.str.) and soprano pipistrelle *Pipistrellus pygmaeus* s.str.]. Although the complex is morphologically relatively uniform, ongoing morphological differentiation is clear using detailed morphometry (Benda *et al.* 2004; A. Evin, unpublished data), especially in Mediterranean demes.

Differences in behaviour, phenology, ecology and other characteristics are presumed to exist and demand further research. Among these populations, *Pipistrellus hanaki* was described as separate species considering its genetic, geographic and morphological distinction (Benda *et al.* 2004). For this study, we accepted the division of this complex into three species: *P. pipistrellus*, *P. pygmaeus* and *P. hanaki* [the latter two comprising the *P. pygmaeus sensu lato* (s.l.) group in some analyses], although the taxonomic status of particular demes is not yet fully resolved.

The studied complex ranks among typical representatives of the pipistrellus-like, or pipistrelloid bats, a large phenotypic group (of c. 140 species) of the family Vespertilionidae, characterized by relatively small size, a shortened rostrum and a specific reduction of tooth number (Tate 1942). Recent molecular studies indicate that this group comprises several parallel radiations, and according to a recent conception (Hooper & Van Den Bussche 2003), the genus *Pipistrellus* Kaup, 1829 s.str. contains c. 30 species with a Palearctic, Afrotropic and Oriental distribution. Interestingly, according to molecular reconstructions based on mtDNA (Hooper & Van Den Bussche 2003), the morphologically distant genus *Nyctalus* was found to have an inner position within the *Pipistrellus* radiation, thus indicating considerable variability within monophyletic group comprising *Pipistrellus* and *Nyctalus*. For example, this taxon is characterized by extremes in body size within the family Vespertilionidae, which include the soprano pipistrelle (*P. pygmaeus*), with a body weight of 4–7.5 g, and the giant noctule bat (*Nyctalus lasiopterus*), with a body weight of 40–75 g and an ability to prey on migrating songbirds by aerial hawking (Popa-Lisseanu *et al.* 2007).

The radiation centre of the group, the Mediterranean Basin, is the largest of the world's five regions with a Mediterranean climate. It stands out among important biodiversity hotspots due to its position at the intersection of the Palearctic, Afrotropic and Oriental faunal regions, its complicated geomorphology and its dramatic palaeoclimatological history (Blondel *et al.* 2010). The region shows a high degree of endemism in many groups of organisms; hence, it is reasonable to conclude that the rate of cladogenesis is higher in the Mediterranean Basin than in other areas, especially among Mediterranean faunal elements. Moreover, the intricate nature of the Mediterranean Basin's biogeography (the repeated occurrence and disappearance of gene flow barriers) enables secondary contacts of temporarily isolated populations.

In our study, we use sampling representatively covering the ranges of particular lineages, enabling a combination of phylogeographic and comparative population

genetic approaches. Because the pattern of intraspecific divergences reconstructed from the cytochrome *b* gene (Hulva *et al.* 2004, 2007) is quite shallow (especially in the *P. pygmaeus* lineage), we used fast-evolving markers (the mitochondrial control region sequence and nuclear microsatellites). To describe proximate mechanisms of differentiation within our model group, we aimed to answer the following key questions based on the respective rationales:

Markers with a different mode of inheritance may provide a different picture of the genetic structure (e.g. Flanders *et al.* 2009). (i) Therefore, do nucDNA microsatellite loci exhibit phylogeographic patterns that contrast with those observed in mtDNA?

During the speciation process, differences among nascent lineages evolve due to neutral evolution in allopatry, spatially variable selection, character displacement, etc. (Coyné & Orr 2004). (ii) Are there differences in the genetic structure of particular lineages, especially between continental siblings? Is it possible to interpret these differences from historical and ecological points of view?

Species with broad distribution often show substructure or clinal variation in connection with different demographic histories and regional adaptations to variable environments, which may graduate to speciation process. Latitudinal differences often exist in the western Palearctic region, especially due to varying impacts of Pleistocene glaciations and different conditions in the Mediterranean basin compared with northern areas (Hewitt 2004). (iii) Therefore, do particular clades (especially continental siblings with large ranges) possess geographical structure of their gene pools?

During the early stages of speciation, hybridization and intergradation of particular lineages may occur. The controversial topic of adaptive potential of these processes in animals has been the subject of recent debate (Seehausen 2004). (iv) Do the investigated lineages form stable genotypic clusters in sympatry and in contact zones between parapatric forms? Are there signs of cytonuclear conflicts and possible historical hybridization and introgression events within the complex?

Materials and methods

Sample collection and DNA isolation

Samples for this study were obtained from field expeditions and from cooperating institutions covering most of the range of the *Pipistrellus pipistrellus* complex, including Europe (focusing on its Mediterranean part), North Africa, the Middle East and Central Asia (Fig. 1, Table S1, Supporting information). We sampled bats

from various environments (caves, space over water bodies, buildings) mostly during the period prior to potential movements to hibernation sites. Each sample was georeferenced. We obtained genetic data for 583 bats from 118 localities; the number of specimens included in the mitochondrial/microsatellite data sets varied depending on the sequencing and genotyping success. The results are based on original data, except for microsatellites from 237 bats from central Europe (Bryja *et al.* 2009). Biopsy samples obtained from the plagiopatagium according to the method of Worthington Wilmer & Barratt (1996) or necropsy samples from pectoral muscle or patagium in cases of museum specimens were stored in pure ethanol at -20°C . Total genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen).

Mitochondrial DNA sequencing

The control region, a major noncoding sequence of animal mtDNA, was chosen for this study. This element consists of two hypervariable domains (HVI and HVII) separated by a central conserved domain. In HVI of *P. pipistrellus*, there is an insertion of R1 tandem repeats consisting of five to nine 81-bp units (Wilkinson *et al.* 1997), making amplification of this region difficult. Therefore, part of the central conserved domain (a GC-rich segment involved in regulating H-strand replication and D-loop formation) and part of the right hypervariable domain [HVII, containing a site for initiating H-strand replication (O_H) and the promoters for H- and L-strand transcription] were analysed. The studied segment was amplified using the primers L16517 (CATCTGGTTCTTACTTCAGG; Fumagalli *et al.* 1996) and HSC (TTGTTTTAGGGGTTTGGCAAGA; Fumagalli *et al.* 1996) or H607 (AGGACCCATCTAAGCATTTCAGTG; Worthington Wilmer *et al.* 1994). Polymerase chain reactions (PCRs) were performed in 20 μL volumes containing 1 \times *Taq* buffer, 2.5 mM MgCl_2 , 200 μM dNTPs, 0.5 μM primers, 1 U *Taq* polymerase (Promega) and 100 ng template DNA. The thermal protocol included predenaturation (94 $^{\circ}\text{C}$, 3 min), 10 cycles of denaturation (94 $^{\circ}\text{C}$, 1 min), annealing (63 $^{\circ}\text{C}$ with a decrease of 0.5 $^{\circ}\text{C}$ in each cycle, 1 min) and extension (94 $^{\circ}\text{C}$, 1 min), followed by 25 analogous cycles with an annealing temperature of 58 $^{\circ}\text{C}$ and a final extension (72 $^{\circ}\text{C}$, 4 min). PCRs were carried out using an iCycler Thermal Cycler (Bio-Rad). Reaction mixtures were separated on 1% agarose gels. Products were excised from gels and purified with the QIAquickGel Extraction Kit (Qiagen), sequenced with the BigDye Terminator v3.1 Cycle Sequencing Kit, and analysed on a 3130 Genetic Analyzer (Applied Biosystems). Results were edited and compiled using SeqMan 5.05 (Swindell & Plasterer

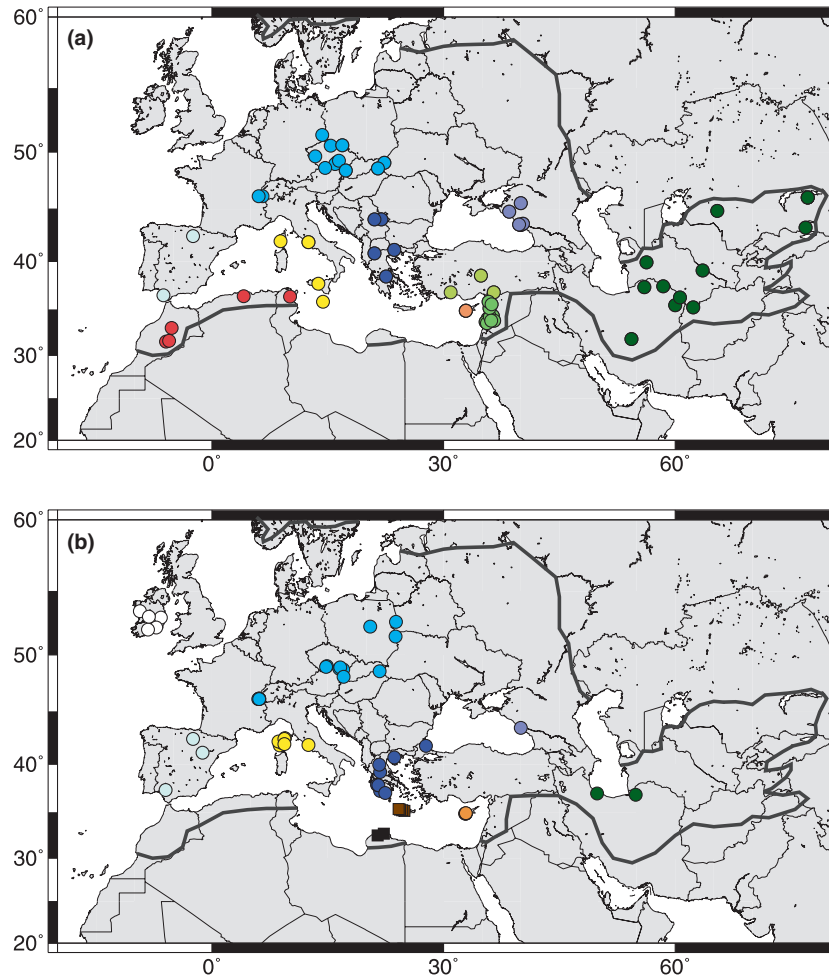


Fig. 1 Map of the current geographic distribution of the species group and sampling localities for this study (a, *Pipistrellus pipistrellus*—circles; b, *Pipistrellus pygmaeus*—circles, *Pipistrellus hanaki*—squares). Colour codes are used to identify the geographic origin of haplotypes in a median-joining network (Fig. 2). Borders of the extant range of the species complex are indicated by the bold line (modified from Courbet 1978). Maps generated using Online Map creation software (http://www.aquarius.geomar.de/omc/omc_intro.html).

1996) then aligned in ClustalW (Thompson *et al.* 1994). Haplotype data were deposited in GenBank (Accession nos: HM105963–HM106128). The sequence was read until the R2 repeat insertion site in the conserved sequence blocks within HVII. The length of the alignment within the ingroup was 378 bp. For outgroup comparison, sequences of *Pipistrellus kuhlii*, *Pipistrellus nathusii*, *Nyctalus noctula*, *Nyctalus leisleri* and *Nyctalus azoreum* were obtained from our material (HM105960–HM105962) or from GenBank (AF054869, DQ887608, AY756612).

Microsatellite genotyping

The investigated bats were genotyped for 11 microsatellite loci using the Multiplex PCR Kit (Qiagen) according to the manufacturer's instructions. The primers were

developed either for other vespertilionid bat genera [EF1, EF4, EF6, Paur05, NN18, NnP217, NnP219 (Kaňuch *et al.* 2007)] or directly for *Pipistrellus* bats [Ppip01, Ppip02, Ppip04 and Ppip06 (Racey *et al.* 2007)]. Fluorescently labelled PCR products were separated by capillary electrophoresis on an ABI 3130 Genetic Analyzer (Applied Biosystems), and electrophoretograms were edited in GeneMapper 3.7 (Applied Biosystems).

Genetic clustering

The sequence evolution model was inferred using ModelTest 3.7 (Posada & Crandall 1998). Visualization of haplotype relationships was performed by a median-joining method (Bandelt *et al.* 1999) with the aid of Network 4.5.1.2 (<http://www.fluxus-engineering.com>). As the tree topology is not the crucial source of

information for shallow divergences, this approach enables visualization of haplogroup structure and alternative genealogical hypotheses. Because the median-joining procedure is sensitive to missing data (Joly *et al.* 2007), the alignment was cut to a length of 335 bp for this analysis.

For the microsatellite data set, an individual-based Bayesian clustering procedure, implemented in Structure 2.3.1 (Falush *et al.* 2003; <http://pritch.bsd.uchicago.edu/structure.html>), was used to infer the number of distinct genetic populations represented in the sample and to assign individuals to these clusters. The Bayesian model assumes K (unknown) populations with different allele frequencies at a set of independent loci. The program was run with 10 independent simulations for each K from 1 to 10, each of 10^6 iterations, following a burn-in period of 10^5 iterations. In all simulations, an admixture ancestry model and correlated allele frequency model (with $\lambda = 1$) were used. We forced the assignments of individuals to clusters beyond the number considered to maximize the posterior probability of the data. This approach can be used to reconstruct the hierarchical relationships among populations as well as to distinguish between historical processes that are likely to shape this structure (e.g. Wang *et al.* 2007). The results of 10 replicate runs for each value of K were combined using the Greedy algorithm of Clumpp 1.1.1 (Jakobsson & Rosenberg 2007), and summary outputs for each value of K were then displayed graphically using Distruct v. 1.1 (Rosenberg 2004).

Comparative population genetics

Descriptive characteristics of genetic variability in the sequence data were computed using DnaSP V5 (Rozas *et al.* 2003) with 378-bp alignment, and number of

segregating sites (S), number of haplotypes (N_h), haplotype diversity (h) and nucleotide diversity (π) were quantified. High values of both h and π signify a constant, large population size, whereas high h and low π indicate recent expansion (e.g. Russell *et al.* 2005).

The population genetic analyses of microsatellite data were restricted to samples containing at least five individuals because most analyses were based on allele frequencies that can be strongly biased in smaller sample sizes. All analyses were performed separately for the two main mtDNA clades (i.e. *P. pipistrellus* s.l. and *Pipistrellus pygmaeus* s.l.). The genetic relationships among selected individuals were first displayed by factorial correspondence analysis (FCA) using Genetix version 4.05.2 (Belkhir *et al.* 2004). The distribution of populations in 2-D space was compared visually with the geographic position of the sampling sites.

To analyse processes affecting continuous continental populations, we conducted more detailed analyses of the intrapopulation variation and interpopulation structure of 12 pooled populations of *P. pipistrellus* s.str. and nine of *P. pygmaeus* s.str. (Table S2). The mean number of alleles (A) and the unbiased estimate of the gene diversity (GD; Nei 1978) were calculated using FSTAT 2.9.3.2 (Goudet 2001). To compare the genetic diversity between populations with unequal sample sizes, we calculated allelic richness (AR) using the rarefaction procedure, also implemented in FSTAT. In other words, we estimated the expected number of alleles at each locus in subpopulations having the smallest number ($n = 4$) of completely genotyped individuals in a population (Table 1). Because of low genotyping success (possible high frequency of null alleles), we excluded the locus EF4 from all analyses of *P. pipistrellus* populations and the locus NnP219 for *P. pygmaeus* populations. Because the time of historical expansion can affect

Table 1 Sequence and microsatellite polymorphism and demographic characteristics for the main lineages from the *Pipistrellus pipistrellus* species complex based on mitochondrial D-loop and 11 microsatellite loci. Lineages defined as haplogroups in median-joining network: *P. pipistrellus* s.str., *P. pipistrellus* from Maghreb and central Mediterranean (shortened to *P. pipistrellus* SW), *Pipistrellus hanaki*, *Pipistrellus pygmaeus cyprius*, *P. pygmaeus* s.str.

Lineage	N_s	S	N_h	h	π	Fu & Li's F^*	Fu & Li's D^*	Fu's F_s	Tajima's D	exp	N_m	GD	A	AS	AR
<i>P. pipistrellus</i> s.str.	109	77	68	0.984	0.047	-0.607	-0.304	-38.845	-0.777	4.334	206	0.87	23	184	11.02
<i>P. pipistrellus</i> SW	19	51	11	0.930	0.063	0.927	0.722	2.955	0.972	2.142	31	0.91	16.1	129	12.76
<i>P. hanaki</i>	28	22	10	0.815	0.028	1.202	1.145	1.805	0.758	2.079	33	0.75	9.5	76	7.71
<i>P. pygmaeus cyprius</i>	17	8	5	0.728	0.012	1.225	0.814	2.072	1.716	1.764	30	0.81	9.3	74	8.05
<i>P. pygmaeus</i> s.str.	158	36	48	0.901	0.010	-2.475*	-2.264	-49.001	-1.774*	9.523	243	0.84	18.5	148	9.20

Sequences: number of individuals sequenced (N_s), number of segregating sites (S), number of haplotypes (N_h), haplotype diversity (h), nucleotide diversity (π), Fu & Li's F^* , Fu & Li's D^* , Fu's F_s , Tajima's D and expansion coefficient (exp).

Microsatellites: number of individuals genotyped (N_m), mean genetic diversity/locus (GD), mean number of alleles per locus (A), sum of alleles at eight loci (AS), mean allelic richness corrected per sample size (AR).

*Significant value.

genetic variation (e.g. due to changes in the effective population size), we compared observed heterozygosity (H_o), GD and AR between populations of *P. pipistrellus* s.str. and *P. pygmaeus* s.str. using two-sided permutation tests (1000 permutations) in FSTAT (loci EF4 and NnP219 were excluded from these tests).

We quantified genetic differentiation between sampling sites by calculating estimators of F_{ST} , as described by Weir & Cockerham (1984). Null alleles are known to overestimate genetic differentiation between populations, and their occurrence is very likely in large-scale studies. We corrected for this effect using the so-called ENA method implemented in FreeNA software (<http://www.ensam.inra.fr/URLB/>) for estimating F_{STcorr} at loci thought to present null alleles. This method efficiently corrects F_{ST} estimates for the positive bias introduced by the presence of null alleles (Chapuis & Estoup 2007). We analysed IBD by regressing pairwise estimates of $F_{STcorr}/(1 - F_{STcorr})$ against ln-distance between sampling sites (Rousset 1997). Finally, we used Mantel tests to test the correlation between matrices of genetic differentiation and geographic distances by 1000 permutations in Genepop 3.4 (Raymond & Rousset 1995).

Demography

Historical demographic trends in particular lineages of the complex, defined as haplogroups in the mtDNA data set, were assessed using several methods. First, to detect signals of population expansion, we used simple expansion coefficients (Peck & Congdon 2004), defined as S/Π , where Π is the average number of pairwise nucleotide differences, and neutrality tests (Ramos-Onsins & Rozas 2002), which encompass a method independent from pairwise comparisons (Fu & Li's F^* , Fu & Li's D^* , Fu's F_S and Tajima's D). We computed these parameters with DnaSP V5. Modern approaches to population dynamics are based on coalescent theory and take into account the stochasticity of processes leading to recent genealogies. Bayesian skyline plots (BSPs) were used to reveal past population size changes (Drummond *et al.* 2005). This method is independent of a priori defined demographic models and tree reconstructions, so it is suitable for taxa with complicated population histories and/or shallow phylogenetic structure. In comparison with simple parametric and older coalescent demographic methods, the smoother estimates and sensitivity of this method, together with a credibility interval, provide a realistic population size function and enable retrieval of more details than just summary statistics. Analyses were run in BEAST 1.4.8 (Drummond & Rambaut 2007) using a GTR model and a strict molecular clock. The Markov chain Monte Carlo simulations were run with 30 000 000 iterations three

times for each lineage, while genealogies and model parameters were sampled, each with 1000 iterations. The first 10 000 000 iterations of each run were discarded as burn-in, whereas the remaining results were combined in LogCombiner and summarized as BSPs after analysing their convergence in Tracer v1.4. For approximate timing of population events, the time to the most recent common ancestor of *P. pipistrellus* s.str. lineages was set to 800 kyr according to the molecular clock analysis of Hulva *et al.* (2004).

Results

Phylogeography

For the entire *Pipistrellus pipistrellus* complex, the R2 repeat motif was the 6-bp-long sequence TACGTG. For outgroups, we found a TACGCA unit inserted in the same site for *Pipistrellus kuhlii*, whereas a TACGCA unit was inserted in the 3' direction within the structurally rearranged HVII in *Pipistrellus nathusii* and the genus *Nyctalus*.

The mitochondrial data set included 128 *P. pipistrellus*, 175 *Pipistrellus pygmaeus* and 28 *Pipistrellus hanaki* sequences. The TrN+I+G model under hierarchical likelihood ratio tests and the TIM+I+G model under the Akaike criterion best fit the data. The network (Fig. 2) was composed of several haplogroups: *P. pipistrellus* s.str., represented by haplotypes from Eurasia, two distant *P. pipistrellus* groups from southwestern and central Mediterranean, *P. pygmaeus* s.str. from Eurasia, a distant *P. pygmaeus* group from Cyprus, and two clades of *P. hanaki* from Libya and Crete, interconnecting the *P. pipistrellus* and *P. pygmaeus* subnetworks. The *P. pipistrellus* s.str. cluster contained 68 haplotypes and possessed an internal structure with geographic localization of partial haplogroups. The SW haplotypes clustered into two separate, partly sympatric lineages with a genetic distance of c. 10% (which is, interestingly, comparable to the separation between *P. pipistrellus* and *P. pygmaeus*). One lineage showed a disjunct range with six haplotypes from Morocco, Sicily, Malta and Corsica. The second, formerly unrecognized lineage, comprised five haplotypes from Morocco, Algeria and Tunisia. The syntopic occurrence of both clades was detected in the Atlas Mountains of Morocco. *Pipistrellus pygmaeus* s.str. showed a shallow, geographically unstructured star-like pattern with 48 haplotypes, dominated by a variant possessed by 49 animals that was widespread from Ireland through Europe to the Caucasus and Iran. In the Cypriot population of *P. pygmaeus*, we found six haplotypes. The Libyan and Cretan lineages of *P. hanaki* comprised 10 haplotypes, and two individuals from Crete possessed a 22-bp-long insertion.

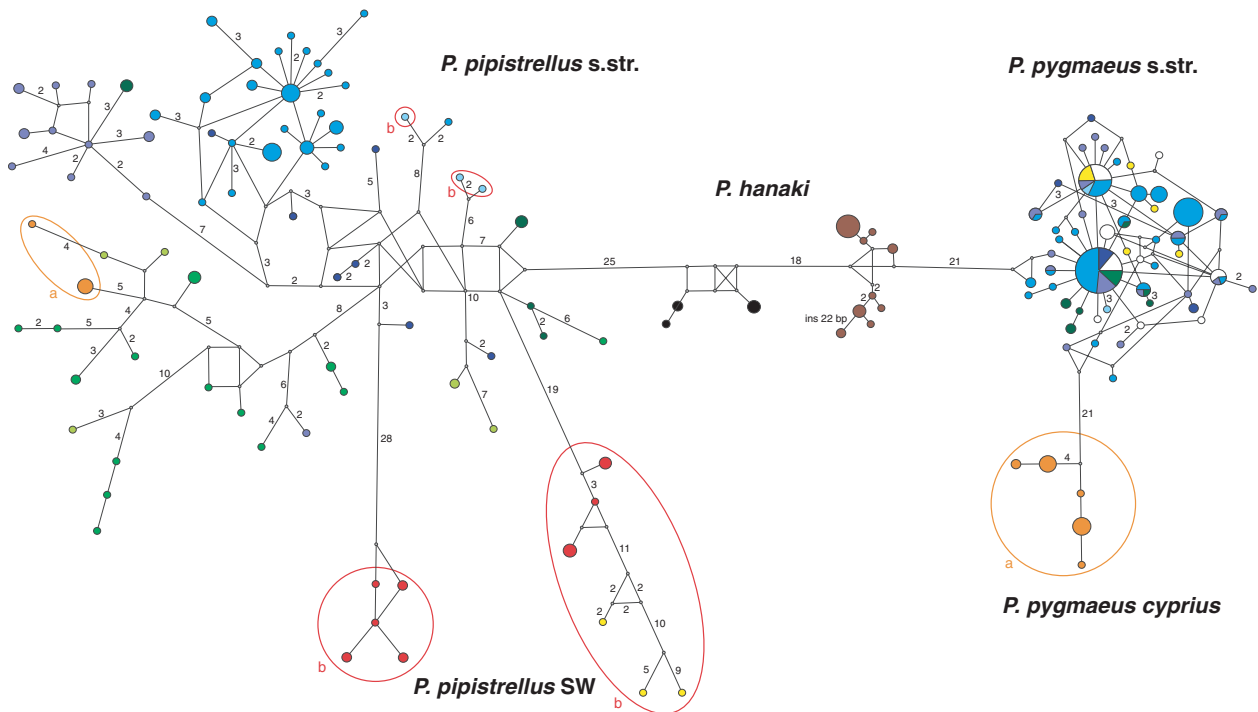


Fig. 2 Median-joining network indicating alternative relationships among haplotypes of the *Pipistrellus pipistrellus* species complex based on D-loop sequences. Haplotypes are denoted as circles, and their size is proportional to the number of individuals with a particular variant. Colours indicate the geographic origin of particular haplotypes (according to the map in Fig. 1). Hypothetical haplotypes are symbolized as grey dots. Numbers at branches represent numbers of mutational steps (displayed for $n > 1$). Areas a (Cyprus) and b (SW of the range) denote distant mt clades sharing the same microsatellite cluster, thus indicating nuclear gene flow between respective lineages.

Hierarchical individual-based clustering analyses applied to 543 microsatellite genotypes revealed an analogous general phylogeographic pattern similar to what is seen for mtDNA (Fig. 3). At $K = 2$, the *P. pipistrellus* individuals were clustered with the *P. pygmaeus cyprius* population, while all remaining *P. pygmaeus* were grouped with *P. hanaki*. In a model with $K = 3$, the SW populations of the *P. pipistrellus* clade (Maghreb, Corsica, Spain, Malta) created a group with *P. hanaki* and *P. pygmaeus* from Cyprus. Increasing the number of putative clusters resulted in complete separation of *P. pygmaeus cyprius*, two different populations of *P. hanaki* (Libya and Crete; completely separated at $K = 9$), and further diversification within the *P. pipistrellus* clade. The European populations first diverged from the Asian populations (at $K = 5$), and subsequently (at $K = 7$) a separate group from the Near East emerged from within the Asian individuals. The populations of *P. pygmaeus* s.str. were much more homogeneous, showing only slight diversification of southern populations (especially from Corsica and Italy) for $K = 6$ and greater. Separate analyses for both *P. pipistrellus* s.l. and *P. pygmaeus* s.l. provided almost identical results (not shown).

Strong separation of the populations from Maghreb and some Mediterranean islands was also confirmed by FCA (Fig. 4). Within the *P. pipistrellus* s.l. clade, the only clearly separated populations were those from Morocco and Corsica. Within the *P. pygmaeus* s.l. clade, the *P. hanaki* populations from two locations (Crete and Libya) were clearly separated, as was *P. pygmaeus cyprius*. All other individuals from this clade formed a genetically very uniform cluster.

Hybridization and introgression

To identify the level of gene flow between particular lineages, the occurrence of cytonuclear conflicts was assessed via comparison of mitochondrial and microsatellite classifications. Ambiguous determination occurred in several individuals, all of which originated from the margins and contact zones of particular lineage ranges. These specimens were reanalysed, confirming the original result in every case. In Cyprus, we analysed 24 samples from four neighbouring localities in the Troodos Mountains, representing the only known population on the island. Based on mtDNA, 17 of these samples formed a separate haplogroup corresponding to the

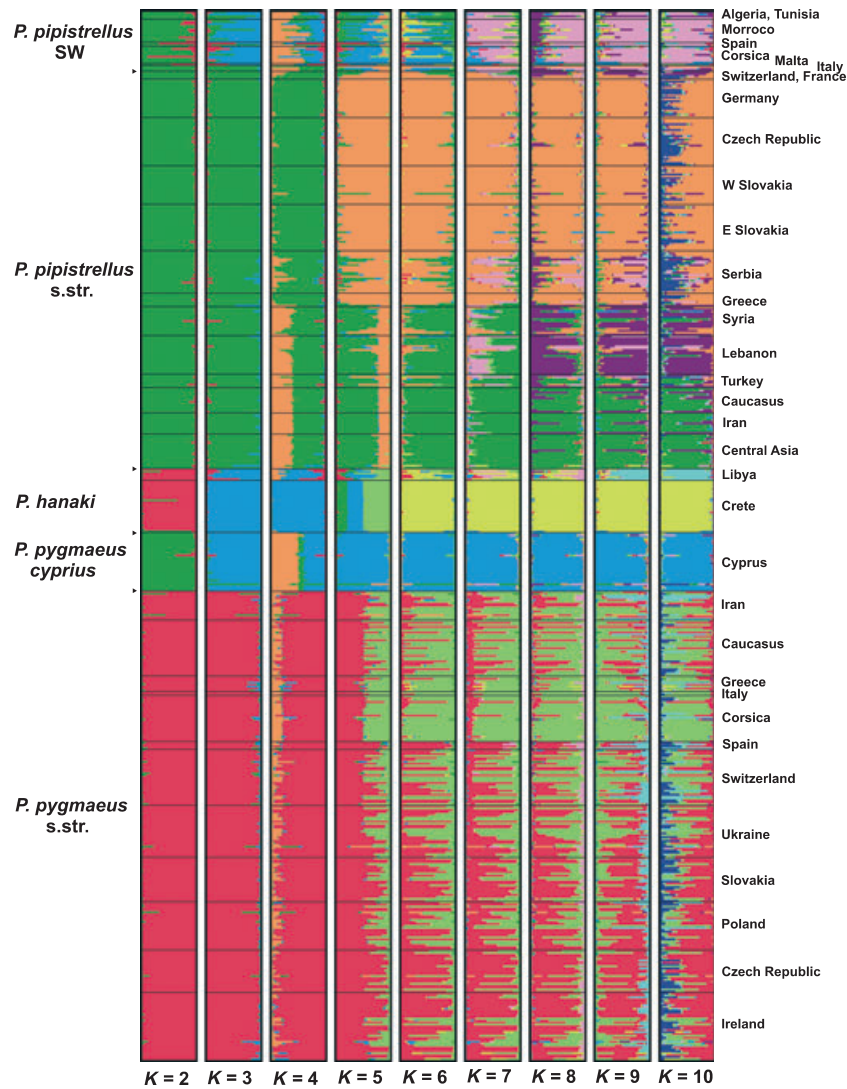


Fig. 3 Population structure estimated using the program Structure based on microsatellite data from 543 individuals from 34 geographic regions. Each individual is represented by a horizontal bar divided into K colour segments. The length of each segment is proportional to the estimated value of the membership coefficient (Q).

P. pygmaeus cyprius subspecies, and six individuals possessed two *P. pipistrellus* haplotypes that were closely related to Middle Eastern (Turkish and Lebanese) sequences. However, all individuals represent one cluster in their microsatellite loci (designated in Fig. 2). In the Moroccan population from the Atlas Mountains (12 samples), a single nuclear gene pool was observed. However, while four animals belonged to a unique Maghrebian mitochondrial haplogroup, eight others clustered with the Italian sequences, thus indicating a mixing of lineages in the southwestern part of the range (marked in Fig. 2). Within *P. pipistrellus* s.l., Spanish animals possessed European mtDNA but clustered with the Maghrebian individuals on the basis of microsatellites. Similarly, a Maltese specimen with an Italian mt

haplotype seemed to cluster with Maghrebian nuclear DNA, but more detailed sampling from this region will be necessary.

Genetic diversity and structure of P. pipistrellus s.str. and P. pygmaeus s.str.

Genetic diversity measures from microsatellite data are given in Tables 1 and S2. AR, unbiased GD and H_O were higher in *P. pipistrellus* s.str. than in *P. pygmaeus* s.str. (AR: 5.124 vs. 4.977; GD: 0.850 vs. 0.842; H_O : 0.747 vs. 0.680), but this difference was significant only for H_O (two-sample permutation test in FSTAT; $P = 0.027$). AR and GD in *P. pipistrellus* s.str. were negatively correlated with longitude (Pearson correlation;

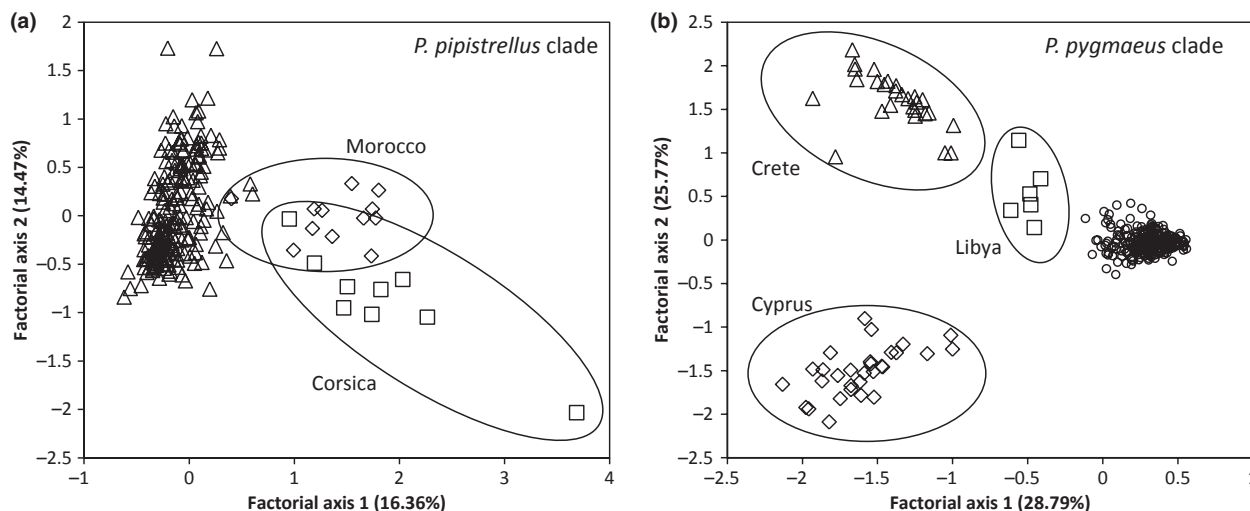


Fig. 4 A two-dimensional plot of the factorial correspondence analysis performed using Genetix based on 11 microsatellite loci for two main clades identified on the basis of mtDNA sequences. Only population samples with more than five genotyped individuals were included. The genetically distant geographic groups are designated by different symbols bounded by grey lines.

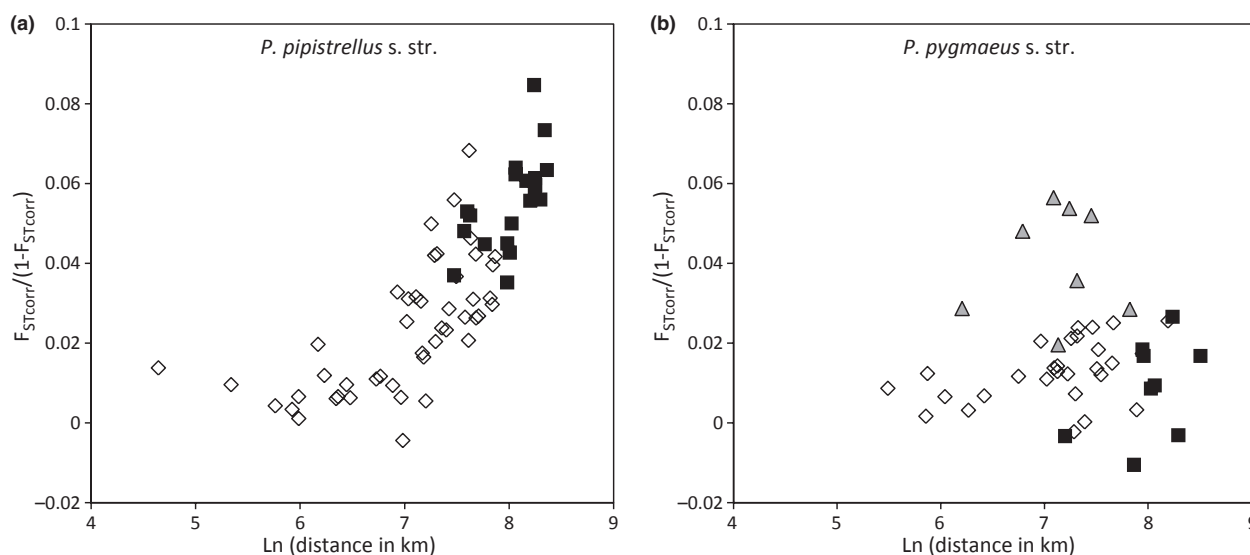


Fig. 5 Correlation between genetic $F_{STcorr}/(1 - F_{STcorr})$ and geographic (ln scale) distances for *Pipistrellus pipistrellus* s.str. (a) and *Pipistrellus pygmaeus* s.str. (b). Black squares, population pairs including Iran (both species) and central Asia (only *P. pipistrellus* s.str.). Grey triangles, population pairs including Corsica.

$P < 0.05$; Fig. S1). This result was due to low genetic variation of eastern populations from the Caucasus, Iran and Central Asia. On the other hand, the absolute greatest GD and AR were found in Turkey, a region that straddles Europe and Asia. No such correlation with latitude or longitude was found in *P. pygmaeus* s.str.

A very significant IBD pattern was found for *P. pipistrellus* s.str. (Spearman rank correlation $R_s = 0.838$; Mantel test, $P < 0.001$). However, the pairwise

F_{ST} -values were very low and relatively constant up to the distance of c. 1000 km, followed by a steep increase of pairwise F_{ST} -values between more distant populations (Fig. 5). In contrast, no IBD pattern was observed for *P. pygmaeus* s.str. ($R_s = 0.098$; Mantel test, $P = 0.300$), for which even the most distant population from Iran showed only moderate genetic differentiation from other populations. The most divergent population within *P. pygmaeus* s.str. was that from Corsica. However, even after exclusion of this population,

the correlation between genetic and geographic distances was not significant ($R_s = 0.235$; Mantel test, $P = 0.135$).

Demography

The values for diversity measures and neutrality tests for the main clades are summarized in Table 1. The strongest signal for population growth was detected in lineages with a northern distribution: *P. pygmaeus* s.str. (expansion coefficient 9.52, negative values of neutrality tests) and *P. pipistrellus* s.str. (expansion coefficient 4.33, negative values of neutrality tests). Although the F_S results were not significant, the values were strongly negative. An excess of low-frequency polymorphisms was also indicated by Tajima's D -statistic, particularly for *P. pygmaeus* s.str. In contrast, populations with a Mediterranean distribution exhibited substantially different demographic patterns with low expansion coefficients (2.14 for the SW *P. pipistrellus*, 1.76 for the Cypriot *P. pygmaeus*, and 2.08 for *P. hanaki*) and non-significant positive values of neutrality tests, thus indicating a rather constant population size. The coalescent approach using BSPs (Fig. 6) are consistent with this pattern. In addition, this sensitive method indicated that summary statistics may provide an oversimplified picture of population trends in our model taxa because the population history may involve several stages with

different characteristics. In *P. pygmaeus* s.str., strong expansion was confirmed by an *c.* 15-fold increase of the population size in the last 90 kyr. However, in *P. pipistrellus* s.str., a more complicated history was revealed. A subrecent wave of population expansion was revealed starting *c.* 180 kyr BP. This expansion was predated by a period of stationarity and a possible second growth phase at *c.* 800 kyr BP, which is less supported due to limitations of the power of the method near the coalescence time and, consequently, a broader confidence interval around that time.

Discussion

Mitochondrial vs. nuclear phylogeographic patterns

As a standard phylogeographic tool, mtDNA has well-known advantages in connection with its high speed of evolution in animals, maternal heredity without recombination, and coalescent genealogy. Nuclear microsatellite assays provide allele frequency data for highly variable, biparentally inherited markers, enabling a comparative population approach. Although a high rate and stepwise mode of mutation may lead to homoplasy, high variability and multilocus character can compensate for these drawbacks (Estoup *et al.* 2002). Microsatellites are thereby becoming increasingly popular in phylogeographic studies (e.g. Koskinen *et al.*

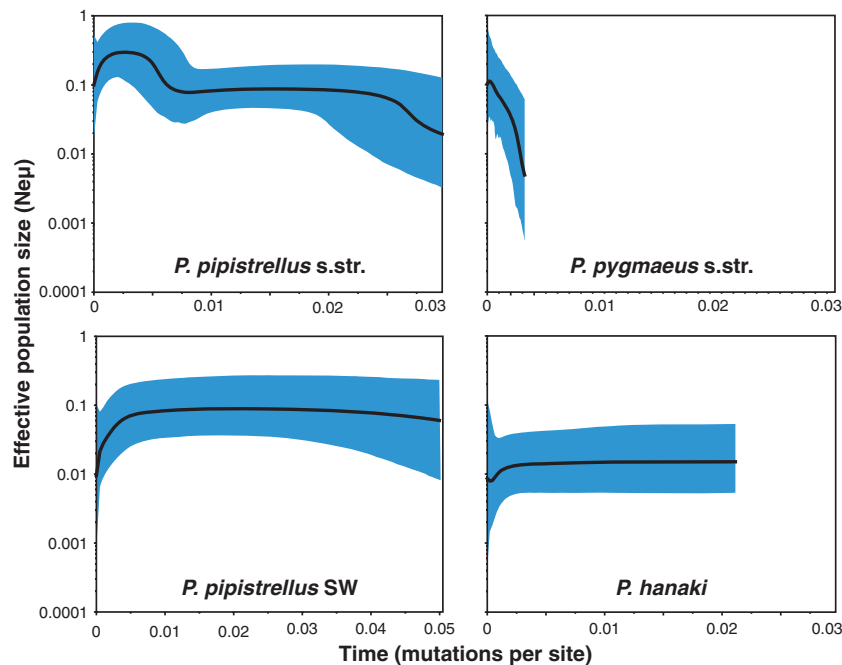


Fig. 6 Bayesian skyline plots for the main lineages of the complex showing changes in effective population size ($N_e\mu$) over time (measured in mutations per site). The thick solid line depicts the median estimate, and the margins of the blue area represent the highest 95% posterior density intervals. The plot for *Pipistrellus pygmaeus cyprius* is not displayed due to short coalescent time.

2002; Rossiter *et al.* 2007; Hu *et al.* 2008). For shallow divergences, microsatellites have proven even more useful than nuclear sequences, permitting avoidance of biases associated with shared ancestral polymorphisms and incomplete lineage sorting (Nichols 2001).

The topology of the mitochondrial D-loop network that we observed was in good concordance with previous cytochrome *b* phylogenetic reconstructions (Hulva *et al.* 2004, 2007), although many more haplotypes were identified due to the relatively higher mutation rate of the D-loop and more extensive sampling. The similarity in the phylogeographical signal revealed by autosomal loci indicates that both gene pools have undergone similar evolutionary pathways. However, several cases of cytonuclear conflict indicate mixing of particular lineages.

Genetic differentiation in the species group

Genetic architecture of this complex was addressed using phylogenetic approach in Hulva *et al.* (2004). The roots of the entire divergence are suggested to be connected with effects of the Messinian salinity crisis, causing fragmentation of Mediterranean habitats and enabling allopatric speciation events. A recent peripatric effect is also obvious in connection with complicated geomorphology of the Mediterranean edge of the range. The isolation effect could be strengthened by lower abundance of the species in the Mediterranean and its patchy distribution, resulting from a preference of mountain habitats and 'islands' of forests or humid ecosystems in dominant maquis shrublands.

The detailed sampling in this study enabled us to compare differences in genetic structure of particular lineages and to address proximate mechanisms of diversification in the group. In the *Pipistrellus pipistrellus* s.str. lineage, the haplotype distribution indicates IBD on a macrogeographic scale and shows that female gene flow was relatively low in relation to the population size, thus enabling lineage sorting and drift to develop shallow genetic substructure. In a microsatellite study in central Europe (Bryja *et al.* 2009), no IBD was observed in either species in the study area (within distances up to 800 km). Our data suggest that a distance of *c.* 1000 km may represent a limit in gene flow for this species in a landscape free of geographic barriers. The negative correlation of genetic diversity with longitude is concordant with presumed range expansion from the western Mediterranean (Hulva *et al.* 2004). The Middle Pleistocene establishment of a longitudinal gradient and a relatively recent northward spread (Hulva *et al.* 2004) is probably also mirrored by the skyline plot. Given that the second expansion event predates the first appearance date of these species in central Europe (*Pipi-*

strellus s.l. fossils are completely absent in mass cave thanatocenoses in central Europe and the Balkans prior to the Holocene and/or late Weichselian; Horáček & Jahelková 2005), it is probable that this phase was related to the Eemian interglacial period in the Mediterranean region, which has been connected with the spread of warm open habitats. In the Mediterranean, the fossil record goes back to the Middle Pleistocene (Spain, Malta; Horáček & Jahelková 2005).

Relationships of *P. pipistrellus* haplotypes within southwestern parts of the range indicate historical connections of the Maghrebian and central Mediterranean populations. The tip position of Maltese, Sicilian and Corsican samples in the network indicates colonization of these territories from North Africa. More detailed sampling of peninsular Italy will be necessary to resolve this pattern and the possible contact zone with *P. pipistrellus* s.str. in this area. The Iberian area shows no genetic exclusivity in pipistrelles and, thus, probably did not play a role as a refugium in this case. In addition, the Gibraltar strait has been repeatedly referred to as a barrier for gene flow in bats (Castella *et al.* 2000; García-Mudarra *et al.* 2009). In pipistrelles, the complete isolation of populations on both sides of the strait was ascertained from matrilineal mitochondrial markers, but sharing of microsatellite alleles implies that gene flow in this group is biased towards males.

The absence of IBD and spatial structure in *Pipistrellus pygmaeus* s.str. could have been caused by very recent (Holocene) colonization of the continental range (especially the Iranian part; Fig. 5) and/or differences in biology from *P. pipistrellus* s.str. (such as different dispersal of subadults, more pronounced migratory behaviour, phenology, metapopulation structure with different gregarious behaviour, colony size, the absence of mass winter hibernacula in Central Europe and social system). These differences could be a result of neutral evolution and regional selection during allopatric stages, as well as the consequence of niche differentiation in sympatry. In a study of an island population of pipistrelles in Great Britain by Racey *et al.* (2007), an IBD pattern was observed in both sibling species at shorter distances than on the continent, and the IBD pattern was more marked in *P. pipistrellus* s.str. However, no effect of the sea channel was observed. In our data, potentiation of the IBD pattern by a sea body was observed in *P. pygmaeus* s.str. in Corsica (Fig. 5). This finding favours ascribing genetic homogeneity of *P. pygmaeus* s.str. more to recent range expansion than to a greater migration capacity. Furthermore, this implication is supported by the BSP, which showed strong population growth (*c.* 15-fold) during the Weichselian-Holocene, thus indicating an even stronger and later range expansion in this species, which could also

contribute to the sudden rise of *P. pipistrellus* s.l. in the fossil record of central Europe.

The Cypriot population, formally described as the subspecies *P. pygmaeus cyprius*, constitutes a relatively distant mitochondrial and nuclear clade, which reflects its relatively isolated island position, with the nearest recently ascertained populations of *P. pygmaeus* on the Balkan Peninsula and in proximity to the Black and Caspian seas. Given that Cyprus was never in contact with the mainland, the origin of this population is likely explained by a colonization event. Different characteristics of the Cypriot orphan deme compared with its continental siblings are probably a consequence of retained ancestral character states corresponding to a relict population located near the radiation centre of the entire complex, and an insular syndrome, involving founder effect, drift and regional selection. Similar factors may play a role in the evolution of genetic distinctness of isolated *Pipistrellus hanaki* populations.

The substantially different nature of the Mediterranean and continental populations was proven not only by general phylogeographic patterns, but also by demographic and population analyses. Restrictions of gene flow and constant population size are characteristic for the south, whereas the opposite is true for the north. This pattern may be correlated with the fact that despite some oscillations also occurred in the Mediterranean region during the Pleistocene (e.g. Dansgaard–Oeschger cyclicity), this region could generally be considered more climatically stable and excluded from glacial fluctuations. The northern siblings have evolved regional migratory behaviour (and other ecological adaptations, such as utilization of caves for hibernation) that is connected mainly with the annual cyclicity of the climate in the part of the range located in the temperate zone.

Spatial behaviour of bats from the *P. pipistrellus* complex remains enigmatic. It is not clear, for example, during which stage of annual cycle mating occurs and whether gene flow should be ascribed to the dispersal of subadults to new territories and/or to seasonal migrations between summer and winter roosts. Our data suggest sex differences in site fidelity with female natal philopatry and male dispersal, as is common among other bats (Kunz 1982). Because the direct evidence for long-distance movements from capture-recapture experiments is scarce due to very low recovery in banding studies, our results represent an important quantification of patterns of gene flow on the rangewide scale, which could not be uncovered by geographically limited studies. A combination of two contrasting features was relevant in shaping the recent phylogeographic patterns of these bat species in the Mediterranean and in the colonization of islands. The first of these relates to the capacity for flying large

distances that is indicated by their wing morphology, their phylogenetic proximity to obligate migrants (*Pipistrellus nathusii*, noctules) and occasional observations of animals moving over the open sea (Ahlén *et al.* 2009). The second contrasting feature is associated with the role of ethological factors and site fidelity as well as ecological adaptations that prevented the development of a panmictic pattern (most pronounced in continental *P. pygmaeus* s.str.) throughout the entire range. This case study suggests a role for allopatry in the early stages of adaptive radiations, which is a phenomenon that is traditionally studied in connection with speciation.

Hybridization and introgression in Mediterranean populations

The most parsimonious interpretation of the occurrence of alien *P. pipistrellus* mitochondria in *P. pygmaeus cyprius* is introgression mediated by *P. pipistrellus* females occasionally migrating to the island from neighbouring Turkish or Levant territories. The interspecific mating was probably facilitated by the abundance asymmetry of both species and the absence or incompleteness of intrinsic reproductive isolating mechanisms (RIMs) between both primarily allopatric lineages. Nevertheless, the clustering of Cypriot microsatellite genotypes with *P. pipistrellus* s.str. in the Bayesian procedure with $K = 2$ suggests a more intense contribution of *P. pipistrellus* s.str. to this mixed nuclear gene pool and the possibility of ongoing hybrid speciation. Similar phenomenon, the admixture among Caribbean species of *Artibeus*, was reported recently in the southern Lesser Antilles (Larsen *et al.* 2010). Genomic approaches will be needed to analyse the Cypriot populations in detail.

Next, we presume that the occurrence of central Mediterranean haplotypes within the Maghrebian population is a consequence of past hybridization and reflects historical range shifts and gene flow between the two lineages. The intergrade classification of the Spanish and Maltese individuals indicates incomplete isolation of particular *P. pipistrellus* lineages, especially in the case of animals that are vagrant over the Gibraltar Strait and the Strait of Sicily.

In contrast, in *P. pipistrellus* s.str. and *P. pygmaeus* s.str., secondary contact of ancestral Mediterranean-born lineages (Hulva *et al.* 2004) was accompanied by the evolution of complete RIMs, as the hybridization between these two forms has never been referred to in genetic studies (Racey *et al.* 2007; Bryja *et al.* 2009). Compared with the initial stages of secondary contacts in other lineages, hybridization and reinforcement could play a role in completing the speciation process. The reproductive isolation of both lineages was associated with the development of a broad zone of sympatry,

presumably accompanied by character displacement (e.g. the shift of *P. pygmaeus* from its ancestral 45 kHz to a 55 kHz echolocation frequency). The contrasting genetic patterns of both sympatric forms indicate that diversifying selection may also act on other traits connected, for example, with ecology and social systems.

The evolution of assortative mate choice is traditionally viewed as an adaptation to prevent production of unfit hybrids. Within the concept of a 'porous genome', the loci connected with adaptations may evolve independently despite hybridization (e.g. Gavrillets & Vose 2005). However, in recent decades, a positive role of introgressive hybridization in speciation and adaptive radiation has been receiving greater attention (Seehausen 2004; Grant *et al.* 2005), and there is growing evidence of its abundance in nature (e.g. Sota 2002), including the evolution of modern humans (Green *et al.* 2010). Higher frequencies of hybridization may have also occurred during species invasions to new environments, coinciding with an increasing rate of adaptive evolution associated with changes in demography and response to selection. New, advantageous genetic combinations could be gained due to partial and unequal gene exchange between hybridizing species, as mediated by transgressive segregation, which has been reported both from breeding programmes (Rieseberg *et al.* 2003) and from field studies (Martinsen *et al.* 2001). In bats, which are the only mammals with powered flight and, thus, a great capacity for movement, there could be frequent chances of secondary contacts between geographically isolated lineages that are an order of magnitude greater than in nonvolant mammals. As multilocus genetic studies are becoming routine, the number of empirical examples of hybridization and introgression in bats is growing (e.g. Mao *et al.* 2010). In pipistrelles, the occurrence of reticulate evolution in nascent and phenotypically distinct Mediterranean demes supports the view of the complex as a syngameon with an important role for the anastomose component in speciation and adaptive radiation.

Although the aim of this work was not taxonomical, it does address the level of gene flow and biologically meaningful subunits defined within the group. Some demes show substantial degrees of isolation and inhabit relatively small ranges, often in popular tourist destinations on the Mediterranean Sea, and their conservation status should be assessed.

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Authors' contribution

PH initiated the study, performed analyses of mitochondrial data and wrote the manuscript. AC undertook the laboratory work for most of the sequences and AF for microsatellites. BA provided samples from Maghreb, PB from North Africa and the Middle East and AE from Corsica. JB performed the analyses of microsatellite data and wrote appropriate parts of the text.

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Supporting information

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

Table S1 List of sampling sites and their coordinates, number of individuals sequenced and/or genotyped, and GenBank Accession numbers

Table S2 Populations used for detailed spatial genetics analyses of microsatellite genotypes. Number of individuals genotyped (N_m), mean number of alleles per locus (A), observed heterozygosity (H_O), mean genetic diversity/locus (GD), mean allelic richness corrected per sample size (AR)

Fig. S1 Correlation of mean allelic richness corrected per sample size (AR) with longitude in *Pipistrellus pipistrellus* s.str.

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