

Environmental margin and island evolution in Middle Eastern populations of the Egyptian fruit bat

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Abstract

Here, we present a study of the population genetic architecture and microevolution of the Egyptian fruit bat (*Rousettus aegyptiacus*) at the environmental margins in the Middle East using mitochondrial sequences and nuclear microsatellites. In contrast to the rather homogenous population structure typical of cave-dwelling bats in climax tropical ecosystems, a relatively pronounced isolation by distance and population diversification was observed. The evolution of this pattern could be ascribed to the complicated demographic history at higher latitudes related to the range margin fragmentation and complex geomorphology of the studied area. Lineages from East Africa and Arabia show divergent positions. Within the northwestern unit, the most marked pattern of the microsatellite data set is connected with insularity, as demonstrated by the separate status of populations from Saharan oases and Cyprus. These demes also exhibit a reduction in genetic variability, which is presumably connected with founder effects, drift and other potential factors related to island evolution as site-specific selection. Genetic clustering indicates a semipermeability of the desert barriers in the Sahara and Arabian Peninsula and a corridor role of the Nile Valley. The results emphasize the role of the island environment in restricting the gene flow in megabats, which is also corroborated by biogeographic patterns within the family, and suggests the possibility of nascent island speciation on Cyprus. Demographic analyses suggest that the colonization of the region was connected to the spread of agricultural plants; therefore, the peripatric processes described above might be because of or strengthened by anthropogenic changes in the environment.

Keywords: Cyprus, Egyptian fruit bat, founder effect, island speciation, microsatellites, Middle East, mitochondrial DNA, range margin

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Introduction

The factors causing the restriction of species ranges remain only partially explored. At environmental margins, large populations in evolutionary stasis often reach the limits of their ecological valence with several mechanisms preventing the evolution of adaptations to

novel environments beyond the edge of the range (Bridle & Vines 2006). In addition to historical constraints, the depletion of genetic variance could play a role in decreasing the adaptive potential of peripheral populations. This may be caused by spatial restriction of the mutational input, and the establishment of viable populations may also be complicated by the Allee effect. If the gene flow between the margin and centre remains intense, the evolution of novelty in the periphery may be prevented by the input of locally deleterious alleles

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from the main population. If the populations at the range margins remain fragmented (e.g. because of the patchiness of suitable habitats), genetic drift in the isolates and, according to the metapopulation model, founder effects in newly established demes can contribute to the loss of genetic variability, particularly in populations that result from expansion compared with the rear edge (Diekmann & Serrão 2012).

However, small peripheral populations can possess some degree of enhanced evolutionary potential and shift to new adaptive peaks, for example, as described in the shifted balance theory (Wright 1932) and models connected with the founder effect, that is, genetic revolutions model (Mayr 1954), founder flush model (Carson 1968) or transience model (Templeton 1980). Potential admixture among different peripheral demes may further facilitate adaptive evolution by outbreeding, combinations of site-specific advantageous mutations and heterosis, the formation of transgressive phenotypes and other mechanisms connected to hybridization (Dowling & Secor 1997). Within the biogeographic perspective of the island model of environmental margin, populations may be influenced by other factors of island evolution, such as the relaxation of competitive, predatory or parasitic pressure, divergence in life-history traits and the occupation of novel niche. The resultant situation depends on the interference of the above-described factors and may culminate in peripatric speciation in some cases. In this study, we aimed to inspect the processes that occurred at the environmental margin by studying the population genetic architecture of the Egyptian fruit bat (*Rousettus aegyptiacus*) in the Middle East, which represents the northern distributional limits of megabats. The study area represents a zone of admixture of Palearctic, Afrotropic and Oriental biotas, and the region has an intricate paleogeology, geomorphology and diverse refugial histories of particular taxa with fragmentary knowledge of general phylogeographic patterns, particularly in mammals and bats (Hulva *et al.* 2007; Furman *et al.* 2009).

Megabats, forming the family Pteropodidae, represent the only frugivorous bats of the Old World. Because of their nutrient dependency on fruit, nectar and pollen, the patterns of distribution are primarily the tropical and subtropical evergreen forest habitats of Africa and Indo-Australasia. According to phylogenetic and paleontological studies, the origin of the family is connected with a Malay-Indonesian biodiversity hotspot (Juste *et al.* 1999). The dispersal to the western part of the range and the origin of hotspot in the Congo basin was presumably connected to three waves of colonization. The ancestors of epomophorines and myonycterines likely occurred in Africa via forested biotopes connecting Asia and Africa in Europe and/or Arabia during the Neogene mammalian

dispersal phases (Thomas 1985) in the Miocene, similar to other rainforest-associated groups (e.g. the bat family Nycteridae; Griffiths 1997). The genus *Eidolon*, which is phylogenetically separate from other African fruit bats, likely colonized Africa in a separate dispersal event. Considering its capacity to fly for long distances, this may have occurred via stepping-stone islands in the Indian Ocean. The genus *Rousettus* Gray, 1821 likely arrived in Africa more recently than other fruit bats (Kirsch *et al.* 1995). The echolocation capacity *sensu stricto*, unique among fruit bats, enabled roosting in caves and spreading after the disappearance of forest blocks between the two continents. However, regardless of the fact that the most basal species of the genus, *Rousettus amplexicaudatus*, has an Asian distribution, the biogeographic analysis by Almeida *et al.* (2011) supported Africa as the ancestral area of the genus. The ecological characteristics of rousettes resulted to a unique distribution pattern among fruit bats, including both Asia and Africa, and also allowed *R. aegyptiacus* and *R. leschenaulti* to spread to relatively high latitudes, characterized by comparatively dry, cold and seasonal climates, which represent conditions close to the limits of the abiotic selective gradient of the group. Recently, *R. aegyptiacus* has an extensive disjunct range and is considered polytypic (Bergmans 1994) with four continental subspecies: *R. a. aegyptiacus* (Geoffroy, 1810) distributed in Cyprus, Turkey, the Levant and Egypt; *R. a. arabicus* Anderson, 1902 in the Arabian Peninsula, Iran and Pakistan; *R. a. leachi* (Smith, 1829) in East Africa; and *R. a. unicolor* (Gray, 1870) in West Africa and two endemic insular subspecies from the Gulf of Guinea, including *R. a. princeps* Juste & Ibáñez, 1993 from Principe island and *R. a. tomentosus* Juste & Ibáñez, 1993 from São Tomé Island.

The cave-dwelling strategy has also affected the population structure of the *Rousettus* species. Tight connections of rousettes with this unequally distributed microhabitat have led to the evolution of large colony sizes and a tendency towards long distance movements among particular cave roosts. This behaviour generates a high level of gene flow and resulted in substantial population genetic homogeneity compared with tree-roosting species with smaller social units and higher site fidelity. This tendency was revealed by molecular ecology studies in the rainforest biome, which is characterized by long-term climatic stability. Therefore, the genetic structure reflects mainly ecological, not historical, factors. These studies include a comparison of the genetic structure in codistributed fulvous fruit bat (*Rousettus leschenaulti*) and the greater short-nosed fruit bat (*Cynopterus sphinx*) in Southeast and East Asia (Chen *et al.* 2010) and comparison of cave vs. tree-dwelling species from microchiropteran genera *Kerivoula* and *Rhinolophus* in Malaysia (Rossiter *et al.* 2012). The gene flow may also be linked to the annual migratory

behaviour, influenced by seasonal changes in the availability of ripe fruits or flowering trees, which is observed in many fruit bat species (Thomas 1983; Palmer & Woinarski 1999). For example, the phenology of plant communities in dry habitats may result in the formation of local patches of food resources that are richer than those in forest ecosystems (van Schaik, Terborgh & Wright 1993; Fleming & Eby 2003). In agricultural landscapes, farming could also play a role in fruit availability.

However, at the edge of the range, the population structure may be complicated by the abovementioned historical factors connected to demography. Additional factors could play a role in the case of the northern populations of *R. aegyptiacus*, such as range pulses raised by climatic oscillations, seasonality and effects caused by population fragmentation and insular evolution. We hypothesize that these phenomena limit the genetic exchange between particular patches, prevent the evolution of population homogeneity on a large geographic scale and lead to peripatric effects. Using detailed sampling of Middle Eastern populations and fast-evolving genetic markers (partial sequence of a mitochondrial control region and 20 nuclear microsatellites), we aim to achieve the following: (i) inspect the genetic variability in the northwestern section of the range, depth of the respective divergences and character of the genealogical patterns; (ii) describe the geographic distribution of genetic variability and identify the potential substructures, respective boundaries and possible barriers to gene flow using a landscape genetics approach; (iii) assess the effect of an island environment on population genetic architecture and (iv) infer the demography of the respective populations using a coalescent approach. We discuss these results together with other data concerning the life history, trophic niche, spatial behaviour, social organization and other traits to achieve a better comprehension of the population structure and microevolution of this unusual element in the eastern Mediterranean.

Materials and methods

Sampling design

The samples were collected during expeditions to the Middle East and East Africa from 2005 to 2011. The populations were sampled mainly in proximity to particular colony roosts (cave entrances or abandoned buildings), and individuals were also captured near feeding sites (e.g. in proximity of fruiting trees), small day roosts (rock crevices) and at flying corridors (above brooks) using mist netting. Each locality was georeferenced. We obtained genetic material from 568 bats from

70 sites, which representatively covered the distribution of the species in the region (Fig. 1, Table S1, Supporting Information, Dryad entry: doi:10.5061/dryad.k68k8). We used wing punch biopsies (Worthington Wilmer & Barratt 1996) or buccal swabs to obtain cellular material. The samples were fixed with pure ethanol and stored at -20°C after transportation to the laboratory.

Sequencing and genotyping

Genomic DNA was isolated using the DNA Blood and Tissue Kit (Qiagen) or NucleoSpin Tissue Kit (Macherey-Nagel). Part of a mitochondrial control region (portion of hypervariable region II and of the central conserved domain) was sequenced. The studied segment was amplified using the forward primers L16517r (5' CAT CTG GTT CTT TCT TCA GG 3'; modified according to Fumagalli *et al.* 1996) or 1211925r (5' ACG CAG ATC ACC ACC AAT GG 3'; modified according to Sun *et al.* 2009), located in the central conserved region, and the reverse primer HSCr (5' CTG TTT TTG GGG TTT GGC GGG A 3'; modified according to Fumagalli *et al.* 1996), located in the Phe-tRNA region. PCRs were performed in 25 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 temperature profile on the iCycler Thermal Cycler (Bio-Rad) consisted of predenaturation (94°C , 3 min), followed by 10 touchdown cycles of denaturation

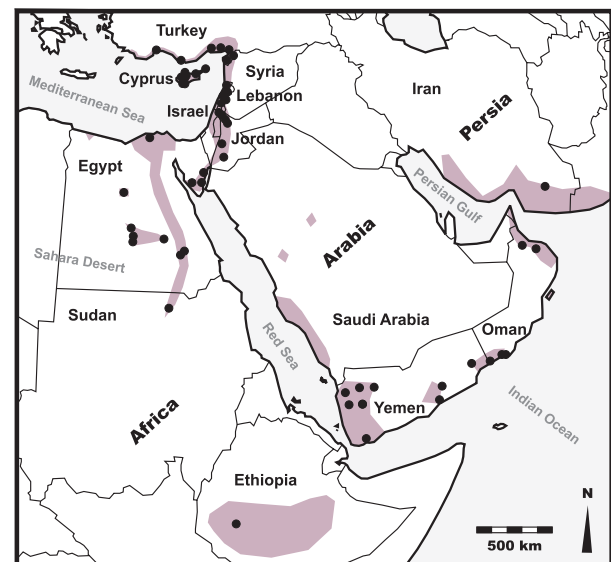


Fig. 1 A map of the Middle East and the sampling design. The extant range of the species is indicated in violet (modified from Kwiecinski & Griffiths 1999; Benda *et al.* 2012 and www.iucnredlist.org) and the sampling localities as black dots. The East African sample is only partially displayed because of space limitations.

(94 °C, 1 min), annealing (68 °C, decreasing by 0.5 °C in each step, 1 min) and extension (72 °C, 1 min), 25 cycles with constant annealing temperature (63 °C, 1 min) and a final extension (72 °C, 4 min). The amplicons were separated on 1% agarose gels, excised and purified using the QIAquick Gel Extraction Kit (Qiagen) and sequenced using the BigDye Terminator version 3.1 Cycle Sequencing Kit and capillary electrophoresis on a 3130 Genetic Analyzer (Applied Biosystems). The chromatograms were compiled in SeqMan 5.05 (Swindell & Plasterer 1996). The sequences were aligned in MAFFT version 6 (Kato *et al.* 2009) using the G-INS-i algorithm. The haplotype data were submitted to GenBank (accession numbers JX198427–JX198505, Dryad entry: doi:10.5061/dryad.k68k8).

For the amplification of the 20 nuclear microsatellite loci, the primers M3 (1, 6, 8, 120, 121), published in the study by Hua *et al.* (2006), and 66HDZ (20, 80, 82, 105, 106, 110, 117, 304, 327, 334, 340, 341, 343, 407, 413), from the study by Andrianaiarivelo *et al.* (2008), were used (Table S2, Supporting Information). The fragments were amplified using fluorescently labelled forward primers in four 10- μ L multiplex reactions with the Multiplex PCR Kit (Qiagen) according to the manufacturer's protocols in an iCycler Thermal Cycler (Bio-Rad). The products were separated on an ABI Prism 3100 Avant Genetic Analyzer using Gene Scan™ 500 LIZ® Size Standard (Applied Biosystems). Allele calling was performed manually using the program GeneMarker v.1.85 (www.softgenetics.com) and taking into account the amplification and electrophoresis artefacts. The binned values of allele sizes were constructed considering discrete breaks in the periodic size classes and off-ladder microvariants using Autobin (Guichoux *et al.* 2011). Frequency plots of the fragment size distributions were visually compared for the raw and transformed data. The genotypes of all individuals are available in the Dryad repository (doi:10.5061/dryad.k68k8).

Genetic variability

The descriptive parameters of sequence polymorphism (the number of haplotypes N_h and haplotype diversity h) were computed using DnaSP 5.10 (Rozas *et al.* 2003). The haplotype relationships were displayed using the Median-Joining network (Bandelt *et al.* 1999) in Network 4.6.0.0 (www.fluxus-engineering.com). This method allows for displaying alternative phylogenetic hypotheses and is useful for the visualization of shallow genealogies. Because the East African haplotypes were recognized as distant, further analyses were performed solely on the Middle Eastern populations. The sequence evolution model was inferred using jModelTest 0.1.1 (Posada 2008). The model of sequence evolution was determined using Akaike (AIC) and Bayesian (BIC) information criteria.

To analyse the demographic history of the Middle Eastern populations in detail, we used Bayesian skyline plots (BSPs), which is a method based on coalescent theory independent of a priori defined models and enables the detangling of complex population dynamics scenarios (Drummond *et al.* 2005). The analysis was performed for the Middle Eastern populations using the BEAST 1.6.2 program (Drummond & Rambaut 2007). The Markov chain Monte Carlo (MCMC) was run three times with 3×10^7 iterations, a piecewise linear demographic function, the model of sequence evolution compatible with the results of the jModelTest and the options available in BEAST (GTR) and a strict molecular clock. The genealogy and model parameters were sampled every 1000 iterations. After discarding 10^7 iterations of each particular run as burn-in, the resulting log files were combined using LogCombiner and summarized as BSPs using Tracer v1.5.

The presence of null alleles or scoring errors, because of stuttering and large allele drop-out, in the microsatellite data set was assessed for each population using a Monte Carlo simulation of the expected allele size differences in MicroChecker (Van Oosterhout *et al.* 2004). The potential occurrence of recurrent sampling of a particular individual was checked by identity analysis in Cervus 3.0 (Kalinowski *et al.* 2007). The observed heterozygosity (H_O) and expected heterozygosity (H_E) were computed using the identical software. The allelic diversity (A), mean allelic richness corrected per sample size (AR) and the inbreeding coefficient (F_{IS}) were analysed using FSTAT 2.9.3 (Goudet 1995).

The population architecture was explored using model-based Bayesian clustering of individual genotypes (Pritchard *et al.* 2000; Falush *et al.* 2003) implemented using Structure 2.3.3. We performed 10^6 MCMC reps after a 10^5 burn-in period 10 times for each K , which varied from 1 to 10 with the admixture model and allele frequencies independent of the populations. The statistical support for the results of each K was then analysed using the method described by Evanno *et al.* (2005) with the aid of Structure Harvester v 0.6.8 (Earl & vonHoldt 2011).

Geographic structure

The occurrence of isolation by distance (sensu Wright 1943) was analysed in a relatively continuous part of the range in the Mediterranean basin and for the entire Middle Eastern region. The matrix of geographic distances was calculated using Geographic Distance Matrix Generator 1.2.3. (Ersts 2011). The genetic distances were measured as (a) Φ_{ST} or Rousset's distance $\Phi_{ST}/(1 - \Phi_{ST})$ for populations with $n > 1$ for the mitochondrial sequences and (b) F_{ST} or Rousset's distance $F_{ST}/(1 - F_{ST})$ for populations with $n > 4$ for the nuclear microsatellite

genotypes. Isolation by distance (IBD) has been illustrated by plotting the genetic distances compared with the geographic distances in km. The correlation between the two matrices was tested using the Mantel test with 1000 randomizations, and a regression line was calculated using the RMA (Reduced Major Axis) across Isolation By Distance Web Service v 3.16 (Jensen *et al.* 2005).

We used a landscape genetics approach implemented in the R package of Geneland (Guillot *et al.* 2005) to analyse the spatial distribution of genetic variability within Eastern Mediterranean. Initially, the number of clusters was determined by running the program five times with 10^6 MCMC iterations, a thinning of 100, the number of populations varying from $K = 1$ to $K = 10$, an uncorrelated allele frequency model and noise blurring of the coordinates set to 5 km. A model assuming a multinomial distribution of the genotypes conditionally on the haplotype frequencies/subpopulation in Hardy–Weinberg equilibrium was used in the case of the haploid/diploid markers. Finally, the model was re-run with 10^7 iterations, a burn-in of 10^4 , the number of subpopulations fixed to K inferred from the initial screening and the remaining parameters were identical to those outlined previously. The inferred Voronoi tessellations were displayed as maps of the posterior probabilities of population memberships. The genetic discontinuities uncovered by the procedure were linked to potential geographic barriers by a visual comparison with maps of the region.

Results

Genetic clustering

The 239 bp alignment of the mitochondrial sequences contained 304 individuals. The visualization of the relationships among the mitochondrial haplotypes revealed

a relatively deep separation between the Middle Eastern and East African branches ($p = 9.7\text{--}18\%$), large genetic distances within the East African haplogroup ($9.2\text{--}17\%$) and a moderately diversified structure in the northern cluster (Fig. 2). The basal position in the northern haplogroup is occupied by Sinaic and Jordan individuals. Within the Mediterranean, widespread dominant haplotypes tend to be partially geographically localized and are often connected to site-specific rare variants. The haplotypes from the Persian and Arabian range patches are localized at the tips; however, they are not reciprocally monophyletic to the rest of the network. The TIM1 + G model was inferred using the Akaike, and TrNef + G model was inferred using the Bayesian information criterion. The Bayesian skyline plot suggested that the population size was approximately constant in the past and indicated a recent pulse in population growth (Fig. 3). The descriptive population genetics parameters for particular colonies are displayed in Table 1.

The identity analysis revealed that no individuals were resampled. Bayesian clustering of 553 microsatellite genotypes (Fig. 4) captured the isolated position of the East African and Cypriot island populations for $K = 2$. This subdivision generated the highest value of ΔK (Fig. 5). The patterns obtained for the increasing values of K showed further substructuring. The East African genotypes were sequentially subdivided into a northern Sudan population (which exhibited similarity to the Egyptian cluster) and a sub-Saharan group. The colonies from southern Jordan, Sinai, the southern Arabian Peninsula and Iran tended to cluster together from $K = 3$, with the emergence of a separate group that included eastern Oman and Iran at $K = 7$. The Egyptian and northern Sudanese individuals formed a unique cluster from $K = 4$. Animals from the Nile basin and delta exhibited similarity with the Levantine populations, whereas isolated demes from the Saharan oases west of the Nile

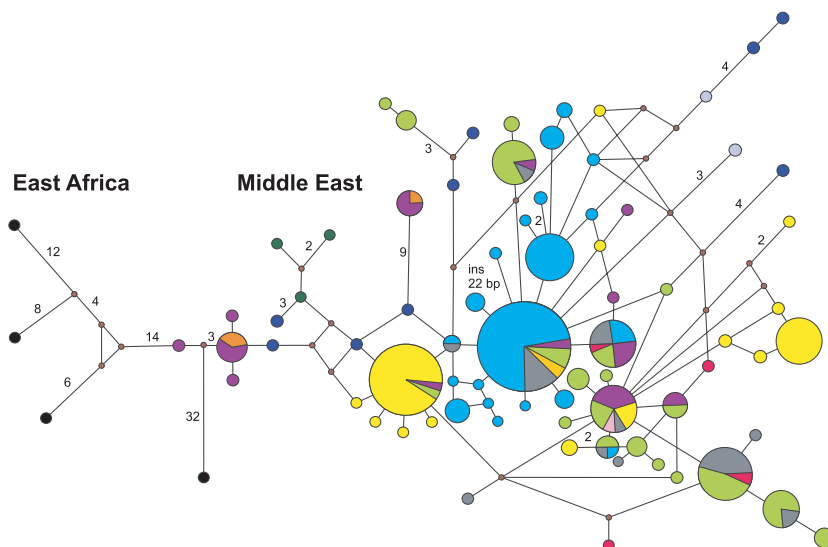


Fig. 2 The Median-joining network for the East African and Middle Eastern samples. The haplotypes are marked as circles, and their size is proportional to the number of individuals with the particular haplotype. The missing haplotypes are denoted as small brown dots. The numbers at the branches represent the number of mutation steps (displayed for $n > 1$). Colour codes: black-East Africa, violet-Jordan, orange-Sinai, dark blue-Yemen, dark green-Iran, yellow-Egypt, bright blue-Cyprus, bright green-Lebanon, dark grey-Turkey, red-Syria, pink-Israel and bright grey-Oman.

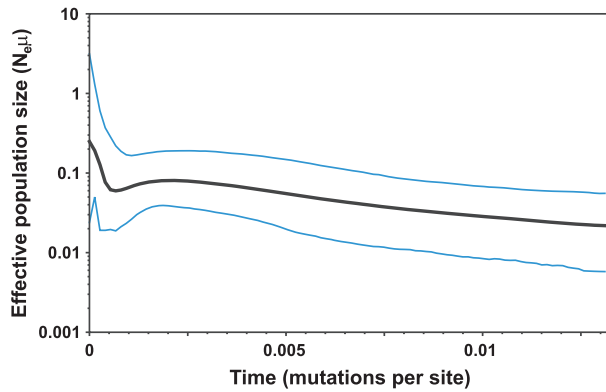


Fig. 3 The Bayesian skyline plot of the Middle Eastern populations showing changes in the effective population size estimate ($N_e\mu$) over time and measured in mutations per site. The black line indicates the median estimate, and the blue lines delimit the highest 95% posterior density interval.

exhibited an exclusive status. Animals from the Levant (northern Jordan, Israel, Lebanon and Syria) and Turkey formed a relatively homogenous group.

Isolation by distance and landscape genetics

Positive correlations between the genetic and geographic distances were reflected using Mantel tests in all of the inspected cases. The results for the Mediterranean basin are displayed in Fig. S1 (Supporting information) ($Z = 129348.3845$, $r = 0.3856$, $P < 0.0001$ for mitochondrial sequences; $Z = 23581104.9530$, $r = 0.2968$, $P < 0.001$ for nuclear microsatellites). For the nuclear data, the F_{ST} of the population pairs, including Cyprus, showed relatively high values of up to $F_{ST} = 0.181$.

The analysis using Geneland generated similar results for the mitochondrial and nuclear data (Fig. 6). Both types of markers resulted in distinguishing the spatial

Table 1 The descriptive genetic parameters for particular localities with at least five individuals analysed for particular data sets. Only the data for sites with at least five individuals genotyped for microsatellites are displayed

Country	Locality	N_s	N_h	h	N_g	A	AR	H_E	H_O	F_{IS}
Cyprus	Ahanas. Androlika	13	5	0.756	16	4.3	1.574	0.574	0.574	-0.004
Cyprus	Pissouri				12	3.7	1.592	0.592	0.473	0.210
Cyprus	Smigies	8	4	0.786	9	3.5	1.554	0.554	0.530	0.048
Cyprus	Ergates	14	7	0.813	14	3.9	1.574	0.574	0.539	0.040
Cyprus	Gerolakkos-Alaykoy				8	3.4	1.579	0.579	0.461	0.204
Cyprus	Mammari	16	5	0.675	25	4.7	1.582	0.582	0.505	0.147
Cyprus	Afendrika	8	5	0.786	8	3.8	1.599	0.599	0.586	0.025
Cyprus	Yedikonuk	12	5	0.727	13	4.2	1.600	0.600	0.563	0.064
Turkey	Antalya	7	3	0.667	16	5.1	1.674	0.674	0.672	-0.006
Turkey	Adana				17	5.2	1.642	0.642	0.640	-0.003
Turkey	Sayköy	8	5	0.786	19	5.6	1.671	0.671	0.656	0.012
Turkey	Cevlik				9	4.8	1.666	0.666	0.674	-0.002
Turkey	Demrek. Dipsiz cave	5	4	0.900	16	5.2	1.647	0.647	0.687	-0.073
Turkey	Harbiye	5	4	0.900	15	5.2	1.659	0.659	0.636	0.022
Lebanon	Adloun				8	4.3	1.636	0.636	0.645	-0.025
Lebanon	Berquayl cave				10	3.5	1.622	0.622	0.425	0.283
Lebanon	Jeita	14	10	0.934	20	5.2	1.648	0.648	0.616	0.071
Lebanon	Jezzine. Pont Al Khalass	7	3	0.762	6	3.9	1.654	0.654	0.563	0.132
Lebanon	Mtal al Azraq	15	8	0.876	24	5.5	1.649	0.649	0.646	0.009
Lebanon	Ouataouite cave				15	5.5	1.661	0.622	0.584	0.044
Lebanon	Aamchit. Saleh cave	10	9	0.978	11	5.0	1.662	0.622	0.584	0.016
Lebanon	Antelias. Kanaan cave	8	8	1	9	4.3	1.644	0.625	0.584	0.096
Jordan	Kufranja. Iraq Al Wahaj cave	5	3	0.800	12	5.2	1.674	0.630	0.593	0.024
Jordan	Iraq al Amir. Wadi As Sir	14	7	0.824	27	7.0	1.682	0.633	0.596	0.027
Jordan	Wadi Dhana	9	4	0.639	12	6.3	1.742	0.636	0.603	-0.024
Egypt	El Qahirah				6	3.8	1.623	0.639	0.608	0.072
Egypt	Aswan	16	8	0.858	22	5.9	1.656	0.641	0.609	0.070
Egypt	El A'aqab				17	5.2	1.648	0.628	0.592	-0.021
Egypt	Dakhla. El Qasr	28	8	0.646	45	5.5	1.571	0.642	0.611	0.071
Egypt	Dakhla. Mut				10	4.0	1.594	0.641	0.608	0.018
Egypt	El Kharga	13	2	0.154	17	4.7	1.607	0.640	0.606	-0.081
Oman	Ain Tabruq				7	5.5	1.696	0.639	0.604	0.027
Oman	Taiq cave				9	6.2	1.735	0.638	0.600	0.083
Oman	Al Nakhar				6	3.9	1.637	0.637	0.596	0.081

Number of individuals sequenced (N_s), number of haplotypes (N_h), haplotype diversity (h), number of individuals genotyped (N_g), allelic diversity (A), mean allelic richness corrected by sample size (AR), expected heterozygosity (H_E), observed heterozygosity (H_O), inbreeding coefficient (F_{IS}).

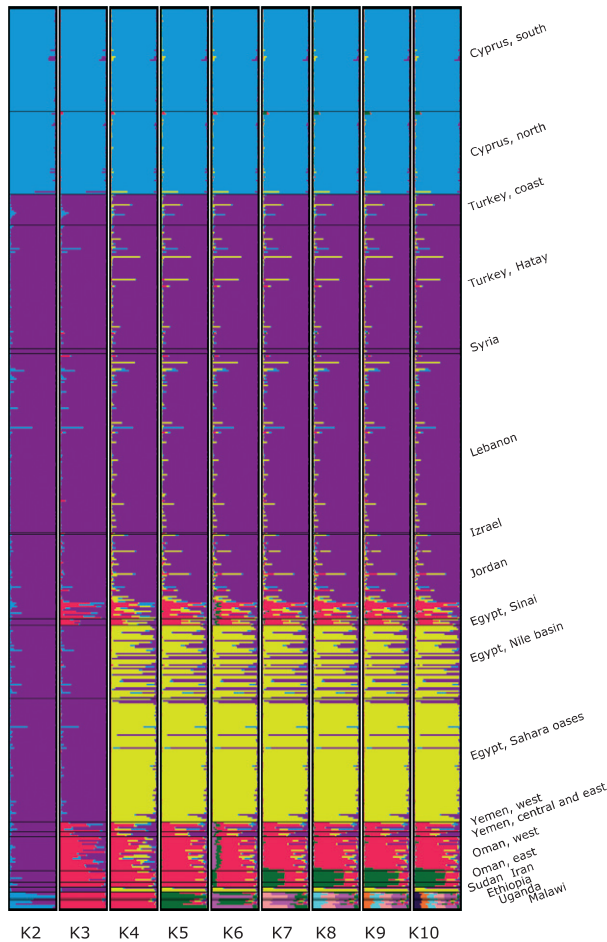


Fig. 4 The population memberships of the inspected individuals for the a priori defined number of clusters $K = 2-10$ inferred by the Structure software. Each genotype is represented by a horizontal line divided into K coloured segments. The length of each segment corresponds to the value of the estimated membership coefficient (Q).

domains comprising the populations from (i) the Nile basin and Saharan oases in Egypt, (ii) Sinai and southern Jordan, (iii) the Levant, including northern Jordan, Israel, Lebanon, Syria and southeast Turkey (Hatay Province) and (iv) the Mediterranean coast of Turkey and Cyprus. The analysis of the microsatellite data revealed an additional domain that included two populations from the Karpas Peninsula, which is the easternmost promontory of North Cyprus (v).

Discussion

The genetic structure at the environmental margin

Different modes of heredity in mitochondrial and nuclear markers have often resulted in different patterns obtained from both types of data in cryptic bat species complexes

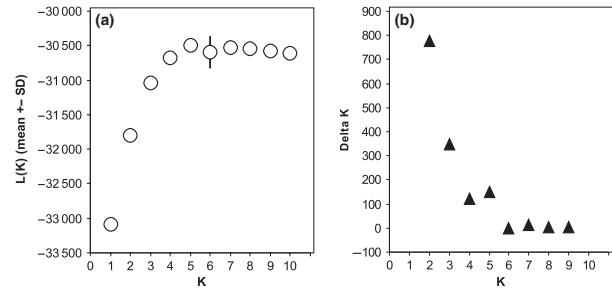


Fig. 5 The detection of the true number of clusters inferred by the Structure software using the method of Evanno *et al.* (2005) and displaying (a) the log probability of the data $L(K)$ and (b) $\Delta K = \text{mean}(|L''(K)|)/\text{SD}(L(K))$ as a function of K .

(Hulva *et al.* 2010) and even in markedly morphologically distinct lineages (Nesi *et al.* 2011). These conflicts can be caused by the hybridization and introgression among particular lineages or differences in the spatial behaviour between the sexes and emphasize the necessity for using a combination of both marker types. However, the results in our model taxon exhibit rough congruence between both data sets within the Middle East, although the nuclear microsatellites provided a more geographically distinct picture and different details because of the higher mutation rate. The absence of mitochondrial substructuring in comparison with the nuclear data suggests an absence of pronounced difference in the spatial behaviour of the sexes.

The results of our study indicate that the genetic variation within the study area is of relatively recent origin. Compared with the cytochrome *b* and NADH dehydrogenase subunit 1 sequence data available to date (Benda *et al.* 2012), which provide insufficient phylogeographic information, detailed sampling and using fast-evolving molecular markers including nuclear microsatellites in this study enabled us to inspect the population structure of the Egyptian fruit bat in detail. Compared with the rather homogeneous population structure of cave-dwelling bat species in climax tropical ecosystems detected using comparable methodology (Chen *et al.* 2010; Rossiter *et al.* 2012), relatively pronounced isolation by distance and population diversification was observed within the studied area in the Egyptian fruit bat, which resembled the situation in tropical tree-dwelling species and indicated the occurrence of peripatric effects. This contrast may be interpreted considering that historical vicariance and gene flow, which are factors with opposing effects on population structures (Bossart & Prowell 1998), may have made different contributions in the two zones. Seasonality and Pleistocene oscillations in the climate could complicate the demographic histories of the species at higher latitudes and may favour the evolution of population substructures. Simultaneously, the cave microclimate in the Middle East was relatively stable during the Quaternary (Frumkin *et al.* 2000;

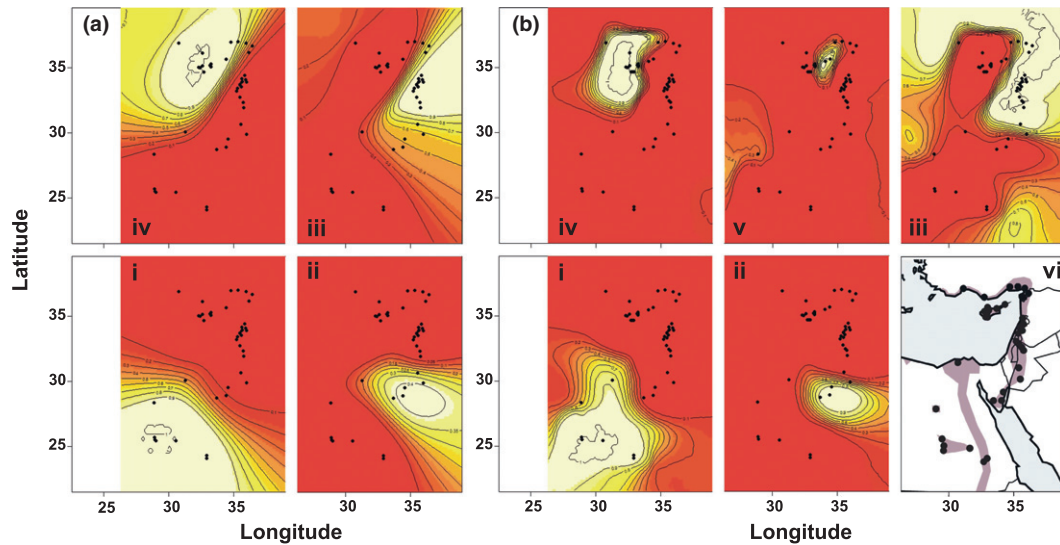


Fig. 6 The tessellation maps inferred using a landscape genetics approach with the Geneland program. The dots represent particular sampling sites, respective subpopulations are displayed by light colours and the contour lines and colour scales denote the values of the posterior probabilities of subpopulation memberships. (a) the mitochondrial sequence data and (b) the nuclear microsatellite data. Particular panels emphasize the following domains: (i) Egypt, (ii) Sinai and southern Jordan, (iii) Levant, (iv) the Mediterranean coast of Turkey and Cyprus, (v) the Karpas Peninsula in North Cyprus and (vi) a map depicting the study region and extant range of the species (violet).

Robinson *et al.* 2006). The varied topography of the Middle East, including uneven allocation of water sources and fluctuating hydrological regime in arid, semiarid and Mediterranean ecosystems, together with the presumably complicated and human-assisted phylogeography of fruit plants (Allen 2009), thus resulting in the mosaic distribution of vegetation associations suitable for fruit bats, could be the main factors responsible for the fragmentation and diversification at the range edge.

Phylogeography and transition zones

The results of both the mitochondrial and nuclear assays indicate a pronounced separation between East Africa and the northern range patches, coinciding with the geographic isolation of the Middle Eastern range offshoot by the Sahara desert. Moreover, the sub-Saharan samples exhibited cryptic variation within this portion of the range. Further sampling is necessary to resolve the population structure within this territory.

Individuals from Sinai and southern Jordan carry ancestral haplotypes within the northern haplogroup, and their nuclear genotypes show relationships with the African ones at low *K* values, which indicate the gateway role of this region in colonizing the Mediterranean basin. Both types of markers indicate the distant status of these populations within the Mediterranean region. This unit is separated from the Levantine population approximately by the territory of the Dead Sea in the

north and from the Egyptian population by the Gulf of Suez and the adjacent arid habitats in the east. However, the genetic relationship to the demes in the southern Arabian Peninsula imply a recent or subrecent gene flow along the coast of the Red Sea in Saudi Arabia, although there is a gap in the species distribution in this region according to the current knowledge. This outcome stresses the role of genetic markers in tracing animal movements, because rare dispersal events may be beyond the traceability by observations, particularly in volatile and nocturnal animals. The evolution of a separate cluster comprising the eastern Arabian and Persian populations might be ascribed to the suture zone intersecting the Middle East, as demonstrated by the phylogeographic structure within the bat genus *Rhinopoma* (Hulva *et al.* 2007).

Island isolation

Within the remainder of the studied area, including the eastern Mediterranean, Nile basin and Saharan oases, a relatively homogenous population structure was detected using mitochondrial data. However, the microsatellites indicated marked differentiation of insular populations in desert oases and an oceanic island. Considering the mutation rate of both types of markers, such patterns indicate the important role of an insular environment in the nascent evolution of genetic substructures in the region.

In Egypt, the relatively basal split in the genotypes from the Saharan oases within the clustering hierarchy indicates a barrier effect of the arid territories and the isolation of particular demes in habitat islands with suitable microclimatic conditions. The population in the Nile basin appears to be composed of individuals with affiliations with both the Saharan oases and the Levant; however, marked admixture was not observed. This outcome suggests a corridor role of the Nile Valley and recent or subrecent contact with the Levantine populations, although there appears to be a recent distributional gap in the north of Sinai and the possibility of migratory behaviour in the Egyptian fruit bat. The observed gene flow between the Saharan oases and the Nile basin, as well as along the Red Sea coast, indicates at least a partial permeability of the desert barriers for the Egyptian fruit bat. One of possible explanations may involve the seasonal enhancement of passability of these region related to the phenology of the respective ecosystems. In the Levant, the species reach relatively high population densities, which are presumably connected to the high degree of gene flow and are mirrored by the homogenous population structure. The highest values of haplotype diversity (Lebanon, Hatay province in Turkey) indicate the possible microrefugial role of this region and metapopulation structure of the Egyptian fruit bat. Cyprus, the only island of oceanic origin within the studied portion of the range, bears the population with the most pronounced separate status at the level of nuclear data observable in the Middle East. The island is separated from the Turkish coast by a 75 km stretch of the Mediterranean Sea. Using the landscape genetics approach in *R. aegyptiacus*, the relationships between these territories indicate the colonization of Cyprus from this region of the mainland.

The native Quaternary mammalian fauna of Cyprus is characterized by a high degree of impoverishment and extreme examples of insular syndrome, including dwarf elephants and pygmy hippopotamuses (Van der Geer *et al.* 2010). Bats represent one of the few mammalian groups capable of naturally colonizing the region (Benda *et al.* 2007), and the island environment may promote peculiar site-specific evolution in some lineages, as demonstrated within the genus *Pipistrellus* (Hulva *et al.* 2010; Evin *et al.* 2011). The capacity of bats, the only mammals with powered flight, to cross sea barriers has been demonstrated by direct observations (Ahlén *et al.* 2009) and the successful colonization of remote islands by the ancestors of many insular species, which often represent the sole native mammals on islands (Fleming & Racey 2009). Genetic markers represent useful tools to measure gene flow across sea barriers. For example, marked genetic differentiation has been reported in the microchiropteran bat *Myotis myotis*

on either side of the Strait of Gibraltar, which is a 14-km-wide sea channel between Europe and Africa (Castella *et al.* 2000). In a taxonomically broader study comprising of microchiropteran species, no correlation was observed between the physical ability to cross the strait (indexed by wing morphology parameters) and the level of gene flow in a particular species (García-Mudarra *et al.* 2009). Within the genus *Rousettus*, a large-scale panmixia across Madagascar, including the neighbouring islands separated by distances of up to 13 km, was demonstrated in *R. madagascariensis* (Goodman *et al.* 2010). A similar pattern involving a substantial degree of genetic mixing was also revealed in *R. obliviosus* in Comoros with water expanses between particular islands ranging from 40 to 80 km (Goodman *et al.* 2010). In our study, the genetic similarity between *Rousettus* populations in the eastern Arabian Peninsula and Iran indicates gene flow over the Strait of Hormuz, which is a 39-km-wide narrowing of the Persian Gulf.

However, island evolution is typical by founder demography, genetic drift and site-specific selection, which are conditioned by the isolation of the population and restriction of gene flow from the mainland (Whittaker & Fernández-Palacios 2007). These processes could lead to changes in genetic composition and decrease the genetic variation, which was the situation revealed in our study. For example, demes from Egyptian oases and Cyprus showed the lowest values of allelic richness among all the studied populations. Cypriot colonies also had the lowest values of expected heterozygosity. Two Cypriot and two Lebanese populations were characterized by inbreeding (higher F_{IS} values, observed heterozygosity lower than expected). Therefore, we concluded that the insular populations of the Egyptian fruit bat were influenced by founding events with sustained or repeated bottlenecks and genetic drift; however, assessing the contribution of a particular effect in concrete cases remains debatable (Clarke & Grant 1996). Founding events under the transience model results in alterations in the gene frequencies (Templeton 1980), which may be the proximate mechanism behind the genetic changes demonstrated in Cyprus and Egypt by the clustering methods. Interisland differentiation and repeated bottlenecks may have occurred on Cyprus in connection with the population fragmentation caused by geomorphology and mosaic distribution of suitable habitats. This result was shown by revealing additional domain in the landscape genetics analysis on Karpas Peninsula using fast-evolving microsatellites compared with mitochondrial sequences. The abovementioned processes may theoretically graduate into founder effect speciation (Barton & Charlesworth 1984; Gavrilets & Hastings 1996). Whereas our study is an empirical illustration of this concept at the

microevolutionary level, the role of the island environment in the diversification of fruit bats may also be demonstrated on larger evolutionary scales. Within the genus *Rousettus*, four of the ten clades with recent species statuses are island endemics, including *R. bidens* and *R. linduensis* from Sulawesi, *R. obliviosus* from Comoros and *R. madagascariensis* from Madagascar (Simmons 2005). Two lineages from islands in the Gulf of Guinea, *R. a. princeps* and *R. a. tomensis* have been granted subspecific statuses (Juste & Ibáñez 1993; Juste *et al.* 1996). The distributional patterns of the diversity, disparity and endemism of megabats on a worldwide scale emphasize the relationships between evolutionary hotspots and island and archipelago environments (Almeida *et al.* 2011). For example, 17 of the 26 total species are endemic to the Philippines (Heaney *et al.* 2005). It is reasonable to conclude that the isolated peripheral populations played important role in the radiation of the entire family Pteropodidae.

The complex relationships between the width and character of the geographic barriers and the degree of genetic isolation indicate that intrinsic biological factors may also play a role in the constitution of island populations and island speciation, including Cyprus, which is situated within a physically traversable distance for fruit bats from the mainland. In bats, which are gregarious and volatile mammals with long lifespans (up to 40 years), factors including the effects of spatial behaviour, navigation cues, trophic niches, population biology, social tradition and the cohesion of particular demes should be considered. Navigation cues have been studied in detail in the Egyptian fruit bat (Tsoar *et al.* 2011). Megabats are unique within the order Chiroptera possessing well-developed eyes with a retinal tapetum lucidum and are macrosomatic mammals with a well-developed rhinarium. The echolocation characteristic of *Rousettus* is useful only over a short range and is not used outside of the cave. Orientation using the Earth's magnetic field has been shown in microbats (Holland *et al.* 2006), but the extent to which this capacity is employed in megabats is not fully understood. Homing experiments in the Egyptian fruit bat have indicated that navigation in this context is guided primarily by distant visual landmarks together with spatial memory (Tsoar *et al.* 2011). Establishing a cognitive map of a visually familiar area within particular demes may promote their cohesion, especially in such markedly delimited territories as islands.

Regarding the extreme position of the eastern Mediterranean regions, including Cyprus, within the distributional pattern of the Pteropodidae family, adaptive responses to the relatively limiting abiotic conditions and island environments cannot be excluded. Adaptive evolution may also play the role in connection with

founder demography (transilience model) and divergence in life-history traits boosted by founding events. In our study, the contribution of selective sweep or background selection to the loss of nuclear variation in insular demes is possible if some of the examined microsatellites were associated with fitness-related loci (Barton 2000). Further research, including genomic approaches, is necessary to test these hypotheses. In terms of the known phenotypic variations, stepped cline in body size metrics along the northwest–southeast axis, with a dominance of large forms in the northern latitudes of the Mediterranean basin, was ascertained (Benda *et al.* 2012). Regarding the proximate context, including the metabolic constraints of a volatile mammal with ancestry in tropical zones, it is possible to imagine the ecogeographic causality behind the evolution of this morphocline, which is interpretable within Bergman's rule.

Within the rising paradigmatic shift, the population biology level and process-based interpretations are key approaches to understanding species phenomenon, rather than static species concepts and diagnoses (Hart 2010). Our study, which contradicts the traditional subspecific alpha taxonomy of the Egyptian fruit bat, provides a useful baseline for describing biologically meaningful units within the northern range of this species. This knowledge may also be useful for frequent studies using Egyptian fruit bats as laboratory model organisms in general biology disciplines.

The role of humans

Because bats, as mammals with powered flight, are characterized by high metabolic rates, the adaptive evolution connected to feeding behaviour has played a key role in the origins of the recent diversity and distributional patterns of the order and in shaping the spatial behaviour within particular populations (Jones *et al.* 2005; Rojas *et al.* 2012). Recently, the diet of *R. aegyptiacus* in the Middle East has included many native or introduced agricultural plants (Korine *et al.* 1999; Del Vaglio *et al.* 2011), for example, the common fig (*Ficus carica*), date palm (*Phoenix dactylifera*), carob tree (*Ceratonia siliqua*) or mulberry (*Morus*). The history of the domestication of many of these species is connected to the Fertile Crescent and Mediterranean basin region and dates from the Neolithic revolution. The occurrence of anthropogenic roosts may also play a role in the distribution of cave-dwelling bats. The temporal coincidence between the onset of human activities in the region and the increase in the effective population size of *R. aegyptiacus* revealed by demographic reconstructions might support the tentative hypothesis regarding the causal relation between these phenomena. From this viewpoint, the

range expansion of the species into the region and the evolution of the abovementioned patterns could have been facilitated by anthropogenic changes in the environment. This hypothesis is also corroborated by the absence of a known fossil record of *R. aegyptiacus* in the Mediterranean region prior to the Holocene, as reported in the Levant (Tchernov 1988). Recent demographic expansions with possibly similar roots have also been suggested by several methods in the abovementioned Comoros and Madagascar *Rousettus* clades (Goodman *et al.* 2010). However, the absence of fossils in the region prevents the calibration of the molecular clock. Further phylogeographic evidence is necessary to explicitly test the hypothesis.

However, the frugivorous bats that coexist with humans in highly populated agricultural areas often become the subject of eradication, thus raising controversial conservation issues (Korine *et al.* 1999). Moreover, the African populations of *R. aegyptiacus* have also become known as reservoirs of emerging zoonoses, that is, the Ebola and Marburg filoviruses (Pourrut *et al.* 2009). Regarding the high migratory capacity of these animals, genetic data shedding light to their subpopulation structure and spatial behaviour may provide valuable information regarding potential transmission routes and specify the vector role of *R. aegyptiacus* in the natural cycles of these viruses. Information concerning the variability within the natural range may help to assess the conservation status of particular populations within this unique faunal element in the Mediterranean basin, a region highly altered by human activities.

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P.H. designed and supervised the study and data analysis and wrote the manuscript; T.M. performed most of the microsatellite and Ch.D. of the sequencing assays; and P.H., R.B., P.B., T.B. and I.H. performed the fieldwork. This study was conducted as a component of P.H.’s postdoctoral projects at Charles University in Prague in the context of long-term multidisciplinary research on mammal model species, which focus on island biogeography, the genetics of small populations and speciation. P.H., T.M., Ch.D. and R.B. are interested mainly in molecular evolution of mammals, T.B. in ecology, and P.B. and I.H. in the biogeography and taxonomy of bats.

Data accessibility

The mitochondrial control region sequences: GenBank accession numbers JX198427–JX198505. Information regarding all analysed individuals including locality, mitochondrial sequence GenBank accession number and microsatellite genotype are available on the Dryad digital repository (doi:10.5061/dryad.k68k8).

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 A list of sampling sites and their characteristics including the coordinates and number of individuals sampled, sequenced and genotyped.

Table S2 A list of the microsatellite markers and their characteristics.

Fig. S1 The relationships between the genetic and geographic distances in km for populations from the Mediterranean basin: (a) the mitochondrial data for populations with $n > 1$ and genetic distance measured using Φ_{ST} and (b) the nuclear microsatellite data for populations with $n > 4$ and genetic distances measured as F_{ST} .

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