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Phylogeography of Barbastelle bats (*Barbastella barbastellus*) in the western Mediterranean and the Canary Islands

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We use two mitochondrial DNA fragments with different substitution rates (cytochrome *b* gene and the control region) to address the following phylogeographic questions about western Palaearctic populations of the barbastelle bat (*Barbastella barbastellus*): 1) Do the Iberian populations of barbastelles show any genetic discontinuity associated with its present fragmented distribution?, 2) Is the Gibraltar Strait an effective barrier to gene flow for barbastelles? and 3) Is the subspecies from the Canary Islands genetically distinct from continental barbastelles? Our molecular survey shows that there is only a shallow genetic structure among populations of the Iberian Peninsula and Morocco, and probably, even across Europe until Thrace, although this last point needs to be confirmed. The Gibraltar Strait has not played any significant role as a biogeographic barrier to prevent the recent passage of European barbastelles to Morocco (or vice versa). Our phylogenetic reconstructions also confirm the taxonomic distinction of *B. barbastellus guanchae* as an endemic subspecies confined to the Canary Islands. The precise origin of this Canarian taxon is, nevertheless, still unclear as its mitochondrial lineage is distinct from any lineage found so far in Morocco and Iberia. This important genetic distinctness suggests either a relatively ancient colonization of the Canary Islands or that the source population of the founders have not yet been identified.

Key words: *Barbastella*, Mediterranean, Canary Islands, phylogeography, cytochrome *b*, control region, colonization, mitochondrial DNA, Gibraltar Strait

INTRODUCTION

The western barbastelle (*Barbastella barbastellus*) is a bat with mainly a European distribution stretching from the Atlantic coast to the Caucasus. Still, it is also found in few scattered localities of northern Africa and the Canary Islands (Rydell and Bogdanowicz, 1997). This species, one of the rarest bats in Europe (Urbańczyk, 1999), selects densely forested areas as foraging grounds (Sierro, 1999). Its current distribution is fragmented, due probably to the shrinking of suitable habitat which is now often restricted to mountain areas. Consequently, many populations are at present geographically isolated from each other, particularly along the southern part of its distribution, e.g., Morocco (Rift and Atlas Mountains) or the southern half of the Iberian Peninsula (Ibáñez *et al.,* 1992; Ibáñez, 1998; Trujillo *et al.,* 2002). It is not known whether this fragmentation has an impact on the remnant genetic structure of

barbastelle bats, or whether major historical barriers such as the Gibraltar Strait have played any role in shaping this genetic structure by impeding genetic exchanges between Iberian and Maghrebian populations.

The Canary Islands are part of the Macaronesian region and support only six distinct genera of bats: *Barbastella, Plecotus, Pipistrellus, Hypsugo*, *Nyctalus* and *Tadarida* (Trujillo, 1991; Mitchell-Jones *et al.,* 1999). In the Canarian archipelago, western barbastelle bats are quite rare as they are known, so far, only from La Gomera and Tenerife islands (Trujillo, 1991). Moreover, these island populations are also morphologically distinct from continental counterparts, and in fact, have recently been proposed to represent a distinct endemic subspecies (Trujillo *et al.,* 2002). If valid, this new taxon would be the only subspecies of *B. barbastellus*, which is otherwise considered as monotypic throughout its distribution (Rydell and Bogdanowicz, 1997; Horáček *et al.,* 2000).

Estimating the degree of intra-specific genetic structure and understanding the forces that determine genetic patterns in a particular species, are of high interest both from evolutionary and conservation perspectives. In relation to the barbastelle bats and using a phylogeographic approach based on mitochondrial DNA (mtDNA), we were interested in focusing on three main questions: (1) Do the Iberian populations of barbastelles show any genetic discontinuity associated with its present fragmented distribution?, (2) Is the Gibraltar Strait an effective barrier to gene flow for barbastelles? and (3) Is the subspecies from the Canary Islands genetically distinct from continental barbastelles?.

Materials and Methods

Samples and DNA amplification

A total of 24 *B. barbastellus* were sampled from the Iberian Peninsula, Central Europe, Thrace, Morocco and the Canary Islands (Fig. 1, Table 1).

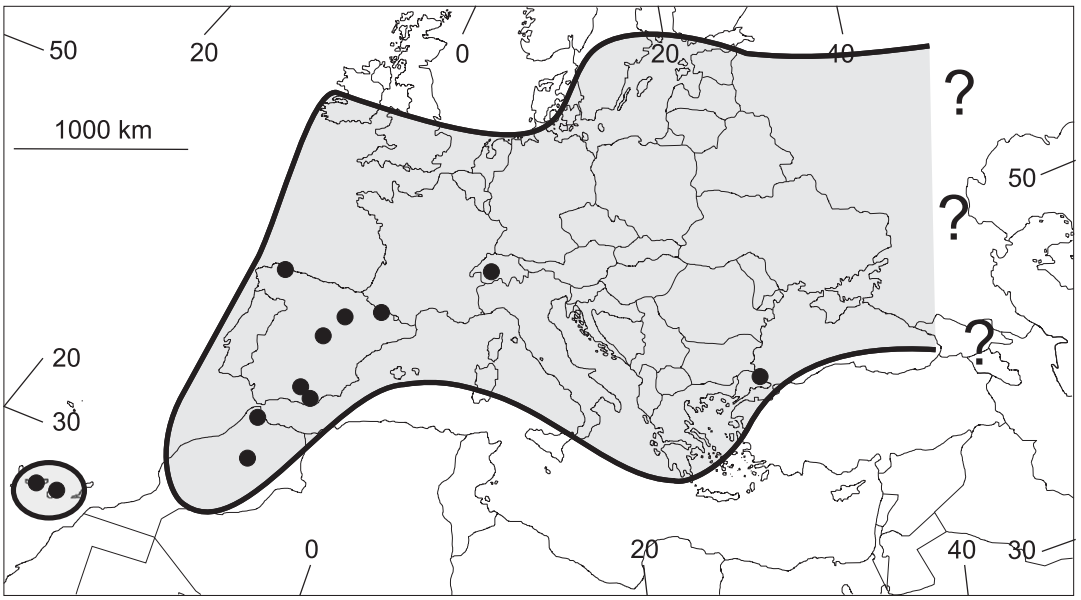

Fig. 1. Map of the approximate distribution of *Barbastella barbastellus* in the western Palaearctic according to Urbańczyk (1999) and localities sampled for this study (see Table 1 for details)

TABLE 1. List of specimens, localities and accession numbers of the samples of *B. barbastellus* used for this study. cyt-*b* – cytochrome *b*, CR – control region

Specimen	Locality	Geographic coordinates	GenBank Acc.N°		Voucher
			cyt-*b*	CR	
Bba9.CAG	La Gomera, Canary Is., Spain	28°06'N, 17°08'W	AF513745	AF515152	EBD16028
Bba10.CAG	La Gomera, Canary Is., Spain	28°06'N, 17°08'W	AF513745	AF515152	EBD16024
Bba150.CAG	La Gomera, Canary Is., Spain	28°06'N, 17°08'W	–	AF515152	MNH110
Bba148.CAT	Santa Úrsula, Tenerife, Canary Is., Spain	28°24'N, 16°28'W	AF513745	AF515152	Biopsy
Bba149.CAT	Santa Úrsula, Tenerife, Canary Is., Spain	28°24'N, 16°28'W	AF513745	AF515152	Biopsy
Bba49.CAT	La Matanza, Tenerife, Canary Is., Spain	28°25'N, 16°26'W	AF513745	AF515152	Biopsy
Bba35.MO	Azrou, Morocco	33°24'N, 05°13'W	AF513752	AF515162	EBD25831
Bba850.MO	Tetouan, Morocco	35°35'N, 05°20'W	AY254570	AY254218	Biopsy
Bba174.IB	Viator, Almería, Spain	36°53'N, 02°25'W	AF513750	AF515158	Biopsy
Bba197.IB	Río Bornova, Guadalajara, Spain	41°10'N, 03°00'W	AF513748	AF515154	Biopsy
Bba11.IB	El Rasillo, La Rioja, Spain	42°12'N, 02°41'W	AF513746	AF515153	EBD15181
Bba12.IB	El Rasillo, La Rioja, Spain	42°12'N, 02°41'W	AF513747	AF515154	EBD15981
Bba145.IB	El Rasillo, La Rioja, Spain	42°12'N, 02°41'W	AF513748	AF515155	Biopsy
Bba146.IB	El Rasillo, La Rioja, Spain	42°12'N, 02°41'W	AF513748	AF515154	Biopsy
Bba47.IB	Belsué, Huesca, Spain	42°19'N, 00°22'W	AF513750	AF515163	Biopsy
Bba48.IB	Belsué, Huesca, Spain	42°19'N, 00°22'W	AF513749	AF515156	Biopsy
Bba147.IB	Belsué, Huesca, Spain	42°19'N, 00°22'W	AF513749	AF515156	Biopsy
Bba277.IB	Ordesa, Huesca, Spain	42°40'N, 00°00'W	AF513749	AF515161	Biopsy
Bba278.IB	Ordesa, Huesca, Spain	42°40'N, 00°00'W	AF513749	AF515156	Biopsy
Bba89.IB	Ortigueira, A Coruna, Spain	43°41'N, 07°51'W	–	AF515164	Biopsy
Bba849.IB	Baza, Granada, Spain	37°23'N, 02°51'W	AF513750	AF515158	Biopsy
Bba198.TK	Sarpdere, Thrace, Turkey	40°52'N, 26°25'E	AF513753	AF515159	Biopsy
Bba199.TK	Sarpdere, Thrace, Turkey	40°52'N, 26°25'E	AF513751	AF515160	Biopsy
Bba165.SW	Martigny, Valais, Switzerland	46°10'N, 07°08'E	AF513749	AF515157	MHNG 1804.94

DNA was extracted either from wing biopsies following Higuchi *et al.* (1988) or from tissue samples preserved in ethanol following standard phenol/chloroform protocols (Maniatis *et al.,* 1989). The primer pair L14724 (Irwin *et al.,* 1991) and MVZ16 (Smith and Patton, 1993) was used to amplify a fragment about 800 bp long of the mitochondrial cytochrome *b* gene (cyt-*b*). Likewise, primers L15975 (Wilkinson and Chapman, 1991) and H16498 (Fumagalli *et al.,* 1996) were used to amplify the left domain of the mitochondrial control region (CR). This non-coding segment includes the first hyper-variable region (HV1) and usually a stretch of variable tandem R1 repeats (Fumagalli *et al.,* 1996; Wilkinson *et al.,* 1997). The PCR profile consisted in 4 min initial denaturation at 94°C followed by 39 cycles of 60 s at 94°C, 30 s at 45–50°C (for the cyt-*b*), and 2 min at 72°C and a final extension of 10 min at 72°C. The annealing temperature for the CR was 55°C. PCR products were purified and sequenced using an ABI 377 or an ABI 3100 automated sequencer (PE Biosystems, Warrington, UK) following the manufacturer's protocols.

Sequence and Phylogenetic Analyses

Alignments were obtained with Sequencher 4.1 (Gene Codes Crop.). No evidence of heteroplasmy was detected in any individual. For the non-coding region, the sequences were further inspected by eye to minimize alignment gaps (indels). Only different haplotypes for each fragment were included in the analyses. For each data set, the best fitting substitution model was selected with hierarchical likelihood ratio tests (LRT) (Modeltest soft., Posada and Crandall, 1998) and levels of genetic differentiation between groups were calculated using MEGA v. 2.1 (Kumar *et al.,* 2001).

After testing for congruence between data sets, the 5' end of the CR fragment was joined to the 3' end of the cyt-*b* sequence in a single string to obtain the most informative phylogenetic reconstruction. A Maximum Parsimony (MP) approach (unweighed heuristic search, TBR branch swapping) was used to build our phylogenetic hypothesis using PAUP* 4.0b10 (Swofford, 2000). Robustness of the topology was assessed through bootstrapping (Felsenstein, 1985) with 5000 replicates and heuristic search. Another approach to infer phylogenetic relationships consisted in the construction of a median-joining network (Bandelt *et al.,* 1999) with all haplotypes found in the joint sequences. This approach combines the topology of a minimum spanning tree with a parsimony-based search of the absent nodes or haplotypes (median vectors) not found in the sampling (Posada and Crandall, 2001). The network was obtained with the software NETWORK 3.1.1 (Röhl, 2003).

RESULTS

A 680 bp fragment of the cyt-*b* and 391 bp of CR were sequenced and aligned for 22 and 24 specimens respectively (Table 1). For the cyt-*b*, a total of 10 different haplotypes were found that showed 45 variable positions (Table 2) of which 19 were parsimony informative. Most of substitutions were transitions and located in third positions (77.8%). For the CR fragment, we found more variability, although less than the expected five times higher variation with respect to the cyt-*b* fragment. In fact, the 24 CR sequences represented a total of 13 different haplotypes with 75 polymorphic sites (Table 2) of which 32 where parsimony informative. The alignment of this fragment required two deletions in the unique haplotype from the Canary Islands (Table 2). All unique haplotypes in both CR and cyt-*b* fragments are deposited in GenBank (accession numbers in Table 1). The overall nucleotide composition was biased towards a deficit of guanine for cyt-*b* sequences (A = 0.283, C = 0.293, G = 0.159, T = 0.264), a result typical for mammalian sequences. On the other hand, the fragment of CR showed cytosine bias (A = 0.248, C = 0.113, G = 0.258, T = 0.380). For the cyt-*b*, the selected model was HKY85 with a Ts:Tv ratio of 5.55, no invariable sites and site heterogeneity (γ-shape parameter = 0.008). Similarly, for the CR a HKY85 model was selected with a Ts:Tv ratio of 15.04 with site heterogeneity (γ-shape parameter = 0.14).

Both partitions of sequences showed high compatibility ($P = 0.91$ in a partition-homogeneity test). The MP reconstruction based on the 14 different haplotypes for the concatenated sequenced resulted in three trees with length of 156 steps and consistency index of 0.823 and retention index of

TABLE 2. Polymorphic sites identified in the mitochondrial DNA fragments sequenced of *B. barbastellus* for the cyt-*b* (n = 45) (above), and for the CR (bellow)

Hap. #	Number of Variable Sites 111111111122222222223333333333444444 123456789012345678901234567890123456789012345
Bba11IB	CCTTTGAAGTTCCTTGCGCACGGCATGTAGAAGAAAATCGTGTTA
Bba12IB	..............C......A.........G....G...C....
Bba48IB	.T...A...C..T.C...T..A..............G...C....
Bba174IB	.T.................................G........
Bba35MO	.TC..A..A...T.C.....TA..............G...C....
Bba850MO	.T...A..A...T.C.....T...............G...C....
Bba165SW	.T...A...C..T.C...T..A..............G...C....
Bba198TK	.T.............................G.....AATCTCCC
Bba199TK	.T...A......T.C....G.A....A...G.....GAATCTCCC
Bba9CAG	T..GCAGGA.CTTCCCTA...AATGC.CTA..AGTGG...C....

Hap. #	Number of Variable Sites 11111111112222222222333333333334444444444555555555566666666667777777 12345678901234567890123456789012345678901234567890123456789012345678901234567
Bba11IB	GGTGTACGGCATAGTACCTCGTTAATTCCGGTAAATTGCGAGGGTACTATACGTAATTTAGTAGGAAAGTACGCACC
Bba12IB	..............T.............A.G......A....C....C..................A.G.A....
Bba47IB	....C.....................
Bba48IB	..............TT........C.....G......A..T.C..................GA..............
Bba145IB	..............T.............A.G......A....C.......................A.G.A....
Bba174IB	................................C.....................................G......
Bba277IB	..C............TT........C.....G......AG.C.C...G.............GA..............
Bba35MO	A.....TAA.......T....A..........G.G...TA.A.AC.T.......G.......G....G...TA....
Bba850MO	A.....TA........T....A..........G.G...TA.A.AC.T.......G..C....G........TA....
Bba165SW	A.C............TT........C.....G......A..C.C..................GA.....C.......
Bba198TK	A..............T........C....A.G......A....C......TA...A.....GA..G.A...A..G.
Bba199TK	A..............T........CC.T.A.G......A....C......TA..GA.....G....G....A..G.
Bba9CAG	AA.-GTAC.TGCGACGTTCTACCGGC.T.AA..G-GGTG.G.AACGTC..G.A.G...CGAC..AG.G.G.T.TGGT

0.759. The consensus tree (Fig. 2) is well structured and indicates a sharp distinction between the lineage from the Canary Islands and all haplotypes of barbastelle bats from the rest of the range (Iberia, Switzerland, Morocco and Turkey). This tree further differentiates Moroccan and Turkish haplotypes in two well-supported groups. Finally, it also supports three clades among the Iberian samples: one (*IB-1*) that is widespread across the Peninsula, one (*IB-2*) restricted to the northern parts, and

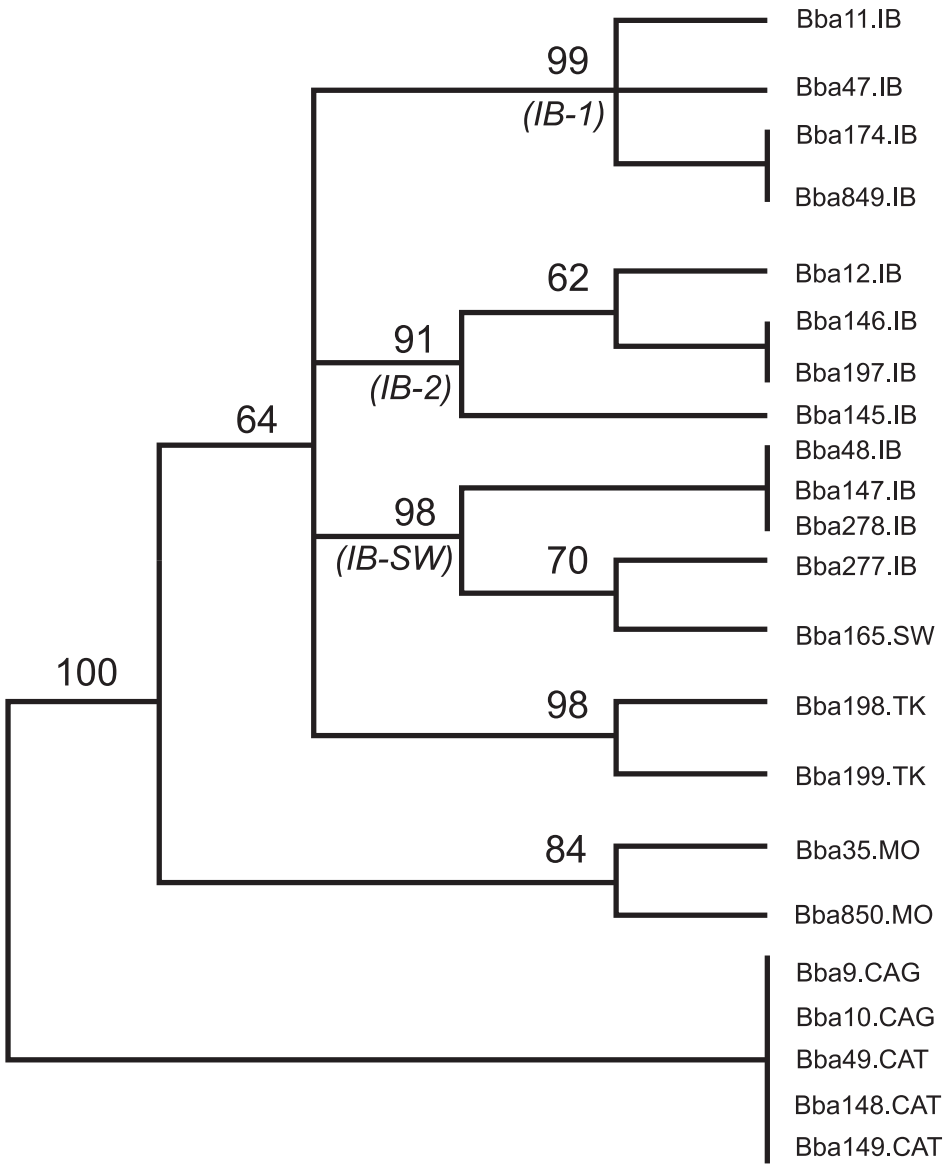

FIG. 2. Maximum Parsimony reconstruction (unweighed heuristic search, TBR branch swapping) of the phylogenetic relationships of western Palaearctic *Barbastella* bats based on the sequences of 14 different haplotypes (in 22 specimens) in a joint fragment of the cytochrome *b* gen (680 bp) and the control region (391 bp). Bootstrap values after 5000 replicates. Location codes: CAG = La Gomera (Canary Islands), CAT = Tenerife (Canary Islands), IB = Iberia, MO = Morocco, SW = Switzerland, TK = Turkey

a third (*IB-SW*) that extends beyond the Pyrenees at least to the Alps. Haplotype divergence for the cyt-*b* between continental samples ranged from 0.7% (between the *IB-SW* group and Morocco) to 2.1% between Morocco and Turkey (Table 3). The genetic structure of European barbastelles is illustrated by a median-joining network (Fig. 3) that shows a good concordance with the geographic origin of samples (Fig. 3). Two Iberian clades (*IB-1* and *IB-2*) cluster together and are link to the third Iberian clade by three median vectors (reconstructed haplotypes). The latter clade (*IB-SW*) also includes the Swiss haplotype.

Discussion

Genetic Divergence

Haplotype divergence found in the barbastelles sampled can be considered very shallow, compared to other bats (Ruedi and Castella, 2003) or to other mammals sampled over a similar range (reviewed in Taberlet *et al.,* 1998).

In fact, when considering only the cyt-*b* gene, the Alpine barbastelle has exactly the same 680 bp sequence (Table 1) as three Iberian samples from two Pyrenean localities (Belsué and Ordesa). This genetic similarity supports the idea that barbastelles from both regions share a common ancestry. In turn, the Turkish samples are placed furthest from other West Palaearctic haplotypes as they differ by at least 15 mutations from other continental sequences. Nevertheless, this connection requires the reconstruction of only one haplotype (Fig. 2). The overall pattern of haplotypic divergence of western barbastelles is therefore broadly concordant with geographic distances and suggests that western and eastern Mediterranean populations may be issued from divergent two subpopulations. Nevertheless, this point needs further research given the limited coverage of our sampling. At more local scale, the three Iberian groups show no clear geographic arrangement, as the same haplotypes are shared in northern and southern populations (Figs. 2 and 3).

Within any given geographic region, sequence variation in the cyt-*b* ranged from 0.3% between northern and central Morocco to 1.3% between the two Turkish samples. Among the 13 Iberian barbastelles, sequence divergence reached only 0.7% in the cyt-*b* and 2% in CR (Table 3). This shallow genetic structure in the Iberian Peninsula with shared haplotypes among relatively distant regions can indicate recent gene flow, or a recent common history of all barbastelle bats from Iberia. As current genetic patterns result from both historical and contemporary factors (see e.g., Avise, 2000; Ruedi and Castella, 2003), denser sampling is necessary to differentiate these alternatives in order to propose a more complete understanding of the population history of barbastelles in the Iberian Peninsula.

Table 3. Corrected genetic distances (HKY85 + Γ model — Hasegawa *et al*., 1985; see text for details) among the main groups of haplotypes of the western Palaearctic samples of *B. barbastellus* in the cyt-*b* (lower half-matrix) and CR (upper half-matrix) fragments

Haplotype Group	IB-1	IB-2	IB-SW	Morocco	Thrace	Canary Is.
IB-1	–	0.024	0.030	0.054	0.048	0.167
IB-2	0.007	–	0.029	0.046	0.032	0.172
IB-SW	0.011	0.008	–	0.049	0.038	0.170
Morocco	0.011	0.009	0.007	–	0.055	0.156
Thrace	0.019	0.018	0.020	0.021	–	0.168
Canary Is.	0.044	0.041	0.040	0.038	0.055	–

Effect of the Gibraltar Strait

Although no haplotypes are shared between Iberian and Moroccan lineages, they diverge only slightly from each other. The average sequence divergence between them is about 1% for the cyt-*b* and 5% for the CR fragments respectively (Table 3), which is again very low compared to other mammals across Europe (e.g., Taberlet *et al.,* 1998) or to other bats across the Strait (Castella *et al.*, 2000 or Juste *et al.,* In press). This small divergence (Table 3) suggests that the common ancestor to these lineages is relatively recent, possibly Holocene, if a common molecular clock is applied (Johns and Avise, 1998). With the available molecular data, it is, however, not possible to determine whether the ancestral lineage expanded from Europe southwards through the Gibraltar Strait to Morocco, or the reverse. The traditional view, based on fossil remains and the current geographic distribution of barbastelle bats (Rydell and Bogdanowicz, 1997) would favour the migration route through the Iberian Peninsula. A third alternative route to the colonization of Morocco could be via eastern North Africa. However, this is unlikely since *Barbastella* has never been reported from any recent locality or fossil deposit in the intervening regions of North Africa.

In any case, the relatively recent common ancestor of the lineages found on either sides of the Gibraltar Strait suggest that this strait did not act as an ancient barrier to gene flow in *Barbastella*, contrary to the situation found in other Plecotine bats (Juste *et al.*, In press) or in the large *Myotis* (Castella *et al.,* 2000). Respective to the Gibraltar Strait, Barbastelles thus behave rather like another patchily distributed

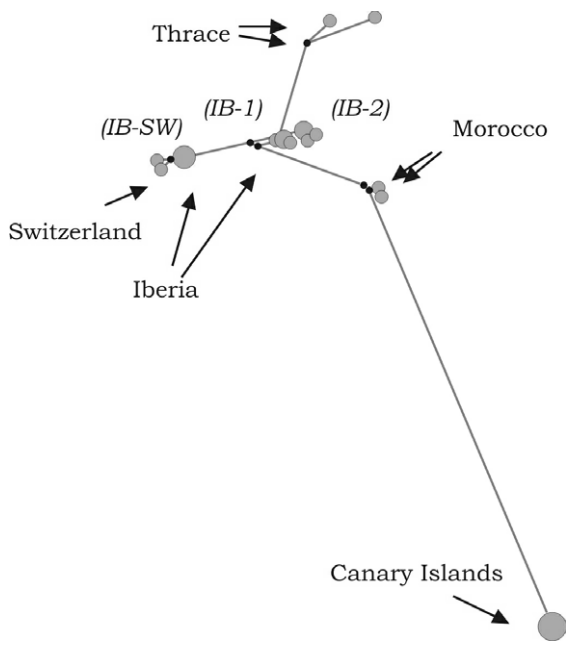

FIG. 3. Median-joining network based on 14 haplotypes found in a joint mitochondrial DNA fragment of 680 bp of the cytochrome *b* and 391 bp fragment of the control region. Black dots represent reconstructed missing haplotypes in the sampling. Size of the circles is proportional to the frequency of each particular haplotype and distance between haplotypes is proportional to the number of mutated positions

bat, *Myotis mystacinus*, which has also similar genotypes on either sides of the strait (Helversen *et al.,* 2001). More species of bats need to be analyzed genetically to identify the reasons why some species do cross the strait more often than others.

The Barbastelles of the Canary Islands

The cyt-*b* sequence divergence between the single haplotype from the Canary Islands and the mainland lineages is relatively high (averaging 3.9 and 4.2% to Moroccan and the Iberian haplotypes respectively; Table 3) and corresponds to the value reported by Pestano *et al.* (2003) for a Canarian population and one specimen from Iberia sequenced at the same gene. Pestano *et al.* (2003), however, found two slightly divergent cyt-*b* haplotypes among seven barbastelles from Tenerife Island, while all our Canarian samples (including three from Tenerife and three from La Gomera) were identical. Our Canarian samples proved to be identical even when they where sequenced for the more rapidly evolving CR (Table 1). Together, these molecular data suggest that the Canary Islands were colonized quite recently by a few, related individuals. The phylogenetic reconstructions did not solve the exact origins of the Canary Island lineages unambiguously (Fig. 2). The network required only two putative intermediate haplotypes to connect the existing sequence from the Canary Islands with those from Morocco (Fig. 3). Thus Morocco is a likely source to the founder population of the Canary Islands, but the exact origins might be difficult to identify either because the source population was actually unsampled, or have gone extinct. From a taxonomic perspective, the low level of cyt-*b* sequence divergence (1–2%) found among samples collected throughout the western Palaearctic region supports the monotypic status of *B. barbastellus* in Europe and in North Africa, as suggested by Rydell and Bogdanowicz (1997) and Horáček *et al.* (2000). On the other hand, the quite high level of cyt-*b* sequence divergence found between the insular and mainland samples (Table 3) supports the sub-specific status of the endemic Canary Islands barbastelles (*B. barbastellus guanchae*) as proposed by Trujillo *et al.* (2002) on the basis of morphological evidence. At present the only known populations from these islands inhabit the western and forested parts of Tenerife and La Gomera (Trujillo, 1991). The low genetic diversity in this endemic taxon needs further scrutiny to evaluate its possible relevance from a conservation perspective. The species is only known from very few localities, and in fact, it can be considered at present the rarest bat in the Canary Islands (Trujillo *et al.,* 2002).

In conclusion, results from two mtDNA fragments show that there is only a shallow genetic structure among populations of the Iberian Peninsula and Morocco, and apparently, even across Europe east to Thrace. Although this needs to be confirmed given the limitations resulting from our incomplete sampling. The Gibraltar Strait did apparently not prevent the passage of European barbastelles to Morocco (or vice versa). Finally, our molecular reconstructions confirm the recent taxonomic distinction at subspecies level of *B. barbastellus guanchae* from the Canary Islands (Trujillo *et al.,* 2002), but their exact origin is still unclear because it is distinct from any lineage found so far in Morocco or in Europe.

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