Secondary contact zones and hybridizations: the case of the lesser white-toothed shrew (Crocidura suaveolens group, Soricidae)

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Received 30 November 2007; accepted for publication 19 March 2008

In the present study, we analyzed 58 samples of the lesser white-toothed shrew group (Crocidura suaveolens) from eastern Europe and Turkey, where, according to previous publications, three different mitochondrial and nuclear lineages are present. We sequenced 799 bp of the nuclear BRCA1 gene and 400 bp of the mitochondrial cytochrome b gene to: (1) determine a potential contact zone between the lineages; (2) detect hybridizations and introgressions between them; and (3) comment on the level of reproductive isolation of the different lineages. We revealed two zones of hybridization in Turkey, of which the first occurred west of the Bosphorus Straits (three hybrids) and the second in Anatolia (twelve hybrids). In the latter, the nuclear markers revealed a large zone of hybridization, of approximately 600 km. It also revealed that hybrids of first, second, and later generations are present within the populations, and therefore that the reproductive isolation between the different lineages is weak. © 2008 The Linnean Society of London, Biological Journal of the Linnean Society, 2008, 95, 557–565.


INTRODUCTION

Numerous phylogeographic studies have shown the impact of Pleistocene climatic fluctuations throughout the world. In the northern hemisphere, mitochondrial DNA (mtDNA) markers reveal general patterns that involve southern refugia and northern recolonization routes (Hewitt, 2000, 2004a, b). Mountain chains and seas are known to have isolated populations in different glacial refugia, leading to the formation of divergent genetic lineages, which are often considered as different subspecies or species. Moreover, the Pleistocene climatic fluctuations have led to various postglacial recolonization patterns of those lineages (Taberlet et al., 1998; Hewitt, 1999). Western Eurasian refugia include the Iberian Peninsula, the Italo-Balkanic region, and eastern areas such as the Caucasus, western Asia, and possibly northern refugia such as the southern Urals and Carpathian Mountains (Santucci, Emerson & Hewitt, 1998; Taberlet et al., 1998; Nesbo et al., 1999; Palme & Vendramin, 2002; Seddon et al., 2002; Michaux et al., 2004; Culling et al., 2006; Dubey et al., 2006, 2007b, b; Saarma et al., 2007). In some cases, the subsequent postglacial expansion led to secondary contact zones and genetic introgressions between populations derived from separated glacial refugia; for example, in the bank vole Clethrionomys glareolus (Tegelström & Jaarola, 1998), in the hares Lepus timidus and L. europaeus (Thulin, Fang & Averianov, 2006), and in the ground squirrel Spermophilus (Ermakov et al., 2002).

Unfortunately, most phylogeographic studies are based on mtDNA and consider at most the phenotype,
whereas the inclusion of nuclear markers can considerably enhance our understanding of population history, as shown recently in several species of Palaeartic shrews of the genus *Crocidura* (Brändli et al., 2005; Bannikova et al., 2006; Dubey et al., 2006, 2007b). Using these techniques, secondary contact zones and mitochondrial introgressions can be more easily detected. In addition, this is the only way to reveal hybridization between different lineages and thus to determine the level of reproductive isolation between taxa.

Within the lesser white-toothed shrew group (*Crocidura suaveolens* Pallas, 1811) numerous subspecies and species have been described based on morphological characteristics (Corbet, 1978; Hutterer, 2005). However, in studies based on mitochondrial and/or nuclear phylogenetics, Dubey et al. (2006, 2007a) have highlighted only ten well differentiated mitochondrial lineages, originating from various Pleistocene refugia. Nuclear and mitochondrial datasets were congruent for the seven different clades identified in Dubey et al. (2006). Moreover, two pairs of morphologically recognized species, namely (1) *Crocidura sibirica* Dukelski, 1930 from Siberia and the European *C. suaveolens* from Crimea (type locality) and (2) *Crocidura monacha* Thomas, 1906 and *Crocidura gueldenstaedtii* Pallas, 1811, both from the Near East, were revealed to be genetically identical and should therefore be considered as synonyms from a genetic point of view (Bradley & Baker, 2001; Baker & Bradley, 2006).

Nevertheless, the specific status of these lineages remains uncertain, and the mean genetic distance between them (Kimura two-parameters distance = 4.7% and 10.8%, respectively) may be situated within a single species, or represent an incipient species or a well separated full species (Bradley & Baker, 2001).

Based on a large sample, the geographic distribution of lineages was well defined, allowing approximate delineation of the potential contact zones. Nevertheless, no sympathy was detected. However, in a study based on nuclear and mitochondrial markers and focused on the Caucasus region, Bannikova et al. (2006) found one hybrid (mitochondrial introgression) between the lineages *gueldenstaedtii* and *suaveolens*. Unfortunately, very few samples were analyzed in the potential contact zones, making it difficult to comment on the level of isolation of the different lineages. Nonetheless, the authors drew taxonomic conclusions, proposing levels of classification such as ‘superspecies’, ‘super-subspecies’, and ‘semispecies’.

In the present study, we analyzed samples of the *C. suaveolens* group from western Turkey, where three different mitochondrial and nuclear lineages are present (Dubey et al., 2006, 2007a). The aims of the study were: (1) to determine a potential contact zone between the lineages; (2) to detect hybridizations and introgressions between them; and (3) to comment on the level of reproductive isolation of the different lineages.

**MATERIAL AND METHODS**

**SAMPLING**

We analyzed 58 samples of the *Crocidura suaveolens* group collected in eastern Europe and western Turkey (Fig. 1, Table 1). We used as an outgroup *Crocidura brunnea* and *Crocidura nigripes* (*cyt-b*: DQ630385 and DQ630384, respectively) from Indonesia, two species that are included within the sister clade of the *C. suaveolens* group (Dubey et al., 2007c). This set of samples included material from the collections of the University of Lausanne, Lausanne, Switzerland (IZEA) and from the collection of the Trakya University, Edirne, Turkey. Some additional sequences were obtained from Dubey et al. (2006; Table 1).

**DNA EXTRACTION AND AMPLIFICATION**

Liver samples from the IZEA collection were frozen in liquid nitrogen in the field and kept for several years at −70 °C before being stored in ethanol for DNA extraction. Samples from the other collections were stored directly in ethanol. The DNA extraction was carried out using the QIAamp DNA Mini Kit (Qiagen). Double-stranded DNA amplifications of the cytochrome *b* gene (*cyt-b*) were performed using the primer pair L14724/H15149 (Irwin, Kocher & Wilson, 1991). Amplifications of the breast cancer susceptibility 1 gene (*BRCA1*) were performed using the primer pair BRCA1f/BRCA1r18 (Dubey et al., 2006). Amplification conditions for *BRCA1* and *cyt-b* consisted of 40 thermal cycles of denaturation at 94 °C for 60 s (30 s for *cyt-b*), annealing at 50 °C for 60 s (45 s for *cyt-b*), and extension at 72 °C for 120 s (60 s for *cyt-b*). The polymerase chain reaction (PCR) products were checked on a 1% agarose gel and then purified using the QIAquick PCR Purification Kit (Qiagen) following the manufacturer’s instructions. DNA sequencing was performed in a total volume of 10 μl containing 1–3 μl of amplified PCR product, 1 μl of 10 μM primer, and 4 μl of ABI PRISM Dye Terminator 1 (Perkin-Elmer). Sequence reactions were visualized on an ABI 3100 genetic analyzer (Applied Biosystems).

**PHYLOGENETIC METHODS**

Nucleotide sequences of *cyt-b* and *BRCA1* genes were edited using Sequence Navigator (Parker, 1997) and
manually aligned. Two methods of phylogenetic analysis were performed for cyt-b, using PAUP*version 4.0b10 PPC (Swofford, 1998). A Neighbour-joining (NJ) tree was constructed using Kimura two-parameter genetic distances (Kimura, 1980). Parsimony analyses (MP) were performed using the options: heuristic search, stepwise addition of sequences, ten replicates of random additions of taxa, and tree-bisection-reconnection branch swapping (Swofford, 1998). Tests were conducted on the complete fragment, all codon positions were used, and trees were rooted using sequences from *C. brunnea* and *C. nigripes*. Fast maximum likelihood (ML) heuristic searches and bootstrap analyses (1000 replicates) were performed using PHYML (Guindon & Gascuel, 2003) with a general time reversible model, which had been selected previously using MODELTEST 3.06 according to the protocol of Posada & Crandall (1998). Bootstrap support values were obtained with 1000 pseudo-replicates.

**RESULTS**

**MITOCHONDRIAL DATA**

In 47 sequences of 400 bp from samples of the *C. suaveolens* group, thirteen haplotypes were found, and were named H1 to H13 (Fig. 2, Table 1). They corresponded to the three different lineages V (H1–H2), VI (H7–H13), and VII (H3–H6) found by Dubey *et al.* (2006, 2007a). The sequences are deposited under the Genbank accession numbers EU271921–EU271933. Lineage V was supported by bootstrap values of 98% for ML, 99% for MP, and 100% for NJ; lineage VI by values of 100% for ML, 97% for MP, and 99% for NJ; lineage VII by values of 81% for ML, 66% for MP, and 90% for NJ.

In the present study, lineage V was found to be distributed from western Turkey (east of the Bosphorus) to Georgia; lineage VI was found in western Turkey (east of the Bosphorus) and on Lesvos Island (Greece); and lineage VII was distributed from...
Table 1. Locality, Location on map, code of the *Crocidura suaveolens* group samples, mitochondrial lineage, Genbank accession of cytochrome b (*cyt-b*) sequences of Dubey *et al.* (2006) and *cyt-b* haplotype of samples of the present study, nuclear lineage(s), and respective nuclear allele(s).

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<th>Mitochondrial lineage</th>
<th>Nuclear lineage(s) and allele(s)</th>
<th>Cyt-b Genbank accession and haplotype</th>
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*See Results section.
Switzerland to western Turkey (west of the Bosphorus). All populations showed only haplotypes of one lineage, except in western Turkey, east of the Bosphorus, where haplotypes of lineages V and VI were found within the same locality (Hamzabey and Soğukpınar; Fig. 1, localities 17 and 18, respectively).

Sequences of cyt-b obtained by Dubey et al. (2006) (Table 1) were not included in the phylogeny shown in Figure 2 because the sequences were not entirely overlapping. Nevertheless, they were used to determine the different existing lineages in the present study (results not shown).

**Nuclear data**

From 799 bp sequences of the BRCA1 gene, we found 11 different alleles within the studied areas, which were named A4 to A18. Six alleles corresponded to alleles previously found in Dubey et al. (2006): A4 to A6 of lineage VII, A7 and A8 of lineage VI, and A13 of lineage V. For convenience, the same nomination was used in the present study, and the five newly identified alleles are named A14 to A18. The number of mutations varied from one to six between the different alleles (Table 2); three characteristic mutations separated the alleles of lineage V from those of lineages VI and VII. Alleles A13 to A15 were found in Turkey and Georgia and were associated with mitochondrial lineage V. Alleles A7, A8, A16, and A17 were found in western Turkey and were associated with mitochondrial lineage VI, and alleles A4 to A6 were found in Europe and were associated with mitochondrial lineage VII. Allele A18 exhibited the characteristic mutations of lineages VI and VII. However, because it was found in only one sample, from the west of the Bosphorus, in association with allele A8 and a haplotype of lineage VII, it was not possible to classify this allele within lineage VI or VII. The eleven alleles are named as follows in Tables 1 and 2 and Figure 1: V-A13, V-A14, V-A15, VI-A7, VI-A8, VI-A17, VI-A16, VII-A4, VII-A5, VII-A6, and VI-VII-A18. The sequences are deposited under Genbank accession numbers EU271910–EU271920.

In Turkey, six different populations showed alleles of two different nuclear lineages, as detailed below.

**Population 17 (Fig. 1; Hamzabey):** one homozygote VI with the haplotype lineage V, and one heterozygote V/VI with the haplotype lineage VI were found (Fig. 1, Table 1).

**Population 18 (Soğukpınar):** one homozygote VI with the haplotype lineage VI, one homozygote V with the haplotype lineage V, two heterozygotes V/VI with the haplotype lineage VI, and one heterozygote with the haplotype lineage V were found.

**Population 25 (Özbek):** nine samples of homozygotes VI with the haplotype lineage VI, and two heterozygotes V/VI with the haplotype lineage VI were found.

**Population 15 (Paşamandıra):** four homozygotes VI with the haplotype lineage VI, one homozygote V with the haplotype lineage VI, and one heterozygote VVI with the haplotype lineage VI were found.

**Population 29 (Balkusan):** three homozygotes VI, one homozygote V, and one heterozygote V/VI with the haplotype lineage V were found.
Population 11 (Edirne): two homozygotes VI with the haplotype lineage VII and one homozygote VI with the haplotype lineage VII were found.

**DISCUSSION**

According to Dubey et al. (2006, 2007a), three different mitochondrial and nuclear lineages, designated V to VII, of the lesser white-toothed shrew are present in eastern Europe and Turkey. The mitochondrial Kimura two-parameter genetic distance between the lineages is 5.8% for V/VI, 4.7% for VI/VII, and 5.1% for V/VII. These lineages are known to have a parapatric distribution: lineage V is distributed strictly in central and eastern Turkey, lineage VI in western Anatolia, and lineage VII in central and eastern Europe.

Based on thorough sampling in this area, the present study has highlighted more complex relationships between the mitochondrial and nuclear data than those reported previously by Dubey et al. (2006, 2007a). Unexpectedly, we revealed hybridizations between lineages VI and VII in western Turkey, west of the Bosphorus, where three samples from different populations possessed haplotypes of lineage VII and nuclear alleles of lineage VI (Fig. 1). By contrast, lineage VI was only found east of the Bosphorus strait. From a biogeographic point of view, this means that lineage VI, during its postglacial expansion from a refugium situated in Anatolia (Dubey et al., 2006), crossed the Bosphorus strait and colonized eastern Europe. This type of colonization from Turkey to Europe is poorly documented by current phylogeographic studies; it has only been described previously for the bicolored shrew Crocidura leucodon (Dubey et al., 2007b). However, the permeability of the Bosphorus for various species has already been demonstrated for the reverse direction in classical zoogeography (Hosey, 1982).

Similarly, hybridizations between lineages V and VI were detected in western and southern central Turkey, with 12 hybrids possessing mitochondrial haplotypes and nuclear alleles of one of the respective lineages, and some being heterozygotes or homozygotes (i.e. hybrids of first, second, and/or later generations). The nuclear data revealed a large zone of hybridization; the most distant populations sharing the two lineages were separated by approximately 600 km (Fig. 1).

Interestingly, the mitochondrial data revealed a much smaller introgression zone: the only two populations sharing the two different haplotype lineages were separated by less than 50 km. Consequently, the mitochondrial introgression is very limited compared with the nuclear one, despite the fact that mtDNA is not linked directly to genes that are involved in reproductive isolation, and may penetrate reproductive barriers more easily than nuclear DNA (Barton & Jones, 1983; Takahata & Slatkin, 1984; Tegelström & Jaarola, 1998).

The present study failed to demonstrate clear reproductive isolation between three different lineages of the C. suaveolens group, despite cyt-b distances of 4.7% to 5.8% between them, and an origin dating from the Lower Pleistocene (Dubey et al., 2006, 2007a). It is probable that the absence of karyotypic rearrangement between the different lineages of the C. suaveolens group supported these types of introgression between relatively distant sister taxa.

In a recent study employing nuclear and mitochondrial markers, Bannikova et al. (2006) translated the different lineages of C. suaveolens s.l. into taxonomic units and attributed levels (superspecies, supersubspecies, semispecies) in accordance with the branching pattern of the resulting phylogeny. By contrast, Dubey et al. (2006, 2007a) preferred to avoid any splitting as long as the degree of genetic isolation at
the contact zones is unknown. In the present study, the results obtained provide evidence of conspecificity between lineages V to VII. It is probable that lineages IX (Crocidura suaveolens aleskiandrosi from Lybia) and X (Crocidura suaveolens cypria from Cyprus Island; Dubey et al., 2007a), which show a similar level of differentiation, should be considered as conspecific, although they are geographically isolated and no contact zones have been identified.

The study of contact zones between deeper branches of the C. suaveolens group, i.e. between lineages II (Crocidura suaveolens suaveolens) and III (Crocidura suaveolens caspica), or of both with lineage V (Crocidura suaveolens gueldenstaedtii), or between lineages IV (Crocidura suaveolens iculisma) and VIII (Crocidura suaveolens mimula), will be a challenge. Nevertheless, no thorough sampling in potential contact zones is currently possible. In conclusion, we have revealed that the clear large-scale biogeographic pattern, shown in Dubey et al. (2006, 2007a), with parapatric distributions of different genetic lineages of lesser white-toothed shrews, is too simplistic. Therefore, sampling in potential contact zones coupled with the analyses of mitochondrial and nuclear markers is the only way to reveal a clear phylogeographic pattern, as well as the level of reproductive isolation between closely-related taxa. Consequently, our results support the argument that both nuclear and mitochondrial markers should be included in phylogenetic studies because the full story can be more complex than the analysis of either category of marker alone might indicate.

ACKNOWLEDGEMENTS

We thank Nelly Di Marco for laboratory facilities and Darron Cullen for linguistic advice. This work was supported by the Herbet Foundation, University of Lausanne.

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