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Short communication

Mitochondrial sequences retrieve an ancient lineage of Bicolored shrew in the Hyrcanian refugium

Ahmad Mahmoudi[a,b,c,*], Jamshid Darvish[a,d], Roohollah Siahsarvie[a,d], Sylvain Dubey[e,f], Boris Kryštufek[g]

[a] Department of Biology, Faculty of Sciences, Ferdowsi University of Mashhad, Mashhad, Iran
[b] Department of Epidemiology and Biostatistics, Research Centre for Emerging and Reemerging Infectious Diseases, Pasteur Institute of Iran, Tehran, Iran
[c] National Reference Laboratory for Plague, Tularemia and Q fever, Research Centre for Emerging and Reemerging Infectious Diseases, Pasteur Institute of Iran, Akanlu, Kabudar Ahang, Hamadan, Iran
[d] Rodentology Research Department, Institute of Applied Zoology, Faculty of Sciences, Ferdowsi University of Mashhad, Mashhad, Iran
[e] Department of Ecology and Evolution, Biophore Building, University of Lausanne, 1015 Lausanne, Switzerland
[f] Hintermann & Weber SA, Rue de l'Eglise-Catholique 9b, 1820 Montreux, Switzerland
[g] Slovenian Museum of Natural History, Prešernova 20, SI-1000 Ljubljana, Slovenia

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ABSTRACT

The bicolored shrew *Crocidura leucodon* consists of two phylogeographic lineages which diverged during the Middle Pleistocene and are roughly separated by the Bosporus Strait at the contact point of Europe and Asia Minor. In this study we analysed mitochondrial cytochrome *b* genes (1137 bp) of nine shrews from the Caspian region in Iran. Phylogenetic trees obtained in Maximum Likelihood and Bayesian analyses retrieved a sister position of our Iranian haplotypes (Iranian lineage) against all the remaining *C. leucodon* samples from Europe, Turkey and Georgia (Euro-Asian lineage). Identical topology was also evident from the unrooted phylogenetic network. The Euro-Asian and the Iranian lineages were separated by a K2P genetic distance of 7.5 ± 0.9 and diverged 1.14 Mya (95% CI: 0.841–1.616). The geographic range of the Iranian lineage is evidently restricted to the Hyrcanian region south of the Caspian Sea which abounds with small range mammalian endemics.

Within its geographic range, extending from the Atlantic coast in the west as far east as the Caspian Sea (Shenbrot et al., 2016), the Bicolored shrew displays a unique phylogeographic pattern with two allopatric lineages. These lineages are roughly, but not strictly separated by the Bosporus strait and presumably diverged during the Middle Pleistocene (Dubey et al., 2007a). Our recent phylogeographic work on shrews from Iran yielded a new phylogeographic lineage in the Hyrcanian refugium south of the Caspian Sea, i.e. in an area which had remained unsampled in Dubey et al. (2007a). Because the new lineage is basal in *C. leucodon*, we believe its discovery is of significance regarding the understanding of the evolutionary history of the species.

Nine Bicolored shrews were captured from three localities in Iran (Table 1) and a further 60 sequences belonging to *C. leucodon* were downloaded from GenBank (Dubey et al., 2007a) to construct phylogenetic relationships among the haplotypes. DNA extraction from tissue samples preserved in 96% ethanol was completed using QIAamp DNA Mini Kit. Partial mitochondrial cytochrome *b* (cyt*b*) sequences (1136bp) were amplified by polymerase chain reaction (PCR) with the following primers; L14727-H15915 (Jaarola and Searle, 2002). Amplification conditions consisted of an initial 7 min denaturation step at 95 C, 30–35 cycles of denaturation at 94 C for 60 s, annealing at 49 or 50 C for 60 s and extension at 72 C for 1–2 min, and a final 10-min extension step at 72 C. PCR products were purified using QIA quick PCR purification Kit (QIAgen), following manufacturer instructions and commercially sequenced using dye-labelled dideoxy terminator cycle sequencing with Big Dye V.3.1 (Applied Biosystems, Inc, South Korea).

Nucleotide sequences of cyt*b* gene were edited using CodonCode Alignment software (CodonCode Corp.) and aligned with the multiple sequence alignment (Clustal O) via Web Services (McWilliam et al., 2013). Kimura-2 parameter-model (K2P) was used to evaluate between and within species genetic divergence in Mega v7 (Kumar et al., 2016). Two sets of phylogenetic analyses were performed to evaluate phylogenetic relationships among

* Corresponding author at: Department of Epidemiology and Biostatistics, Research Centre for Emerging and Reemerging Infectious Diseases, Pasteur Institute of Iran, Tehran, Iran.
E-mail address: a.mahmoudi.bio@gmail.com (A. Mahmoudi).

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Table 1
Sample location, geographic coordinates, haplotype acronyms and GenBank accession numbers of cyt*b* sequences for *Crocidura leucodon* collected from Iran.

Location	Sample location	Longitude, latitude	Haplotype name	Accession No.
1	Zanjan-Soltanieh	36°22′N / 48°43′E	ZS1	MH602279
2	Semnan-Jashlobar	35°52′N / 53°06′E	SJ1	MH602278
3	Bojnord-Darkesh	37°26′N/ 56°43′E	BD1	MH602276
			BD2	MH602277
			BD3	MH602272
			BD4	MH602274
			BD5	MH602275
			BD6	MH602271
			BD7	MH602273

the sequences. Maximum Likelihood (ML) analysis was performed using PhyML 3.0 (Guindon et al., 2010) including the model parameters previously obtained with jModeltest (Posada, 2008). Node supports for ML analysis were estimated using 500 non-parametric bootstrap replicates. Bayesian analysis was performed in MrBayes v3.2 (Ronquist et al., 2012). Two simultaneous analyses were run with four Markov Chain Monte Carlo (MCMC) models, which were started from random trees and run for 4,000,000 iterations. The trees were sampled every 100th generation and the first 5000 trees were discarded as the burn-in stage. Branch support in the BI tree was assessed by Bayesian posterior probabilities (BPP). Trees were rooted with two species of red-toothed shrews (subfamily Soricinae), *Sorex araneus* (EU122225, Meegaskumbura et al., 2007) and *S. minutus* (DQ065610; Dubey et al., 2007b). The topologies resulting from these two methods were compared using a Shimodaira and Hasegawa (1999) test implemented in PAUP* 4.010b (Swofford, 2003) with 1000 bootstrap replicates. To further examine the phylogenetic relationships among our cyt*b* haplotypes and those from the literature (Dubey et al., 2007a), we constructed a Neighbor-Net phylogenetic network (Bryant and Moulton, 2004) from the same dataset used to produce the ML/BI trees (above). The default parameters of uncorrected P distance and the equal-angle algorithm were implemented in the SplitsTree 4.10 software (http://www.splitstree.org).

We used two calibration points to assess the most recent common ancestor (TMRCA) of the lineages retrieved in our analysis: (i) 1.94 Myr BP [1.58–2.52] for separation between *C. shantungensis* (clade I sensu Dubey et al., 2006) and all the remaining *suaveolens* lineages, and (ii) 0.691 Myr BP [0.510–0.980] for separation between the Western and the Eastern sub-lineages of *C. leucodon* (Dubey et al., 2007a). An uncorrelated lognormal relaxed-clock (Drummond et al., 2006), under assumption of the GTR+G+I substitution model, was used to estimate the timing of divergences with 95% highest posterior density (HPD). Two independent chains were run for 50 million generations with sampling every 5000 generations. The first 10% of generations were discarded as burn-in based on evaluated values using Tracer v1.6 (Rambaut et al., 2013). Two independent runs were combined in LogCombiner 1.8.2 (Rambaut and Drummond, 2015a) after removing the appropriate burn-ins. The maximum clade credibility tree obtained with TreeAnnotator 1.8.2 (Rambaut and Drummond, 2015b) was visualized using FigTree 1.4.2 (Rambaut, 2014).

Our sequencing yielded nine new cyt*b* haplotypes of *C. leucodon* generating a total dataset of 50 unique cyt*b* haplotypes of Bicolored shrews from throughout their range. Of the 1137 bp long sequences considered here, 109 sites were variable and 74 sites were parsimony informative. No stop-codons, insertions or deletions were observed in the alignment.

Phylogenetic relationships among cyt*b* sequences were reconstructed by two different probabilistic methods (BI and ML) which showed a very similar tree topology. The Shimodaira–Hasegawa test did not reveal significant differences between these trees ($P = 0.069$); consequently, only the BI tree is shown (Fig. 1a). Haplotypes of the *C. leucodon* clustered in two strongly supported (BPP = 1.00, BP = 100%) lineages. The widespread Euro-Asian lineage encompassed all the published haplotypes from Europe, Asia Minor and Georgia (hereafter Euro-Asian lineage), and the Iranian lineage contained all our new haplotypes from Iran. In line with published results (Dubey et al., 2007a), the Euro-Asian lineage consisted of two supported sublineages (BPP = 1.00, BP = 99), the Western, and the Eastern. Within the Iranian lineage, the only haplotype from the westernmost sampling site in Zanjan (ZS1) held a supported (BPP = 1.00, BP = 100%) sister position against the remaining samples collected further east. The three *C. leucodon* cyt*b* lineages were also evident in the Neighbor-Net network (Fig. 1b), which likewise shows a close relationship to the West and East sublineages, and a distinct position for the Iranian lineage.

The corrected K2P genetic distance between the Euro-Asian and the Iranian lineages of Bicolored shrews (7.5 ± 0.9) was within the range of pairwise divergences among the lineages of the *suaveolens* group (3.9–11.5%; Dubey et al., 2007c). Heterogeneity within the *leucodon* sublineages was the lowest for the Western sublineage (0.5), intermediate for the Eastern sublineage (0.6), and the highest for the Iranian lineage (1.2). Again, these values were inside the range of heterogeneities within the lineages of the *suaveolens* group (0.4–1.5; Dubey et al., 2007c).

Application of the calibration points developed by Dubey et al. (2006, 2007a) estimated the separation between the outgroups (subfamily Soricinae) and ingroups (subfamily Crocidurinae) at 20 million years ago (Mya). In line with this metric, the divergence between the Euro-Asian and the Iranian lineages was 1.14 Mya (95% CI: 0.841–1.616 Mya), therefore dating back to the Early Pleistocene.

An earlier phylogeographic study of the Bicolored shrew retrieved the major evolutionary divergence on the contact point between Europe and Asia Minor (Dubey et al., 2007a). Our phylogenetic reconstruction, however, yielded the most divergent lineage at the eastern periphery of the species' range. This finding substantially alters our understanding of the evolutionary history of the Bicolored shrew and suggests a split in the Caspian region as the most plausible initial step in evolution of *C. leucodon*. This also foresees the Asiatic origin of the ancestor to the Euro-Asian and the Iranian lineages of Bicolored shrew. Such a scenario does not violate paleontological evidence. The earliest fossils of *C. leucodon* are from the Middle Pleistocene in Europe (Rzebik-Kowalska, 1998) and therefore postdate the estimated basal dichotomy, which was estimated in our study to occur in the Early Pleistocene.

K2P genetic distance between the Euro-Asian and the Iranian lineage (7.6) is above the upper cut-off value ($\leq$6.5) for intraspecific heterogeneity tentatively outlined by Baker and Bradley (2006) and therefore most likely points to a cryptic species. The taxonomic name for the Iranian lineage is already available (*Crocidura leucodon persica*; Thomas, 1907). Gureev (1979) claimed for *persica* a status of full species. If subsequent analyses of nuclear markers and craniodental morphology will confirm our results, then the Iranian lineage of Bicoloured shrew will classify as yet another species

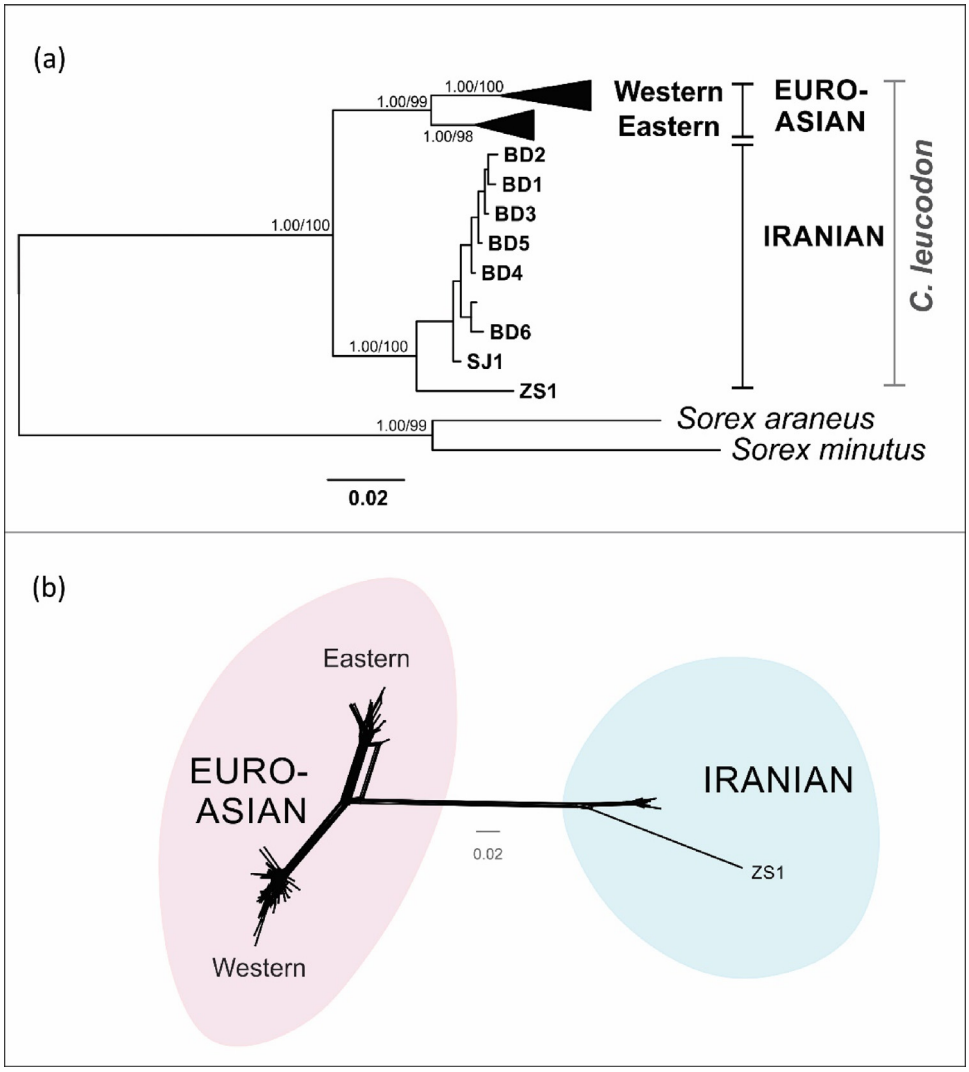

Fig. 1. (**a**) Mitochondrial (cytb) tree inferred for *Crocidura leucodon* with Bayesian Posterior Probabilities and Bootstrap values, respectively. The triangles represent the lineages based on published haplotypes (Dubey et al., 2007a). (**b**) Neighbor-Net network of the cyt*b* haplotypes for *Crocidura leucodon*. Euro-Asian lineage is further subdivided into two sub-lineages (Western and Eastern). Note the divergent position of the Zanjan haplotype (ZS1) in the Iranian lineage.

endemic to the Hyrcanian region (Dubey et al., 2007c; Naderi et al., 2013; Bannikova et al., 2015; Darvish et al., 2015; Mahmoudi et al., 2017).

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