

# Phylogeny and systematics of the *Crocidura suaveolens* species group: corroboration and controversy between nuclear and mitochondrial DNA markers

Phylogénie et systématique du groupe d'espèces *Crocidura suaveolens*: coordination et contradiction des marqueurs nucléaire et mitochondriaux de l'ADN

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## Abstract

Despite obvious advances in systematic research on Palaearctic white-toothed shrews (*Crocidura*), phylogenetic relationships and species diagnosis of 40-chromosome species (*suaveolens* sp. group) remain poorly understood. Phylogenetic relationships of these shrews were analyzed on the basis of two independent molecular markers: interspersed repeat PCR fingerprints (inter-SINE-PCR) and complete (1140 bp) or partial (~400 bp) sequences of the mtDNA *cyt b* gene. According to these data, *C. suaveolens* from Western Europe (Italy) appeared distinct from samples of *C. suaveolens* from Eastern Europe and Mongolia, as well as a Siberian sample. mtDNA introgression of Eastern European *C. suaveolens* with *C. gueldenstaedtii* in their contact zone in the Tuapse region was revealed. Hybridization between *C. gueldenstaedtii* and *C. suaveolens* resulted in the formation of a population, nuclear DNA and morphological characteristics typical for *C. gueldenstaedtii*, while the mitochondrial genome is assimilated from *C. suaveolens*. The population of the Talysh region of the Caucasus (*C. caspica*) represents a separate entity that is clearly distinguished from the populations of Georgia and Tuapse (*C. gueldenstaedtii*) and *C. suaveolens*. Therefore, the position of *C. caspica* as a full species is supported. The present analysis of both inter-SINE-PCR and *cyt b* sequence data revealed two major clades in Palaearctic 40-chromosome *Crocidura*. The eastern clade is formed by true *C. suaveolens*/*C. sibirica*, together with *C. caspica*, and the western clade is formed by Western European *C. suaveolens*, which should be treated as a distinct species, *C. mimula* and the closely related *C. gueldenstaedtii*.

**Keywords:** *Crocidura suaveolens*; cytochrome *b*; phylogeny; SINEs; systematics.

## Résumé

Bien que la systématique des musaraignes paléoarctiques du genre *Crocidura* paraisse aujourd'hui mieux maîtrisée, le diagnostic et les relations phylogénétiques du complexe d'espèces à 40-chromosomes (groupe *C. suaveolens*) demeurent peu connus. Dans le présent article, les relations phylogénétiques entre les espèces de ce groupe sont analysées à partir de deux marqueurs moléculaires indépendants: (inter-SINE-RPC) et la succession complète (1140 pb) ou partielle (~400 pb) du gène mitochondriale *cyt b*. Conformément aux données de inter-SINE-RPC et à l'analyse de la séquence de *cyt b*, les *C. suaveolens* d'Italie diffèrent de ceux de l'Europe de l'Est, de la Mongolie et de la Sibirie. On a découvert une introgression mtADN entre *C. suaveolens* d'Europe de l'Est et *C. gueldenstaedtii* dans la zone de contact aux alentours de Tuapse. L'hybridation *C. suaveolens* et *C. gueldenstaedtii* dans cette région semble avoir abouti à la formation d'une population dans laquelle l'ADN nucléaire et les signes morphologiques sont typiques de *C. gueldenstaedtii*, tandis que le génome mitochondrial correspond à *C. suaveolens*. La population de *C. caspica* de la région de Talyshe (Caucase) représente une unité particulière, qui se distingue bien de celles de *C. gueldenstaedtii* de Géorgie et de Tuapse et des populations de *C. suaveolens*. La position de *C. caspica* comme une espèce indépendante et soeur de *C. suaveolens* se trouve donc confirmée. En général, l'analyse présentée inter-SINE-RPC ainsi que l'analyse du séquence de *cyt b* révèle deux clades paléoarctiques majeurs des *Crocidura* à 40 chromosomes: une branche orientale formée par l'espèce type *C. suaveolens*/*C. sibirica* et *C. caspica* et une branche occidentale formée par l'espèce «*C. suaveolens*» d'Europe Occidentale. Cette dernière devrait être traitée comme une espèce particulière (*C. mimula*) en même temps que *C. gueldenstaedtii* dont elle est la plus proche.

**Mots clés:** *Crocidura suaveolens*; cytochrome *b*; phylogénie; SINEs; systématique.

## Introduction

Nearly 15 years have passed since the last taxonomic revision of the major part of Palaearctic white-toothed

shrews (*Crocidura*) by Zaitsev (1991) on the basis of comprehensive morphological and karyological data. Despite obvious advances in systematic research on white-toothed shrews, many aspects of the systematics of the Palaearctic 40-chromosome species group (= *suaveolens* species group) from species diagnosis to phylogenetic relationships within and among lineages remain poorly understood.

Based on morphological criteria, Zaitsev (1991) demonstrated that *C. suaveolens* Pallas 1811, *C. sibirica* Dukelsky 1930, *C. gueldenstaedtii* Pallas 1811 and *C. caspica* Thomas 1907 were distinct and should be treated as independent species. Jiang and Hoffmann (2001) found that small *Crocidura* from East Asia were different from *C. suaveolens* from Middle and Central Asia, and assigned them to *C. shantungensis* Miller 1901. Moreover, Hoffmann (1996) previously revealed some differences between shrews from Asia Minor, Afghanistan and most of Iran on the one hand, and those from Kazakhstan, Central Asia and western China on the other hand, suggesting that regard the latter be regarded as the distinct species *C. gmelini* Pallas 1811. The author believed that this species is distributed extensively in Kazakhstan, Middle and Central Asia, being sympatric with *C. suaveolens* in northeast Iran, where two forms are known to co-occur: a larger and darker form that inhabits mountain forests, and a smaller and paler steppe-dweller described as *C. hyrcania* Goodwin 1940. Hoffmann (1996) assigned the former to *C. gueldenstaedtii*, treating it as just a subspecies of *C. suaveolens* based on the opinion of Hutterer (1993) and the results of Catzeflis et al. (1985). The latter northeast Iranian *Crocidura* was considered conspecific with *C. gmelini*, along with other putative forms, including *C. lignicolor* Miller 1900 from Xinjiang, *C. ilensis* Miller 1901 from East Kazakhstan, *C. lar* Allen 1928 from Mongolia, and *C. mordeni* Goodwin 1934 from Central Kazakhstan. In a subsequent study (Jiang and Hoffmann 2001), an extended data set was analyzed that provided evidence of sympatry of *C. gmelini* and *C. suaveolens* in northeast Pakistan and northwest Afghanistan.

Thus, the size of the *C. suaveolens* taxon, inhabiting a huge range from the Atlantic coast of Europe to Japan (Hutterer 1993), is still debatable. Many authors suggested that all populations with  $2n=40$  within this range may be lumped into a single species, *C. suaveolens* (Catzeflis et al. 1985; Vogel et al. 1986; Hutterer 1993). However, some morphological and recent molecular data indicate a surprisingly high cryptic diversity in this group (Zaitsev 1991; Tembotova 1999; Bannikova et al. 2001; Jiang and Hoffmann 2001; Ohdachi et al. 2004; Vogel et al. 2003), emphasizing the need for a comprehensive taxonomic revision using additional data. It is clear that general consensus on the phylogenetic relationships and taxonomy of *Crocidura* may be obtained only on the basis of a molecular study of genetic variability. The issue is complicated by the huge range of *C. suaveolens*, which requires a representative sample to cover all the geographic variability of the species. Limited sampling may lead to new errors and biases. Recently, a few molecular studies produced results that led to more questions than answers.

Based on mitochondrial cytochrome *b* (*cyt b*) gene sequences, Ohdachi et al. (2004) showed that *C. shan-*

*tungensis* is distinct from *C. suaveolens* in Europe and Central Asia, while shrews from Central Europe (Austria) were significantly different from a rather compact cluster representing Mongolia and Xinjiang. However, according to their data, *C. sibirica* was undistinguishable from all other samples from Central Asia, which questions the validity of this taxon. The authors believed that at least a fraction of *C. sibirica* might be a synonym of *C. gmelini*, which occurs in Central Asia, and that true *C. suaveolens* is distributed only in Europe. These conclusions seem to be in agreement with the *cyt b* gene findings of Vogel et al. (2003), whereby *C. suaveolens* from Greece and Italy appeared to be phylogenetically distinct from *C. suaveolens* in Turkmenistan and the whole *C. suaveolens* sample appeared to be paraphyletic with respect to *C. gueldenstaedtii*. Thus, Vogel et al. (2003) placed in doubt the validity of *C. gueldenstaedtii*. However, the level of variability within *C. suaveolens* was too high, leading to the suspicion that multiple species were lumped under the nomen *C. suaveolens* s.str. Neither of the two molecular studies invoked material from *terra typica* of *C. suaveolens* (Crimea) or any other data on Eastern European shrews. Hence, the recognition of two taxa within the former *C. suaveolens* (*C. suaveolens sensu stricto* and *C. gmelini*), with ranges contacting or even overlapping somewhere in the west of Middle Asia, should be regarded as merely a provisional decision that requires additional confirmation.

In the recent study of Crocidurinae nuclear DNA using interspersed repeat PCR fingerprints, a notable difference between *C. suaveolens*, *C. sibirica* and *C. gueldenstaedtii* was found (Bannikova et al. 2005). However, the *C. suaveolens* sample was limited to Eastern European specimens and the *C. gueldenstaedtii* sample comprised only specimens from a very narrow locality in the Krasnodarskii region.

Hence, it is clear that the above-cited studies were based not just on geographically different samples, but different molecular markers as well. Consensus might be attained on the basis of extended sampling and a combination of methods.

In the present study, we analyzed the molecular phylogenetic relationships of 40-chromosome *Crocidura* in Europe, Caucasus and East Asia on the basis of two independent molecular markers: interspersed repeat PCR fingerprints (inter-SINE-PCR) and complete (1140 bp) or partial (~400 bp) sequences of the mtDNA *cyt b* gene. One of the Caucasus forms, *C. caspica*, has never been examined in any genetic studies.

## Materials and methods

### Specimens

Tissue samples were taken from 51 shrew specimens. *C. leucodon* and *C. lasiura* were used in the phylogenetic analysis as an outgroup. Most of the shrews were collected by the authors during expeditions held from 1994 to 2003. All specimens collected are deposited in the Zoological Museum of Moscow (Lomonosov) State University (ZMMU). For *cyt b* gene sequence analysis, seven sequences were retrieved from GenBank. The list of species, collection sites, number of specimens, and museum

catalogue numbers are shown in Table 1. The geographic locations of the specimens (both original and cited in the literature) used in our study are presented on the map in Figure 1. It should be noted that species identifications for this study were based on morphological criteria and were performed prior to the molecular analysis.

### DNA isolation

Genomic DNA was isolated from ethanol-fixed liver, kidney or muscles by proteinase K digestion, phenol-chloroform deproteinization and isopropanol precipitation (Sambrook et al. 1989).

### PCR using SINE-specific primers (IS-PCR)

The method of inter-SINE-PCR (IS-PCR) is based on amplification of DNA fragments flanked by copies of SINEs (short interspersed elements) located 100–1000 bp apart. This study used a SINE family named the mammalian interspersed repeat (MIR), which has 10<sup>5</sup> copies in all mammalian genomes (Jurka et al. 1995; Smit and Riggs 1995; Gilbert and Labuda 2000). In addition to the MIR element, we studied a short retroposon SOR of 179 bp in length, which is specific only to the family Soricidae (Borodulina and Kramerov 2001).

Inter-MIR-PCR was carried out with primers complementary to the most conserved regions of the central core sequence of the MIR element (Jurka et al. 1995). Four primers were used in the following combinations: MIR17/MIL17 and OMIL17/OMIR17. For inter-SOR-PCR, we used a primer complementary to the beginning of the SOR element. The sequences of the primers were developed in a previous study (Bannikova et al. 2005). Primers (100 pmol each) were labeled with [ $\gamma$ -<sup>32</sup>P]-ATP (1 MBq) by polynucleotidekinase (Sambrook et al. 1989). PCR was carried out in a reaction mixture of 20  $\mu$ l containing 67 mM Tris-HCl buffer, pH 8.6, 16.6 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2.5 mM MgCl<sub>2</sub>, dNTPs, 0.2 mM each, 4 pmol of each primer, 1 U of Taq polymerase, and 25 ng of DNA template. PCR was performed according to Jurka et al. (1995). SOR-specific PCR annealing was conducted at 65°C using an MJ Research (USA) thermal cycler. PCR products were denatured and separated by electrophoresis in a 6% polyacrylamide gel (50 cm×28 cm×0.4 mm) containing Tris-borate buffer and 8 M urea (Slatko and Albright 1992) for 7 h at a constant power of 73 W. The dried gel was autoradiographed by exposure to X-ray film for 48 h.

### Cytochrome *b*

The near-complete *cyt b* gene (1010–1140 bp) was sequenced in 12 shrews, including three specimens from Talysh, single specimens from Georgia and Abkhazia, and single specimens from Tuapse, Crimea, Baskunchak, Novosibirsk, Kemerovo, Gobi-Altai and North Italy (Table 1). A gene region that included the whole of the mitochondrial *cyt b* gene was amplified by PCR with the forward/reverse primer combination L14734/H15985 (Ohdachi et al. 2001). The forward internal primer was designed as 5'-GTAATAGCAACAGCCTTTATAGGTTA-3' (L15136). In 11 additional specimens (Table 1) we se-

quenced a portion of the gene using the L14734 primer (mean fragment length ~400 bp, range 200–500 bp). Amplification consisted of 36 thermal cycles of 30-s denaturation at 94°C, 1-min annealing at 57°C and 1-min extension at 72°C. PCR products were visualized on 1% agarose gel and then purified using ammonium-ethanol precipitation. Approximately 30 ng of the purified PCR product was used for sequencing with each primer on an ABI 3100-Avant autosequencing system using ABI PRISM<sup>®</sup> BigDye<sup>™</sup> Terminator v. 3.1.

### Phylogenetic analysis

IS-PCR bands were scored for their presence/absence. To infer molecular phylogenetic relationships among Crocidurinae species of our data set, binary matrices were analyzed by neighbor-joining (NJ; Saitou and Nei 1987) and maximum parsimony (MP) analyses using PAUP, version 4.0b10 (Swofford 1998). The statistical confidence of groupings in the NJ and MP trees was evaluated by bootstrap tests based on 1000 replications. To estimate the dissimilarity among individual patterns, Nei-Li genetic distances (Nei and Li 1979)  $D_{NL}$  were calculated.

*cyt b* gene sequences were visually aligned. Final alignment of the mitochondrial regions included 1140 bp. Phylogenetic NJ, ML and MP analyses were conducted using PAUP\* version 4.0b10 (Swofford 1998). Unweighted maximum-parsimony analysis was performed using a heuristic search, starting with stepwise addition trees and employing TBR branch-swapping. Bootstrap analysis with 1000 pseudoreplicates was used to measure the support of the resulting MP and NJ topologies. To reconstruct the ML tree, an appropriate model of sequence evolution was chosen on the basis of hierarchical likelihood ratio tests (LRT) as implemented in Modeltest version 3.04 (Posada and Crandall 1998). In the course of subsequent heuristic searches with fixed model parameters, the initial NJ topology was subject to TBR swapping. The ML bootstrap analysis was based on 100 replicates (NJ tree as the start and SPR swapping). Taking into account the low level of difference between taxa (no more than 15% for outgroup comparison and less than 10% for ingroup), saturation problems were hardly expected. The assumption of rate constancy was tested using an LRT test for best ML trees with and without a clock. Divergence dates were calculated using the depths of internal nodes in the ML tree, assuming a molecular clock. To obtain standard errors for the divergence dates, the variances of both the divergence rate index and the node depths were taken into account. The latter source of error was estimated on the basis of 100 bootstrap replicates, while the former was assessed using the delta-method according to Waddell et al. (1999) with modifications.

## Results

### Inter-SINE-PCR

**Intraspecific variability** The levels of intraspecific variability appeared to be comparable for inter-MIR- and inter-SOR-PCR data sets, as indicated by the mean with-

**Table 1** List of specimens analyzed.

Specimen	Location (map code)	Museum catalog number, tissue or field code	IS-PCR and <i>cyt b</i> code	<i>cyt b</i> sequence	
				Length, bp	GenBank acc. no (reference)
<i>C. suaveolens</i>	Feodosia, Crimea (1)	10/05 fc03-1	su Crimea	1110	AY994377
<i>C. suaveolens</i>	Moscow, Russia (2)	S-177831	su Moscow 8	429	AY994378
<i>C. suaveolens</i>	Madzhalis, Dagestan (3)	S-177834	su Dag 7	–	–
<i>C. suaveolens</i>	Madzhalis, Dagestan (3)	S-177833	su Dag 6	–	–
<i>C. suaveolens</i>	Madzhalis, Dagestan (3)	S-177832	su Dag 5	–	–
<i>C. suaveolens</i>	Madzhalis, Dagestan (3)	S-177815	su Dag 3	230	AY994379
<i>C. suaveolens</i>	Chernozemelsk, Kalmykia (4)	S-177822	su Kalm 9	379	AY994381
<i>C. suaveolens</i>	Chernozemelsk Kalmykia (4)	S-177823	su Kalm 10	–	–
<i>C. suaveolens</i>	Nalchik, Kabardino-Balkaria (5)	T-N2	su Nalchik 2	476	AY994380
<i>C. suaveolens</i>	Nalchik, Kabardino-Balkaria (5)	T-N3	su Nalchik 3	–	–
<i>C. suaveolens</i>	Salsk, Rostov region (6)	05/14 fc1547	su Salsk1 547	481	AY994383
<i>C. suaveolens</i>	Tzimplansk, Rostov region (7)	05/14 fc1546	su Tzimla 1546	–	–
<i>C. suaveolens</i>	Tzimplansk, Rostov region (7)		su Tzimla 1548	–	–
<i>C. suaveolens</i>	Tzimplansk, Rostov region (7)	05/14 fc1549	su Tzimla 1549	458	AY994384
<i>C. suaveolens</i>	Tzimplansk, Rostov region (7)	T-emb	su Tzimla emb	–	–
<i>C. suaveolens</i>	Baskunchak, Astrakhansk region (8)	S-177842	su Baskunchak	1140	AY994386
<i>C. suaveolens</i>	Diakovka, Saratov region (9)	S-177828	su Diak 4	464	AY994385
<i>C. suaveolens</i>	Diakovka, Saratov region (9)	S-177830	su Diak 10	–	–
<i>C. suaveolens</i>	Shkili, Astrakhansk region, Ahtubinsk (10)	S-177827	su Shkili 3	276	AY994382
<i>C. suaveolens</i>	Shkili, Astrakhansk region, Ahtubinsk (10)	S-177825	su Shkili 4	–	–
<i>C. suaveolens</i>	Ih-Bogdyn-Nury, 44°58' N, 100°38' E, Mongolia (11)	44/04 fc320	su Ih Bogdo	1119	AY994387
<i>C. suaveolens</i>	Mosuowan, Xinjiang (12)	–	su Mosuowan	1140	AB077087 (Ohdachi et al. 2004)
<i>C. suaveolens</i>	Qarqan, Xinjiang (13)	–	su Qarqan	1140	AB077084 (Ohdachi et al. 2004)
<i>C. suaveolens</i>	Latisana, Venezia, Italy (14)	T-sult*	su Venezia	1120	AY994388
<i>C. suaveolens</i>	Vienna, Austria	–	su Vienna	1140	AB077280 (Ohdachi et al. 2004)
<i>C. sibirica</i>	Krapivinsk region, Kemerovo, (16)	S-177772	sib 195	–	–
<i>C. sibirica</i>	Krapivinsk region, Kemerovo, (16)	S-177773	sib 624	–	–
<i>C. sibirica</i>	Krapivinsk region, Kemerovo, (16)	S-177775	sib 1650	–	–
<i>C. sibirica</i>	Krapivinsk region, Kemerovo, (16)	S-177776	sib 1651	1137	AY994389
<i>C. sibirica</i>	Krapivinsk region, Kemerovo, (16)	S-177777	sib 1688	–	–
<i>C. sibirica</i>	Novosibirsk (17)	T-Nov1	sib Nov 1	713	AY994390
<i>C. sibirica</i>	Novosibirsk (17)	T-Nov2	sib Nov 2	–	–
<i>C. gueldenstaedtii</i>	Vishnevka village, Tuapse (18)	S-177817	gu 11	392	AY994371
<i>C. gueldenstaedtii</i>	Vishnevka village, Tuapse (18)	S-177818	gu 12	479	AY994372
<i>C. gueldenstaedtii</i>	Vishnevka village, Tuapse (18)	S-177819	gu 13	–	–
<i>C. gueldenstaedtii</i>	Vishnevka village, Tuapse (18)	S-177816	gu 6	–	–
<i>C. gueldenstaedtii</i>	Vishnevka village, Tuapse (18)	S-177820	gu 20	1129	AY994373
<i>C. gueldenstaedtii</i>	Vishnevka village, Tuapse (18)	S-177821	gu 40	–	–
<i>C. gueldenstaedtii</i>	Adler, Russia (19)	S-167371	gu Adler	286	AY994374
<i>C. gueldenstaedtii</i>	Ahaltsikhe, Georgia (20)	S-177771	gu Ahaltsikhe 1	–	–
<i>C. gueldenstaedtii</i>	Ahaltsikhe, Georgia (20)	S-177770	gu Ahaltsikhe 2	1114	AY994376
<i>C. gueldenstaedtii</i>	Alazani, Georgia (21)	fc23608*	gu Alazani 1	–	–
<i>C. gueldenstaedtii</i>	Cnori, Alazani, Georgia (21)	S-169130	gu Alazani 2	–	–
<i>C. gueldenstaedtii</i>	Gagra, Abkhazia (22)	S-131494	gu Gagra	1052	AY994375
<i>C. caspica</i>	Siov, Astara, Talysh, Azerbaidjan (23)	S-177796	casp 1	1120	AY994369
<i>C. caspica</i>	Siov, Astara, Talysh, Azerbaidjan (23)	S-177797	casp 2	–	–
<i>C. caspica</i>	Siov, Astara, Talysh, Azerbaidjan (23)	S-177799	casp 4	–	–
<i>C. caspica</i>	Siov, Astara, Talysh, Azerbaidjan (23)	S-177800	casp 5	1140	AY994368
<i>C. caspica</i>	Siov, Astara, Talysh, Azerbaidjan (23)	S-177801	casp 6	–	–
<i>C. caspica</i>	Siov, Astara, Talysh, Azerbaijan (23)	S-177802	casp 7	–	–
<i>C. caspica</i>	Siov, Astara, Talysh, Azerbaijan (23)	S-177803	casp 8	–	–
<i>C. caspica</i>	Burdjala, Lenkoran, Azerbaijan (24)	S-177805	casp 21	–	–
<i>C. caspica</i>	Burdjala, Lenkoran, Azerbaijan (24)	S-177806	casp 22	–	–
<i>C. caspica</i>	Burdjala, Lenkoran, Azerbaijan (24)	S-177809	casp 27	–	–
<i>C. caspica</i>	Burdjala, Lenkoran, Azerbaijan (24)	S-177812	casp 31	1140	AY994370
<i>C. shantungensis</i>	Popov Is.	–	sha Popov	1140	AB077278 (Ohdachi et al. 2004)
<i>C. shantungensis</i>	Putjatin Is.	–	sha Putjatin	1140	AB077081 (Ohdachi et al. 2004)

(Table 1 continued)

Specimen	Location (map code)	Museum catalog number, tissue or field code	IS-PCR and <i>cyt b</i> code	<i>cyt b</i> sequence	
				Length, bp	GenBank acc. no (reference)
<i>C. lasiura</i>	Kedrova Pad, Primorye	S-177824	la 29	–	–
<i>C. lasiura</i>	Ussuriisk, Primorye	–	la 1	1140	AB077071 (Ohdachi et al. 2004)
<i>C. lasiura</i>	Kraskino, Primorye	–	la 2	1140	AB077072 (Ohdachi et al. 2004)
<i>C. leucodon</i>	Madzhalis, Dagestan (3)	S-177814	le 90-7A	–	–

\*These specimens were kindly preserved by Dr. Peter Vogel (Lausanne University).

in-population interindividual  $D_{NL}$  values. For inter-MIR-PCR and inter-SOR-PCR, the value of this index was 0.047 (range 0.010–0.113) and 0.046 (range 0.000–0.196), respectively.

The overall amount of evolution per character measured as the standardized tree length (NJ topology and ME method of length estimation) was approximately equal for both types of markers (0.741 and 0.728 for inter-MIR- and inter-SOR-PCR data sets, respectively). Moreover, the homoplasy index is very similar for the two data sets (0.45 and 0.46).

Given the observed concordance in patterns of intra- and interpopulation variation, joint analysis of inter-MIR- and inter-SOR-PCR data sets is warranted (Table 2). Based on these results, the minimum level of individual variability for samples from the same locality was found for the Lenkoran population of *C. caspica* (0.02) and the maximum level was for *C. suaveolens* from Shkili (0.097). The  $D_{NL}$  value for samples from different localities reached a minimum between the geographically close Astará and Lenkoran populations of *C. caspica* (0.037) and a maximum between the Ih-Bogdyn-Nury and Baskunchak specimens of *C. suaveolens* (0.178).

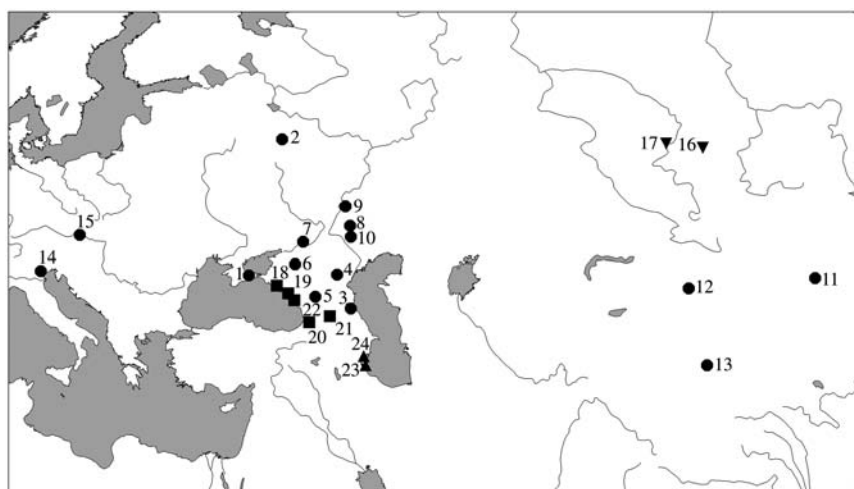
**Interspecific variability** Mean interspecific genetic distances (excluding *C. lasiura*) for the combined results

of inter-MIR- and inter-SOR-PCR ranged from 0.166 (between *C. suaveolens* and *C. sibirica*) to 0.6 (between *C. suaveolens* from Italy and *C. caspica*; Table 2).

### Phylogenetic analysis

**Inter-SOR-PCR** Among 140 characters considered in the parsimony and NJ analyses of the inter-SOR-PCR data set, 84 were parsimony-informative. The topology of the NJ tree (Figure 2) is characterized by the monophyly of *C. suaveolens* (bootstrap value 76%) and *C. sibirica* (92%) samples. *C. suaveolens* from Italy appears to be a sister group to *C. gueldenstaedtii* (NJ 69%, MP 82%). Other groupings have high support in both MP and NJ analyses, but their relationships are still unresolved.

**Inter-MIR-PCR** The overall number of characters produced by inter-MIR-PCR of *Crocidura* for the two combinations of primers is 361. The number of parsimony-informative characters is 201. The resulting topologies of both NJ and MP trees are basically the same (Figure 3). Contrary to the inter-SOR PCR results, inter-MIR-PCR analysis segregated *C. gueldenstaedtii* and *C. suaveolens* from Italy, but did not show the monophyly of Eastern European *C. suaveolens* with respect to

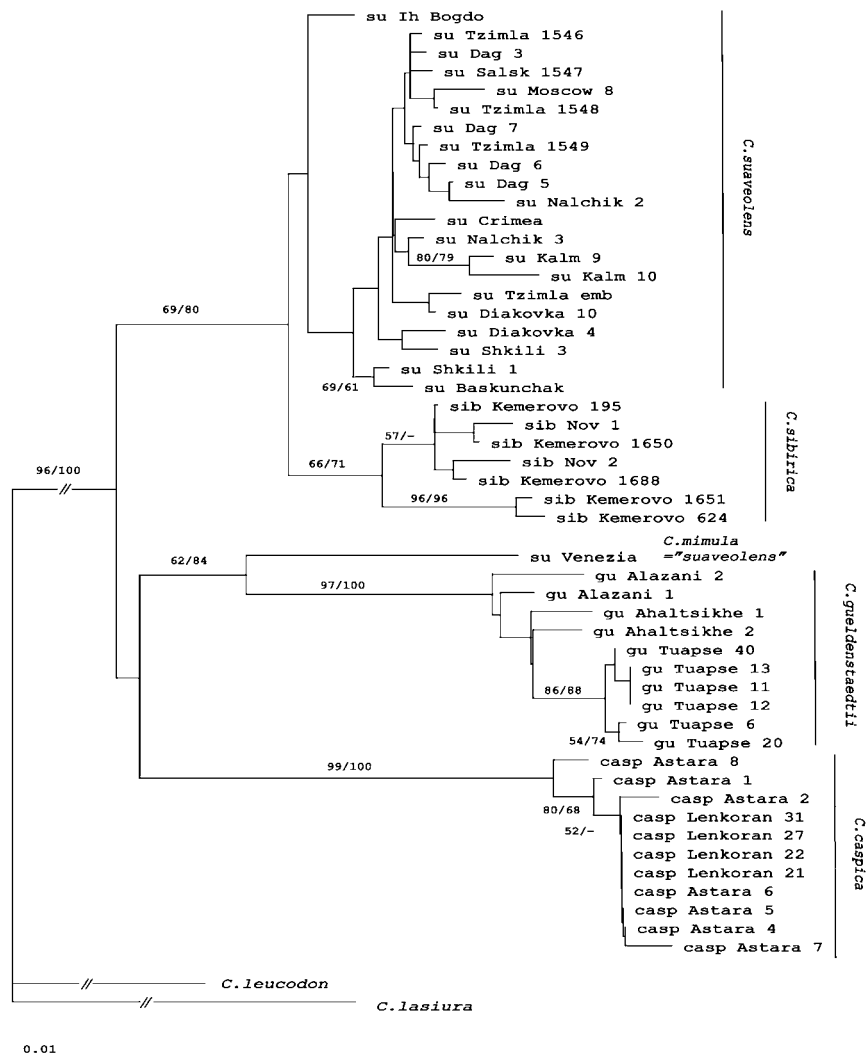


**Figure 1** Collection locations for specimens in Europe and Siberia used in the study. ● *Crocidura suaveolens*; ■ *C. gueldenstaedtii*; ▼ *C. sibirica*; ▲ *C. caspica*. 1, Feodosia, Crimea; 2, Moscow, Russia; 3, Madzhalis, Dagestan; 4, Chernozemelsk, Kalmykia; 5, Nalchik, Kabardino-Balkaria; 6, Salsk, Rostov region; 7, Tzimlansk sands, Rostov region; 8, Baskunchak, Astrahansk region; 9, Diakovka, Saratov region; 10, Shkili, Astrakhansk region; 11, Ih-Bogdyn-Nury, Bayan-Khongor region, Mongolia; 12, Mosuowan, Xinjian; 13, Qarqan, Xinjian; 14, Latisana, Venezia, Italy; 15, Vienna, Austria; 16, Krapivinsk district, Kemerovo region; 17, Novosibirsk; 18, Vishnevka village, Tuapse, Krasnodar region, Russia; 19, Adler, Krasnodar region, Russia; 20, Ahaltsikhe, Georgia; 21, Alazani, Georgia; 22, Gagra, Abkhazia; 23, Siöv, Astará, Talysh, Azerbaidjan; 24, Burdjala, Lenkoran, Azerbaidjan.

**Table 2** Mean inter-individual Nei-Li genetic distances (Nei and Li 1979) for the combined results for both inter-MIR and inter-SOR PCR data sets.

Specimen location (map code)	1	2	3	4	5	6	7	8	9	10	11	14	16	17	18	20	21	23	24	la	le	
Su Crimea (1)	-																					
Su Moscow (2)	0.094	-																				
Su Dagestan (3)	0.065	0.064	<b>0.054</b>																			
Su Kalmykia (4)	0.065	0.088	0.074	<b>0.043</b>																		
Su Nalchik (5)	0.082	0.098	0.093	0.098	<b>0.078</b>																	
Su Salsk (6)	0.067	0.067	0.069	0.090	0.089	-																
Su Tzimla (7)	0.066	0.083	0.080	0.088	0.079	0.058	<b>0.049</b>															
Su Baskunchak (8)	0.103	0.151	0.117	0.128	0.134	0.129	0.111	-														
Su Diakovka (9)	0.084	0.115	0.094	0.108	0.105	0.083	0.073	0.102	<b>0.061</b>													
Su Shkili (10)	0.106	0.119	0.106	0.127	0.124	0.098	0.098	0.102	0.096	<b>0.097</b>												
Su Ih-Bogdo (11)	0.145	0.162	0.160	0.175	0.149	0.140	0.134	0.178	0.143	0.132	-											
Su Venezia (14)	0.520	0.561	0.520	0.554	0.546	0.519	0.565	0.542	0.567	0.543	0.546	-										
Sib Kemerovo (16)	0.156	0.172	0.161	0.170	0.177	0.150	0.150	0.181	0.149	0.169	0.219	0.594	<b>0.073</b>									
Sib Novosibirsk (17)	0.156	0.152	0.159	0.166	0.170	0.158	0.154	0.174	0.156	0.167	0.201	0.588	0.084	<b>0.052</b>								
Gu Tuapse (18)	0.407	0.407	0.399	0.435	0.442	0.404	0.433	0.446	0.441	0.441	0.446	0.552	0.451	0.445	<b>0.036</b>							
Gu Ahaltsikhe (20)	0.436	0.450	0.434	0.457	0.458	0.435	0.462	0.435	0.469	0.453	0.473	0.476	0.484	0.484	0.127	<b>0.044</b>						
Gu Alazani (21)	0.413	0.427	0.434	0.442	0.429	0.413	0.443	0.423	0.452	0.441	0.462	0.493	0.482	0.471	0.174	0.091	-					
Casp Astara (23)	0.372	0.397	0.369	0.404	0.397	0.354	0.375	0.377	0.388	0.369	0.365	0.618	0.384	0.374	0.532	0.545	0.551	<b>0.040</b>				
Casp Lenkoran (24)	0.361	0.392	0.363	0.396	0.393	0.342	0.367	0.378	0.382	0.362	0.350	0.599	0.370	0.361	0.515	0.527	0.534	0.037	<b>0.020</b>			
C. lasiura (la)	1.320	1.402	1.327	1.319	1.306	1.303	1.298	1.331	1.339	1.331	1.361	1.356	1.278	1.322	1.449	1.418	1.398	1.413	1.406	-		
C. leucodon (le)	1.358	1.382	1.345	1.309	1.343	1.338	1.341	1.338	1.395	1.396	1.402	1.508	1.386	1.430	1.479	1.508	1.487	1.490	1.483	1.614	-	

Distances between *Crocidura* specimens from different geographic locations are below the diagonal. Distances between individuals from the same location are on the diagonal in bold font.



**Figure 2** NJ tree of the relationships of the *Crocidura suaveolens* species group inferred from the Nei-Li genetic distances based on the results of inter-SOR-PCR. The bootstrap values ( $bv \geq 50\%$ ) obtained from 1000 replications in NJ and MP analysis are given above the branches (NJ/MP). *C. lasiura* and *C. leucodon* are used as an outgroup.

*C. sibirica*. The latter formed a monophyletic group within the sample of *C. suaveolens*.

### Combined inter-MIR- and inter-SOR-PCR results

The number of characters examined in the combined phylogenetic analysis of inter-SINE-PCR-derived data with both MIR and SOR systems of primers totals 501. The number of parsimony-informative characters is 284. Compared to the separate analyses, the combined data set generally produced the same trees with 85%/94% NJ/MP bootstrap support for *C. sibirica*/*C. suaveolens*+*C. caspica*, and <50%/65% NJ/MP support for *C. gueldenstaedtii*+*C. suaveolens* from Italy (Figure 4). Hence, this topology and inter-SOR-PCR tree are rather similar in terms of the reciprocal monophyly of *C. sibirica* (95%/100%) and *C. suaveolens* (56%/68%) and the association of *C. gueldenstaedtii* with *C. suaveolens* from Italy. There is no differentiation within the Eastern European *C. suaveolens*, but bootstrap values support the division of *C. gueldenstaedtii* into the population of the northeast Black Sea coast (Tuapse) and the Transcaucasus population (Alazani and Ahaltsikhe).

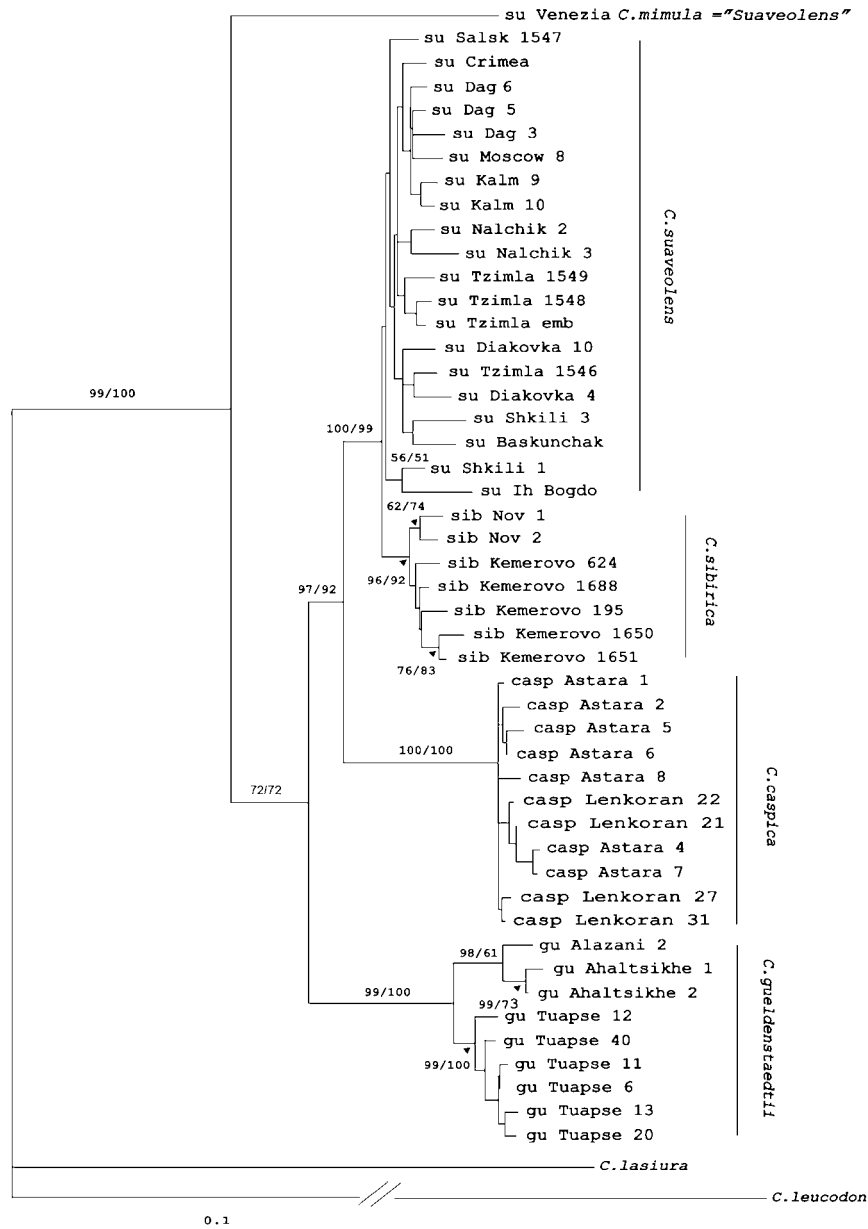
### cyt b

Among 23 individuals sequenced in this study, 12 haplotypes were identified. No insertions or deletions were observed. Most nucleotide substitutions were synonymous transitions.

The near-complete haplotypes of *C. suaveolens* from Crimea (1110 bp) and *C. gueldenstaedtii* from Tuapse (1129 bp) are almost identical, demonstrating just one third-position replacement. At the same time, they are notably distant from *C. gueldenstaedtii* from other localities.

No difference was observed among partial sequences from Moscow, Salsk, Kalmykia, Dagestan, Nalchik, Tzimlanskie sands, Tuapse and near-complete sequences from Crimea and Tuapse. The haplotypes of shrews collected on the left bank of the Volga River are identical and differ by one transitional change from other Russian haplotypes.

The difference between shrews from Eastern Europe and those from Central Asia (Mongolia and Xinxiang) ranges from 3 to 8 changes (0.5% on average). The difference between *C. sibirica* and *C. suaveolens* haplo-



**Figure 3** NJ tree of the relationships of the *Crocidura suaveolens* species group inferred from the Nei-Li genetic distances based on the results of inter-MIR-PCR. The designations are as in Figure 2.

types is approximately of the same order of magnitude as interpopulation distances within *C. suaveolens* (0.8% on average).

While no sequence variation was found in the Tuapse population of *C. gueldenstaedtii*, all three specimens of *C. caspica* from the Talysh region had their own haplotypes, differing by 4–7 mutational changes.

ML, MP, and NJ trees were inferred on the basis of 19 complete or almost complete sequences (both original and retrieved from GenBank). Taking into account the outcome of the Modeltest analysis (LRT), an ML tree was reconstructed using the TrN+G+I model (Figure 5). Unweighted maximum parsimony analysis of *cyt b* sequences resulted in two equally parsimonious trees (380 steps,  $ci=0.776$   $ri=0.883$ ) differing in minor details of branching within the *C. suaveolens* clade.

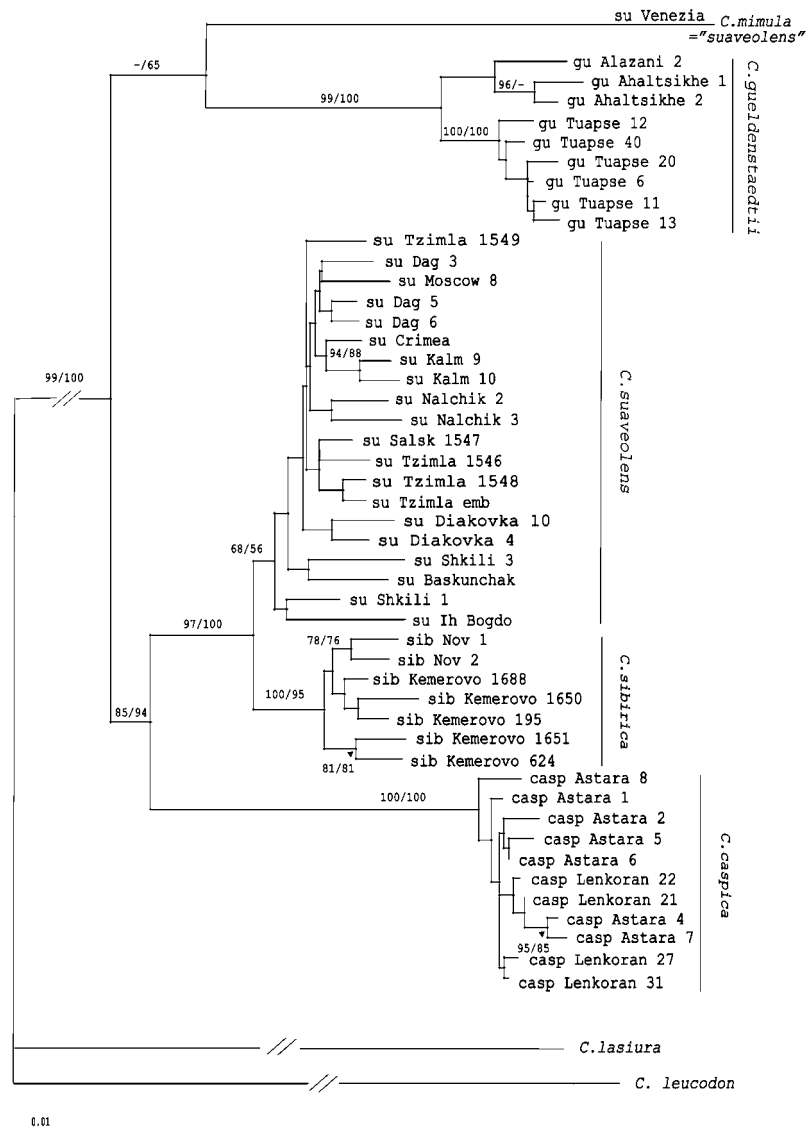
The topologies of both the MP and NJ trees were principally the same as that of the ML tree (Figure 5). Within

the 40-chromosome species group, five monophyletic assemblages with the exclusion of *C. lasiura* were recognized: (1) *C. suaveolens* from Western Europe; (2) *C. gueldenstaedtii* from Georgia; (3) *C. caspica*; (4) *C. suaveolens* from Eastern Europe and Central Asia, together with *C. gueldenstaedtii* from Tuapse and *C. sibirica*; and (5) *C. shantungensis*.

The latter taxon branches basally with high bootstrap support, while the others form two clusters: *C. suaveolens* (Western Europe)+*C. gueldenstaedtii*; and +(C. *suaveolens* (Eastern Europe, Asia), *C. sibirica*)+*C. caspica*. While the first of these clades is 100% supported in all analyses, bootstrap values for the second grouping are only moderate in ML and MP analyses (49% and 63%, respectively) but reach 97% for NJ.

The two *C. sibirica* haplotypes always cluster together; however, in the ML tree the haplotype of *C. suaveolens* from Kashgar tends to occupy a basal position in the





**Figure 4** NJ tree of the relationships of the *Crocidura suaveolens* species group inferred from the Nei-Li genetic distances based on the combined results of inter-MIR- and inter-SOR-PCR. The designations are as in Figures 2 and 3.

clade combining these two species, and thus makes *C. suaveolens* paraphyletic relative to *C. sibirica*.

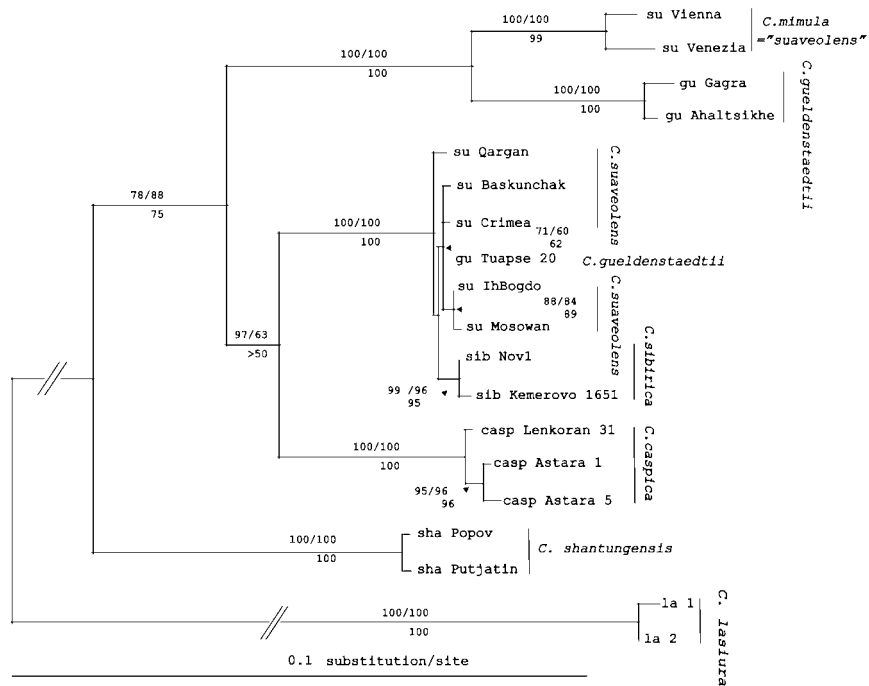
Thus, the *cyt b* study revealed interspecific variation that generally conforms to the pattern revealed by the inter-SINE-PCR data, apart from (1) the monophyly of *C. sibirica* with respect to Eastern Eurasian *C. suaveolens* and (2) the unexpected heterogeneity within *C. gueldenstaedtii*.

#### Estimation of times

The result of the LRT does not allow rejection of the assumption of rate constancy for our *cyt b* data set ( $\chi^2=2\Delta\ln L=22.9$ ; d.f.=17;  $p=0.15$ ), although the tip-to-root distances in *C. gueldenstaedtii*+*C. suaveolens* (Western Europe) are apparently longer. Estimates of divergence dates for the main clades (Table 3) were based on node-to-tip distances in the ML tree, assuming a molecular clock. A single primary calibration point was used corresponding to separation of *C. gueldenstaedtii* from western *C. suaveolens*, which, we suggest, took

place at the end of the lower Pleistocene (~0.5 Mya) due to transgression of the Aegean Sea (Keraudren and Mercier 1977). The average intrapopulation distance, estimated to be ~0.0027, was used to correct for ancestral polymorphism. The estimate of the divergence rate for *cyt b* (all codon positions) was 10.9% ( $\pm 1.5$ ), which is nearly two-fold greater than the rate obtained for *Crocidura* species by Cosson et al. (2005).

Another set of estimates was obtained using a secondary calibration date for *C. lasiura*/*C. suaveolens* s.l. divergence ( $2.95\pm 0.8$  Mya) inferred on the basis of an estimate of 20 Mya for the Crocidurinae/Soricinae split. Following Fumagalli et al. (1999), calibration was performed considering *cyt b* sequence divergence based on third-position transversions. ML analysis of a data set comprising complete sequences for 10 species of Soricidae and three species of Talpidae (details available from the authors) provided an estimate for the divergence rate at third-position transversions in shrews of 3.74% ( $\pm 0.59$ ) per Mya. The evident discrepancy with an earlier



**Figure 5** ML tree generated using a TrN+G+I model of the *Crocidura suaveolens* species group based on near-complete mitochondrial cytochrome *b* sequences. Bootstrap values  $\geq 50\%$  obtained from 100 replications are given above the branches (NJ/MP) for NJ and MP analyses and below branches for ML analysis (100 replicates). *C. lasiura* is used as an outgroup.

**Table 3** Estimates of divergence times.

Clade	Mean net divergence ( <i>cyt b</i> ML)	Divergence time (Mya)	
		<i>gueldenstaedtii/suaveolens</i> from Italy calibration point	<i>lasiura/suaveolens</i> s.l. calibration point
<i>C. lasiura/rest</i>	0.2967	2.713 $\pm$ 0.508	<b>2.952<math>\pm</math>0.956</b>
<i>C. shantungensis/rest</i>	0.1327	1.214 $\pm$ 0.216	1.321 $\pm$ 0.421
<i>C. suaveolens</i> + <i>C. sibirica</i> + <i>C. caspica</i> / <i>C. gueldenstaedtii</i> + <i>C. suaveolens</i> from Italy	0.1079	0.986 $\pm$ 0.165	1.073 $\pm$ 0.335
<i>C. gueldenstaedtii</i> / <i>C. suaveolens</i> from Italy	0.0547	<b>0.500<math>\pm</math>0.097</b>	0.544 $\pm$ 0.179
<i>C. caspica</i> / <i>C. suaveolens</i> + <i>C. sibirica</i>	0.0681	0.623 $\pm$ 0.133	0.678 $\pm$ 0.230
Basal divergence in <i>C. suaveolens</i> + <i>C. sibirica</i> clade	0.0039	0.036 $\pm$ 0.020	0.039 $\pm$ 0.024
<i>C. sibirica</i> / <i>C. suaveolens</i>	0.0030	0.027 $\pm$ 0.018	0.030 $\pm$ 0.021

Divergence times are presented as mean $\pm$ SE. Calibration dates are given in bold.

result (1.36%) reported by Fumagalli et al. (1999) is most probably associated with significant among-site rate heterogeneity ( $\alpha \approx 0.4$ ) observed for third-position transversions, which was not taken into account in the cited study.

Despite relatively large errors, analyses based on the two calibration dates provide concordant sets of divergence dates.

## Discussion

The present analysis of both inter-SINE-PCR and *cyt b* sequence data revealed two major clades in the western/central Palearctics that reflects the eastern and western geographic structuring within the 40-chromosome *Crocidura* shrews. The eastern clade is formed by *C. suaveolens*/*C. sibirica*, together with *C. caspica*, and the western clade is formed by Italian *C. suaveolens*, together with the closely related *C. gueldenstaedtii*. Within

these clades the level of genetic divergence of different forms is not equal and their taxonomic status is not evident.

### *C. suaveolens*-*C. mimula*

In the present study, *C. suaveolens* from Italy was distinct from samples of *C. suaveolens* from Crimea, Moscow, Dagestan, the Volga region, Kalmykia and Mongolia, as well as the Siberian sample. Thus, our inter-SINE PCR and *cyt b* data are congruent with the *cyt b* results of Vogel et al. (2003) and Ohdachi et al. (2004) regarding the deep division between Western European and Asian *C. suaveolens*. However, it is now evident that shrews from Eastern Europe form a single taxon with Asian, but not Western European populations. All these findings raise a question regarding the separate taxonomic status of European and Asian *C. suaveolens*. Both mitochondrial *cyt b* and nuclear inter-SINE-PCR data indicated that these two taxa could be treated as separate species.

Whereas *C. suaveolens* was first described and named from Crimea by Pallas in 1811, this name should be reserved for the populations of Eastern Europe and part of Asia.

We believe that if the Western European form is recognized as a valid species, the proper name for it is *C. mimula* Miller 1901 (*terra typica* Switzerland), as this is the senior synonym among all nomens attributable to *suaveolens*-like shrews in this region. The location of the contact zone between *C. mimula* and *C. suaveolens* remains to be determined, although the Carpathian Mountains are one of the plausible areas. It should be mentioned that *C. mimula* is perhaps not the only European *C. suaveolens*-related form that deserves recognition as a separate species, since according to the results of Vogel et al. (2003), shrews from Spain might be significantly divergent from both *C. mimula/C. gueldenstaedtii* and *C. suaveolens* s.str.

Regarding the status of *C. gmelini*, a few remarks should be made. It is evident from the combined genetic data that many of populations that were previously included in *C. gmelini* should be attributed to *C. suaveolens* s.str. No genetic data from Dasht (Bujnurd vicinity, northwest Chorassan, to which the neotype of *C. gmelini* belongs) are available so far, but if the haplotype of the shrew from the relatively close west Turkmenistan examined by Vogel et al. (2003) is rather similar to haplotypes from southern Russia and Central Asia, then there is no reason to suspect that *C. gmelini* is anything more than a synonym of *C. suaveolens*. Returning to the two sympatric forms in northeast Iran assigned by Hoffmann (1996) to *C. suaveolens* (*C. gueldenstaedtii*) and *C. gmelini*, a supposition might be proposed that in fact these refer to *C. caspica* (or *C. gueldenstaedtii*) and *C. suaveolens*, respectively. However, taking into account the lack of genetic data from Southwest Asia, any conclusions concerning the systematics of *suaveolens*-like shrews in this region are not yet warranted.

### ***C. gueldenstaedtii***

All specimens from the western Caucasus studied in the inter-SINE-PCR analyses belonged to a single, highly supported cluster and should be recognized as *C. gueldenstaedtii*. Within this grouping, specimens from Tuapse always clustered together with Georgian samples. However, *cyt b* sequence analysis revealed significant heterogeneity within *C. gueldenstaedtii*. These results clearly indicate that all examined representatives of the Tuapse sample join the *C. suaveolens* cluster, sharing identical haplotypes with *C. suaveolens* from the right bank of the Volga River and, thus, given *cyt b* data alone could have been classified as members of the latter species. Georgian haplotypes formed a distinct cluster differentiated from the others at a level consistent with that obtained by inter-SINE-PCR. It might be concluded that this cluster represents the "true" mitotypes of *C. gueldenstaedtii*, which forms a well-supported association with European *C. suaveolens* from Italy. The lack of congruence between nuclear and mtDNA data for *C. gueldenstaedtii* may be explained by possible hybridization between *C. gueldenstaedtii* and *C. suaveolens* near the eastern shore of the Black Sea, resulting in a replacement of *C. gueldenstaedtii* mtDNA haplotypes with *C. suaveolens* ones

at some sites. Previously we suggested that at the Black Sea bank the border between north Caucasian *C. suaveolens* and Transcaucasian *C. gueldenstaedtii* is located at 44°30' N, 38°60' E (Bannikova et al. 2001). The current results confirm this conclusion, indicating the hybrid origin of the Tuapse population. Given the absolute lack of divergence between the haplotypes of *C. gueldenstaedtii* from Tuapse and *C. suaveolens* from an adjacent population of the northern Caucasus, we might suggest a recent origin of the hybridization zone. This finding contributes to an ever-increasing collection of examples of similar events in the evolutionary history of various groups of mammals that are detectable due to assimilation of mitochondrial haplotype of one species by another (Tegelstrom 1987; Ermakov et al. 2002; Alves et al. 2003)

### ***C. caspica***

Populations of 40-chromosome white-toothed shrews from Astará and Lenkorán of the Talysh region of the Caucasus represent a separate entity that is clearly distinguished from populations from Georgia and Tuapse (*C. gueldenstaedtii*) and *C. suaveolens*. Thus, inter-SINE-PCR and *cyt b* results both confirmed the conclusions of the morphological study by Zaitsev (1991) suggesting the species validity of *C. caspica*. Although the contemporary population of *C. caspica* occupies a restricted range, it shows relatively high *cyt b* diversity, which might be a result of stable population numbers throughout its history.

### ***C. sibirica***

Both *cyt b* data and inter-SINE-PCR trees indicate a distinct cluster comprising all *C. sibirica* specimens, which is, nevertheless, rather close to *C. suaveolens*. However, while nuclear DNA data provide some support, albeit weak, for reciprocal monophyly of *C. sibirica* and *C. suaveolens*, the sequence data suggest that the latter is just as a paraphyletic assemblage relative to *C. sibirica* and that the level of divergence of the *C. sibirica* cluster falls well within the range typical for interpopulation variation (0.5–0.8%). Thus, although the sample of *C. sibirica* was augmented with specimens from a Kemerovo population, the results of Ohdachi et al. (2004) are fully confirmed in terms of *cyt b* sequence variation.

We cannot consider the discrepancy between inter-SINE-PCR and *cyt b* analysis as highly significant, since: (1) the levels of *C. sibirica/C. suaveolens* divergence according to inter-SINE-PCR are much lower than those between *C. suaveolens/C. caspica* and *C. mimula/C. gueldenstaedtii*; and (2) the sample for inter-SINE-PCR includes just a single specimen from Central Asia (Mongolia), and hence clade support could be even lower if an extended sample were analyzed. In this context it is worth mentioning that the results of the present study are in apparent contradiction to our recent analysis of phylogenetic relationships of *Crocidura* on the basis of inter-SINE-PCR (Bannikova et al. 2005), which suggested sister-group relationships between *C. suaveolens* and *C. gueldenstaedtii* relative to *C. sibirica*, although bootstrap support of that pattern in MP analysis was only moderate. The discrepancy between the previous and present

inter-SINE-PCR results may be due to the limited size of *C. suaveolens* and *C. gueldenstaedtii* samples included in our earlier studies, representing just few localities within central and south Russia.

The results obtained prompt the conclusion that the species status of *C. sibirica* is doubtful, which is rather surprising since the level of its morphological divergence within the *C. suaveolens* group is only second to *C. lasiura* (Zaitsev 1991). Moreover, *C. sibirica* demonstrates some specific features in Y-chromosome structure (Grifodatsky et al. 1988), which is otherwise stable elsewhere within the group. For this reason, the validity of this species was seldom questioned until *cyt b* data (Ohdachi et al. 2004) became available.

Considering the nuclear DNA divergence of Novosibirsk and Kemerovo populations in inter-SOR PCR analysis and bearing in mind that it is suspected that mitochondrial DNA may easily introgress in *Crocidura* shrews, one possible explanation is that *C. sibirica* is a result of ancient hybridization between *C. suaveolens* and some previously existing form endemic to southwest Siberia. As we have found, the haplotypes of *C. suaveolens* were assimilated by *C. gueldenstaedtii* in the Caucasus, so both mitochondrial and nuclear genes from *C. suaveolens* could have been assimilated by *C. sibirica* in a similar manner.

An alternative hypothesis suggests that *C. sibirica* is a young species that has recently diverged from *C. suaveolens*. The increased rate of morphological evolution might be a consequence of isolation in an enclave in the northernmost part of the distribution range of the species group, which might entail adaptive shifts.

### Taxonomic implications

The results of our study and other recent publications suggest that the status of currently recognized populations/species of 40-chromosome Palearctic shrews has to be revised. The taxon comprising all *suaveolens*-like shrews can be regarded as a superspecies or a species complex, while all distinct allopatric forms constituting it (*shantungensis*, *casgica*, *gueldenstaedtii*, *mimula* and *suaveolens* s.str., but not *sibirica*) should then be treated as semispecies (or supersubspecies). Although some well-supported groupings are evident among five semispecies, it is hard to draw a line separating "true species" from subspecies or subspecies groups. One possible solution is to regard each form as a valid species. Otherwise, a set of intercalary taxonomic levels could be suggested to allow structuring of phylogenetic relationships.

Here we suggest an example of such a system:

- C.* (superspecies *suaveolens*)
- C.* (*suaveolens*) *suaveolens* Pallas 1811
  - C. s.* (supersubspecies *suaveolens*)
    - C. s.* (*suaveolens*) *suaveolens* Pallas 1811 =syn. *C. s. gmelini* Pallas 1811
    - C. s.* (*suaveolens*) *sibirica* Dukelsky 1930
  - C. s.* (supersubspecies *casgica*)
    - C. s.* (*casgica*) *casgica* Thomas 1907
- C.* (*suaveolens*) *gueldenstaedtii* Pallas 1811
  - C. g.* (supersubspecies *gueldenstaedtii*)
    - C. g.* (*gueldenstaedtii*) *gueldenstaedtii* Pallas 1811

*C. g.* (supersubspecies *mimula*)

*C. g.* (*mimula*) *mimula* Miller 1901

*C.* (*suaveolens*) *shantungensis* Miller 1901

Another possible solution is to retain *C. casgica* as a full species, while treating *C. gueldenstaedtii* as a polytypic species with two semispecies.

### Possible speciation scenario

Palaeontological data indicate that *C. suaveolens* was known in Eurasia from the early Pleistocene (Rzebik-Kowalska 1998), i.e., approximately 1 Mya. The nucleotide divergence of the mitotypes of *C. shantungensis* (~13%) from other species of the *suaveolens* group suggests its pre-Pleistocene or Eo-Pleistocene separation, probably due to late Pliocene climatic changes that resulted in disruption of the previously continuous range of *C. suaveolens* in the south of Siberia and/or Central Asia. Nucleotide divergence between the mitotypes of western and eastern clades of European white-toothed shrews (~10%) suggests separate glacial refugia in the early Pleistocene. It is tempting to suggest that exactly at that moment the original west European population was subdivided into two lineages restricted to the Spanish and Italy/Balkan refugia (Vogel et al. 2003). The Asian populations of *pro-suaveolens* may have remained in some refugial biocenoses of Middle Asia and Iran. One of these, associated with Talysh Pleistocene refugia, gave rise to *C. casgica*. The expansion of white-toothed shrews from other Asian refugia during the last interglacial and postglacial warm periods resulted in the contemporary range of *C. suaveolens*. Long-distance colonization resulted in a loss of diversity, so that just a few closely related mitotypes are now found in Crimea, north Caucasus, middle Russia, the Volga region, Mongolia and east China. Besides, the presence of a single non-specific mitotype in the north Caucasus indicates that contemporary *C. suaveolens* populations of this region are recent colonizers rather than relics from some local refugia.

*C. gueldenstaedtii* was probably formed at the beginning of the Middle Pleistocene due to Balkan/Anatolian disruption in the range of *pre-mimula*; it could survive colder periods in the putative refugia in northern Turkey in a narrow band along the southern shore of the Black Sea, which persisted there through the entire Pleistocene (Sinitin 1965). During postglacial expansion it colonized the west and north Caucasus, where it formed a secondary contact zone with *C. suaveolens*. The substantial level of genetic divergence and difference in ecologic affinities between the two species, however, cannot preclude limited gene exchange, as evidenced by fixation of alien mitochondrial haplotypes in the northernmost population of *C. gueldenstaedtii*.

### Conclusions

Our study includes four major findings. (1) Both nuclear and mtDNA support the existence of two distinct lineages within *C. suaveolens*: the eastern lineage, which is actually true *C. suaveolens*, and the western lineage,

which should be treated as a distinct species, *C. mimula*. (2) Close genetic affinity of the western lineage (*C. mimula*) and *C. gueldenstaedtii* was found, whereas the eastern lineage (*C. suaveolens*) is a sister group to *C. caspica*. (3) The position of *C. caspica* as a full species is resolved. (4) Our study suggests that hybridization between *C. gueldenstaedtii* and *C. suaveolens* in the vicinity of Tuapse resulted in the formation of a population with nuclear markers and morphological characteristics that are typical for *C. gueldenstaedtii*, while the mitochondrial genome is assimilated from *C. suaveolens*.

For future work, it is important to sample areas of sympatry or contact between different Asian populations to evaluate the molecular history of *C. sibirica*. Besides, given the evident ease with which mitochondrial DNA crosses species boundaries in *Crociodura*, it appears essential to use independent nuclear markers in future phylogenetic and phylogeographic studies.

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