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Multilocus Analysis of Phylogenetic Relationships in the *Crocidura suaveolens* Sensu Lato Species Complex: A Comparison with Mitochondrial Data

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Abstract—Multilocus analysis was for the first time used to study the phylogeny of the *Crocidura suaveolens* s. l. species complex. Sequencing data for 16 nuclear genes indicated that several distinct forms exist within the species complex. The structure of the complex did generally not contradict its mitochondrial phylogeny. Siberian shrew showed certain specificity of the nuclear genome, but the degree of its genetic differentiation did not correspond to the species level. Relationships of *Crocidura aff. suaveolens* from South Gansu and Sichuan with other forms of the species complex were clarified. Shrews from Buryatia and Khentei also belong to this form, but their mtDNA apparently introgressed from *C. shantungensis* in the past. Hybridization of *C. suaveolens* s. str. with *C. aff. suaveolens* and *C. güeldenstaedtii* occurred recently. Due to multiple introgression events in the history of *C. suaveolens* s. l., a far larger set of loci is necessary for the analysis of the phylogenetic relationships between its forms.

Keywords: species complex, lesser white-toothed shrew, interspecies hybridization, multigene analysis, *Crocidura*

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INTRODUCTION

The evolutionary history of multispecies groups of closely related species with a relatively recent origin are now at the focus of phylogenetic research because its investigation helps to better understand the initial speciation processes. However, ancestral polymorphism, past hybridization events, and current gene flow may complicate molecular phylogenetic reconstruction of cladogenesis for closely related species.

The lesser white-toothed shrew *Crocidura suaveolens* sensu lato species complex has a huge species range, which spans throughout Eurasia from the Iberian Peninsula to the Tsushima Island and across several biomes. The karyotype is identical in all lesser white-toothed shrew forms other than *C. sibirica* [1], giving origin to the idea that all populations with $2n = 40$ should be combined in a single species, *C. suaveolens* [2, 3]. However, there is evidence for morphological and high molecular diversity of the group [4–10]. At least six forms are now recognized within the lesser white-toothed shrew species group by morphology or mitochondrial DNA (mtDNA), but the views on their species status vary among different researchers. The forms recognized commonly include *C. suaveolens* Pallas, 1811; *C. sibirica* Dukelsky, 1930; *C. güeldenstaedtii* Pallas, 1811; *C. caspica* Thomas, 1907; *C. shantungensis* Miller, 1901; and *C. zarudnyi* Ognev, 1928 [4, 8, 11]. The species status is sometimes assigned to *C. mimula* Miller, 1901 from Western Europe [8] and *C. aleksandrisi* Vesmanis, 1977 from Cyrenaica on the Mediterranean coast of North Africa [11]. Some researchers include *C. mimula* as a subspecies in *C. güeldenstaedtii* together with *C. g. iculisma* Mottaz, 1908 and *C. g. cypria* Bate, 1903 [11]. All of these forms (except *C. sibirica* [12]) and populations of the Aegean coast and central and eastern Iran constitute separate phylogroups based on mitochondrial data [10]. Data on nuclear DNA marker variation are nearly absent, and it is therefore unclear to which extent mtDNA reflects the overall genome differentiation of the forms.

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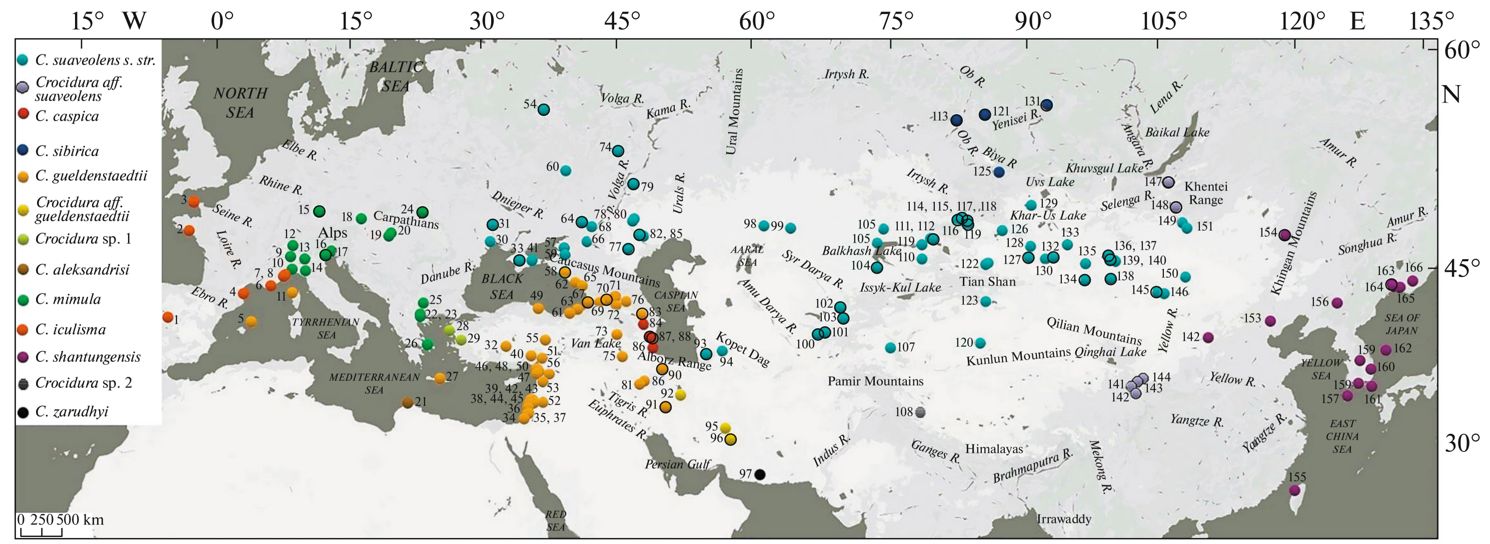

Fig. 1. Localities of the samples used in this work: 1, Candelaria, Spain; 2, Hoedic Island, France; 3, Sark Island, the United Kingdom; 4, Figuerasses, Spain; 5, Menorca, Spain; 6, Porquerolles Island, France; 7, Ventimiglia, Italy; 8, Fraitusa, Badalucco, Italy; 9, Vercelli, Italy; 10, Varazze, Italy; 11, Corsica Island, France; 12, Gordevio, Switzerland; 13, San Nicolo, Piacenza, Italy; 14, Fivizzano, Italy; 15, Germany; 16, Venice, Italy; 17, Latisana, Italy; 18, Vienna, Austria; 19, Fülöpháza, Hungary; 20, Hungary; 21, Wadi el-Huf, Libya; 22, Epanomi, Greece; 23, Thessaloniki, Greece; 24, Volovets, Ukraine; 25, Sandanski, Bulgaria; 26, Athens, Greece; 27, Crete, Greece; 28, Lesbos, Greece; 29, Yukarikizilca, Izmir, Turkey; 30, Odessa, Ukraine; 31, Nikolaev oblast, Ukraine; 32, Konya, Turkey; 33, Crimea; 34, Tse'elim, Israel; 35, HaShfela, Israel; 36, Israel; 37, Mata, Israel; 38, Achziv, Israel; 39, Ein Ya'akov, Israel; 40, Adana, Turkey; 41, Feodosia, Crimea; 42, Tsiv'on, Israel; 43, Safed, Israel; 44, Moshavat Kinneret, Israel; 45, Sea of Galilee, Israel; 46, Ras al-Bassit, Syria; 47, Jableh, Syria; 48, Rabia, Syria; 49, Chakally, Samsun, Turkey; 50, Slinfah, Syria; 51, Asagikarafakili, Turkey; 52, Qanawat, As-Suwayda, Syria; 53, Qattinah, Syria; 54, Moscow oblast, Russia; 55, Tanir, Kahramanmaraş, Turkey; 56, Hama, Syria; 57, Sea of Azov, Russia; 58, Tuapse, Krasnodar oblast, Russia; 59, Krasnodar oblast, Russia; 60, Voronezh, Russia; 61, Altindere, Turkey; 62, Gagra, Abkhazia; 63, Rize, Turkey; 64, Rostov oblast, Russia; 65, Sukhumi, Abkhazia; 66, Sal'sk, Russia; 67, Batumi, Adjara, Georgia; 68, Tsimlyanks, Russia; 69, Akhaltsikhe, Georgia; 70, Kakheti, Georgia; 71, Dusheti, Georgia; 72, Shulaveri, Georgia; 73, Bastam, West Azerbaijan, Iran; 74, Penza oblast, Russia; 75, Mohammadyar, Iran; 76, Alazani Valley, Georgia; 77, Chyornye Zemli Nature Reserve, Kalmykia, Russia; 78, Peski Shkili, Astrakhan oblast, Russia; 79, D'yakovka village, Saratov oblast, Russia; 80, Lake Baskunchak, Russia; 81, Bisotun, Iran; 82, Astrakhan oblast, Russia; 83, Ghizil-Agaj, Azerbaijan; 84, Azerbaijan; 85, Dosang, Russia; 86, Alanjin, Hamadan, Iran; 87, Siyov, Astara, Azerbaijan; 88, Burjdali, Lankaran, Azerbaijan; 89, Esalem, Gilan, Iran; 90, Faizabad, Qazvin, Iran; 91, Bakhshabad, Bakhtiari, Iran; 92, Esfidan, Isfahan, Iran; 93, Imer, Golestan, Iran; 94, Darkesh, Khorasan, Iran; 95, Kerman, Iran; 96, Jebal Barez mountain range, Kerman, Iran; 97, Pir Sohrab, Iran; 98, Aralsk, Kazakhstan; 99, Koskol, Kazakhstan; 100, Hissar Nature Reserve, Uzbekistan; 101, Saritag River, Tajikistan; 102, Chatkal Nature Reserve, Uzbekistan; 103, Pokhtapor, Tajikistan; 104, Chu-Ili Range, Kazakhstan; 105, northwestern Balkhash, Kazakhstan; 106, northern Balkhash, Kazakhstan; 107, Tashkurgan, Xinjiang, China; 108, Zanda, China; 109, Bel'-Saksaul, Kazakhstan; 110, Kapal, Kazakhstan; 111, Almaty oblast, Kazakhstan; 112, Shibindy, Kazakhstan; 113, Novosibirsk, Russia; 114, Tassay, Kazakhstan; 115, Irtysh River, Kazakhstan; 116, Aigyrkum, Kazakhstan; 117, Aksuat, Kazakhstan; 118, Shekelmes, Kazakhstan; 119, Tugyl, Kazakhstan; 120, Cherchen, Xinjiang, China; 121, Kemerovo, Russia; 122, Xinjiang, China; 123, Korla, Xinjiang, China; 124, Mosowan, Xinjiang, China; 125, Lake Teletskoye, Altai, Russia; 126, Altay County, Xinjiang, China; 127, Baitag Bogd Uul, Mongolia; 128, River Bungal Gol, Mongolia; 129, Shaazgai-Nuur Lake, Mongolia; 130, Takhin-Shara-Nuru Range, Mongolia; 131, western Krasnoyarsk Pillars, Russia; 132, Gun-Tamga-Bulag, Mongolia; 133, Sharga, Govi-Altai Province, Mongolia; 134, Barun Shargiin Gol, Mongolia; 135, Ikh Dzarmag, Mongolia; 136, Ba-Tsagaan, Bayanknongor Province, Mongolia; 137, southern lope of Baga Bogd Uul, Bayanknongor Province, Mongolia; 138, Dzulganai Oasis, Mongolia; 139, Orog Lake, Mongolia; 140, Ikh Bogd Uul, Mongolia; 141, Goinba Monastery, southern Gansu, China; 142, Zoige, Sichuan, China; 143, Lianhuashan, southern Gansu, China; 144, Lianhuashan Nature Reserve, southern Gansu, China; 145, Gashuun Bulag, Gobi Desert, Mongolia; 146, Tavan-Aldyn Khudun, Mongolia; 147, Buryatia, Russia; 148, Selenge Province, Mongolia; 149, Khar-Yamat, Bayan-Ovoo District, Mongolia; 150, Hatan Bulag District, Mongolia; 151, Bayan Uul, eastern Khantey, Mongolia; 152, Ningwu, Shanxi, China; 153, Zunhua, Hebei, China; 154, Khingan Range, Dornod Province, Mongolia; 155, Taichung, Taiwan; 156, Liaoning, Xinbin, China; 157, Jeju Island, South Korea; 158, coastal islands, South Korea; 159, South Korea; 160, Gyeongju, South Korea; 161, Tsushima Island, Japan; 162, Ulleungdo Island, South Korea; 163, southern Primorsky Krai, Russia; 164, Popov Island, Primorsky Krai, Russia; 165, Putyatin Island, Primorsky Krai, Russia; 166, Lazovsky Nature Reserve, Primorsky Krai, Russia. The localities of samples included in nuclear gene analysis have black outlines.

MATERIALS AND METHODS

In total, 420 samples were used in our work; their geographic localities are shown on a map (Fig. 1). The *cytb* mitochondrial gene sequence (1140 bp) was obtained for 11 samples. For 91 samples, we sequenced 16 nuclear loci: *ABHD11, ApoB, BDNF, BRCA1, GHR10, ITPK1, MCGF, NHSL1, PALLD, PTGER4, RAG1, RAI14, ROGDI2, SORBS2, SPECC1L,* and *vWF* (GenBank accession nos. OP599577–OP599587, OP599602–OP599612, OQ374926–OQ376279). To perform a *cytb* phylogenetic analysis, 398 sequences were retrieved from GenBank (accession nos. AB077075–AB077090, AB0770278–AB077280, AY843448–AY843461, AY843487–AY843500, AY843502, AY843511, AY994368–AY994370, AY994372, AY994373, AY994375–AY994377, AY994386–AY994389, DQ059023, DQ242541, DQ630057–DQ630061, DQ630064–DQ630106, DQ630108, DQ630110–DQ630112, DQ630114–DQ630118, DQ630120, DQ630121, DQ630395, DQ641270, EU742583–EU742594, EU742605–EU742614, HM586991–HM586996, KX354172–KX354178, KX354180, KX354181, LR536317–LR536326, LR536367, LR536372, MF136304–MF136385, MF152782, MN690925–MN690944, MN691017, MW297680–MW297698, MW297700–MW297721, MW297723–MW297727, MW297729–MW297733, MW297735–MW297757, and MW297759–MW297791). Sequences of the following species were used as outgroups in mitochondrial and nuclear sequence analyses: *C. dsinezumi* (AB077274 and AB077277), *C. lasiura* (AB077072, MW381915, MW381936, MW381956, MW381979, MW382002, MW382020, MW382042, and MW410130), *C. leucodon* (MW381916, MW381937, MW381980, MW382003, MW382021, MW382043, and MW410131), and *C. zaitsevi* (OL451379, OL451380, OL451373, OL451417, OL451434). Additional material was included in the mitochondrial gene analysis; i.e., sequences of larger intraspecific samples and sequences of *C. suaveolens* s. l. forms (*zarudnyi*, *aleksandrisii*, and *iculisma*), which were absent from our sample, were used to assess the diversity of mitochondrial lineages in the group.

DNA was isolated, amplified, and sequenced as described previously [12, 13]. Primers to amplify *cytb* and *BRCA1* were as in [13]; primers to amplify the other loci were as in [14, 15]. A substantial part of the data was obtained by Sanger sequencing of the PCR products. The *ABHD11, ITPK1, NHSL1, PALLD, RAI14, ROGDI2, SORBS2,* and *SPECC1L* sequences of the vast majority of the samples and the *ApoB, BDNF, BRCA1, GHR10, MCGF, PTGER4, RAG1,* and *vWF* sequences of fewer samples were obtained by sequencing on the Illumina MiSeq platform. Libraries were obtained using the PCR products and an Illumina kit as recommended by the manufacturer; the PCR products of all loci were pooled in equal proportion for each sample separately. The primer and adapter sequences and low-quality bases were removed from the resulting reads using the program Trimmomatic v. 0.33 [16]. The programs bowtie v. 1.1.2 [17] and samtools [18] were used to map the reads to reference sequences of *C. suaveolens* s. l. or, in the case of individual loci sequenced for the first time in this work, GenBank sequences of closely related species (*C. indochinensis* and *Sorex araneus*) with their subsequent replacement with consensus sequences of the respective loci. Variant calling was performed using GATK 4.1.2.0 [19]; consensus sequences were constructed using bcftools [20]; positions where indels were observed were completely replaced with N in sequence alignments. Bases covered with less than 40 reads were considered not read. Consensus sequences were verified using IGV 2.11.2 [21]. Sequences were aligned using BioEdit 7.2.5 and MEGA 11.0.8.

To determine the allelic composition of the species, nuclear sequences were phased using the Phase module of the package DnaSP 5.10.1 [22] and refined using IGV. Population groups were delimited in STRUCTURE 2.3.4 [23] (Admixture model), using 2.5 million MCMC iterations and 250 000 burn-in iterations. The diversity structure was tested by varying the number of clusters from 4 to 10; the optimal number of clusters was then determined using Structure Harvester [24].

Phylogenetic trees were constructed with the maximum likelihood (ML) method in IQTREE 1.6.9 [25] and Bayesian inference (BI) in Mr.Bayes 3.2.6 [26]. Individual gene trees and 5000 bootstrap replicas at all of the 16 nuclear loci were used to construct a species tree in ASTRAL 5.15.5 [27].

RESULTS

Mitochondrial Phylogeny of C. suaveolens s. l.

Twelve mitochondrial lineages were observed in *C. suaveolens* s. l. in a *cytb* tree (Fig. 2a): (1) *C. suaveolens* s. str. + *C. sibirica*; (2) *Crocidura aff. suaveolens,* which included haplotypes from southern Gansu and Sichuan; (3) *C. caspica*; (4) *C. iculisma*; (5) *C. aleksandrisi;* (6) *güeldenstaedtii*; (7) *C. aff. güeldenstaedtii,* which included haplotypes from central and eastern Iran; (8) *Crocidura* sp. 1, which included haplotypes from islands of the Aegean Sea and nearby Anatolia regions; (9) *C. mimula;* (10) *C. shantungensis,* including haplotypes from northern and central Mongolia (Khentei) and Buryatia; (11) *C. zarudnyi*; and (12) *Crocidura* sp. 2, which was a sister lineage to the previous one and included haplotype from Zanda of western Tibet. *Crocidura shantungensis* and *C. zarudnyi/Crocidura* sp. 2 were the closest to the root, but their relationships were not resolved. The other species showed distinct separation into two clades associated with particular geographical areas, western and eastern.

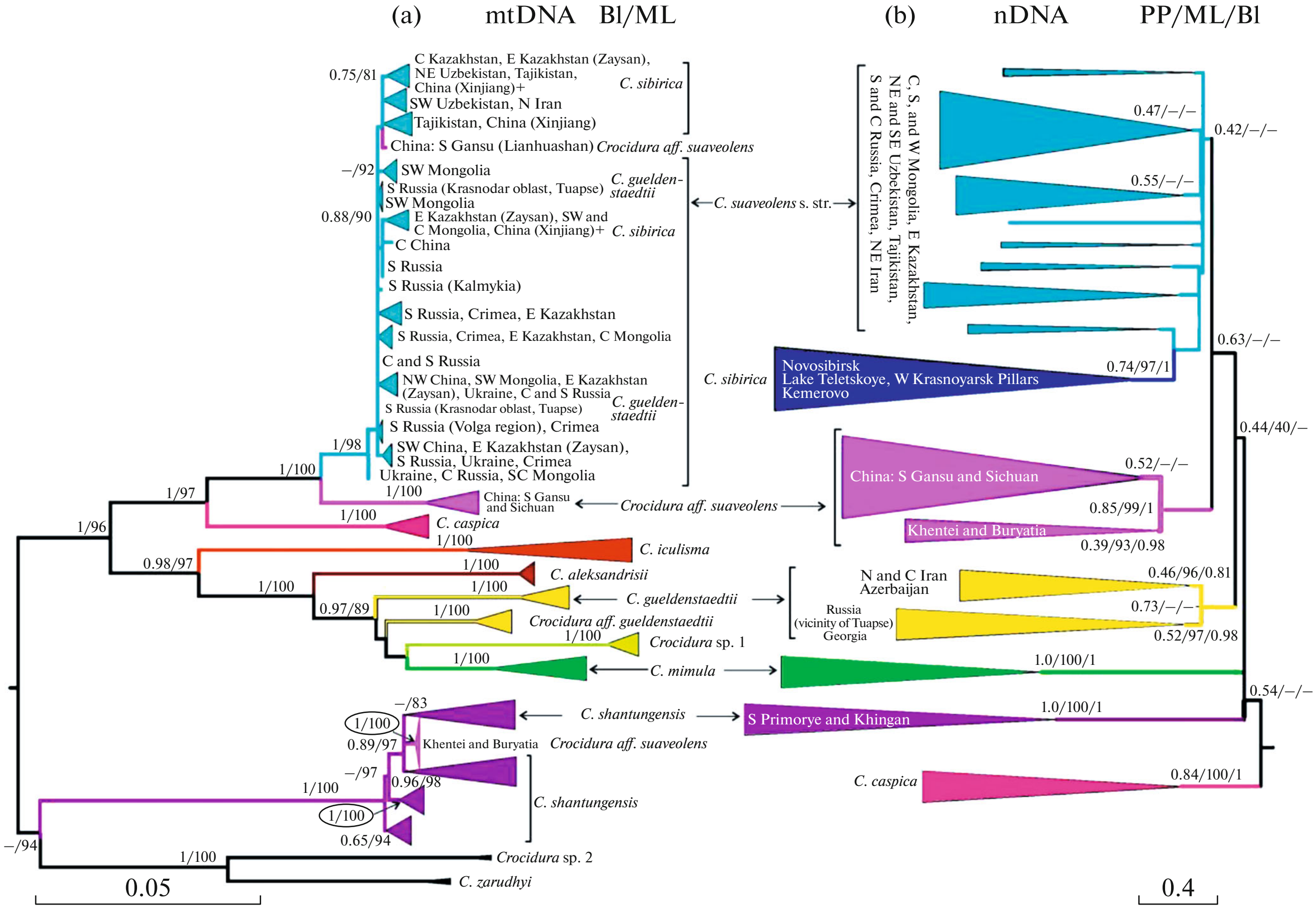

Fig. 2. (a) Phylogenetic tree constructed for the *C. suaveolens* s. l. species group by BI analysis of the *cytb* mitochondrial gene. Bayesian posterior probability (BPP) and bootstrap support obtained in ML analysis are shown at respective nodes. (b) Species tree constructed for the *C. suaveolens* s. l. group in the program ASTRAL 5.15.5 on the basis of data on the 16 nuclear genes. Local posterior probability (PP) calculated by the program, bootstrap support obtained in ML analysis, and BPP obtained in an analysis of concatenated nuclear gene sequences (PP/ML/BPP) are shown at respective nodes. The outgroup was removed from each tree.

The eastern clade included East European and Asian populations of *C. suaveolens* s. str., *C. aff. suaveolens, C. sibirica,* and *C. caspica*. *Crocidura caspica* formed confidently a sister branch to the group *C. aff. suaveolens*/(*C. suaveolens* s. str. + *C. sibirica*). Haplotype from the *C. sibirica* range fell within the *C. suaveolens* s. str. haplogroup without forming a monophyletic group; the same was observed for *C. güeldenstaedtii* haplotypes from the Black Sea coast of the Caucasus (Tuapse).

The western clade included shrews from West and Central Europe, Middle East, islands of the Aegean Sea, and nearby Anatolian regions. They formed several haplogroups related to *C. güeldenstaedtii* and *C. mimula*. A basal position in the western clade was occupied by *C. iculisma* (the Iberian Peninsula, southern and western France).

Nuclear Data Analysis with the STRUCTURE Program

Unstable results were obtained with the STRUCTURE program; i.e., different grouping patterns were produced in repeated analyses at the same number of clusters (K). The optimal K value yielded by Harvester corresponded to subdivision into five groups (Fig. 3). In the best solution (with maximum Ln Prob) obtained with this K value, *C. shantungensis*, *C. aff. suaveolens,* and *C. güeldenstaedtii* each formed a separate cluster, and two other clusters combined *C. suaveolens* s. str. + *C. sibirica* and *C. mimula* + *C. caspica*. In the best solution obtained at K = 6, *C. mimula* and *C. caspica* formed separate clusters. At K = 7, *C. sibirica* was separated from *C. suaveolens* s. str. At K = 8, the clustering pattern was the same as at K = 7. A noticeable gene exchange signal was observed only between *C. sibirica* and *C. suaveolens* s. str.

Phylogenetic Analysis of Nuclear Data

Species tree. An analysis with the Astral program was carried out to construct a species tree in the case where gene trees might differ significantly. Six groups with unresolved between-group relationships were observed in the resulting tree (Fig. 2b): (1) *C. shantun-*

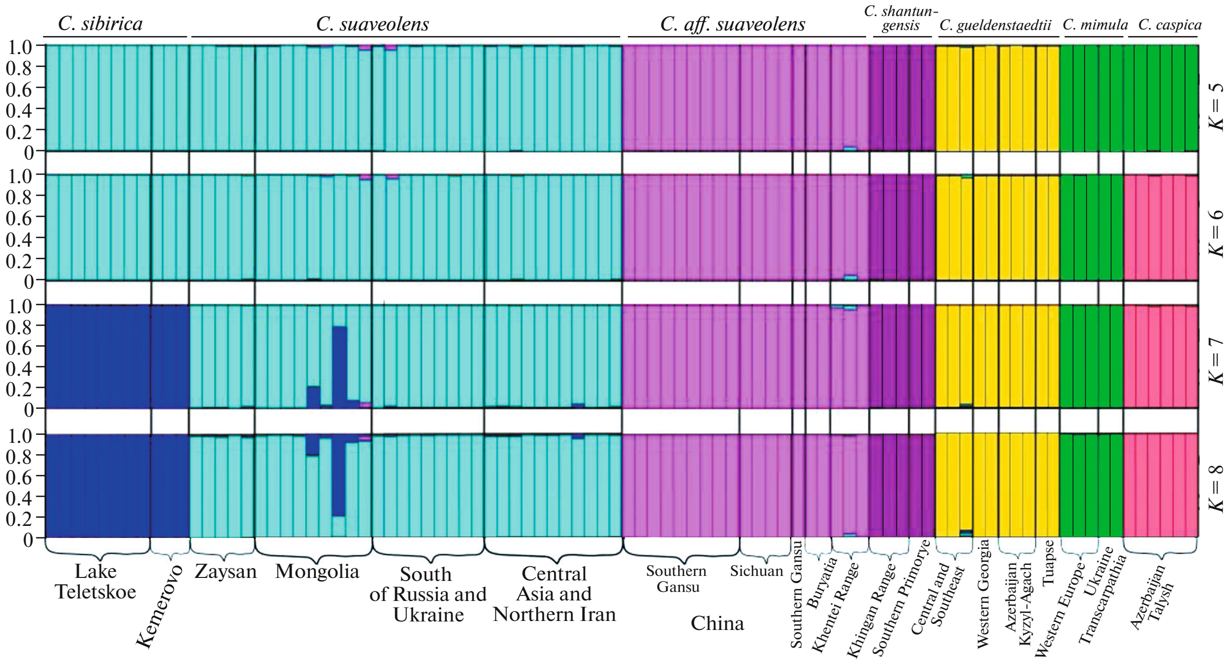

Fig. 3. Analysis of the allele frequencies of 14 nuclear genes in the *C. suaveolens* s. l. species complex with the program Structure 2.3.4.

gensis (southern Primorskii Krai and Khingan); (2) *C. mimula* (Ukrainian Transcarpathia and Western Europe); (3) *C. güeldenstaedtii*, including two sister lineages: one from northern and central Iran and Azerbaijan and the other from Georgia and the Black Sea coast of the Krasnodar oblast of Russia; (4) *C. caspica*; (5) *C. aff. suaveolens* (China, Buryatia, and Khentei); and (6) *C. suaveolens* s. str. + *C. sibirica*. Monophyly of group (6) has a low support; *C. sibirica* forms a well-supported cluster within the group.

ML and BI analyses of a concatenated sequence of the nuclear genes. In a phylogenetic tree based on ML and BI analyses of the concatenated nuclear gene sequence (the tree is not shown), the group composition within *C. suaveolens* s. l. only slightly differed from that observed in the species tree (Fig. 3b). Two *C. güeldenstaedtii* branches did not form a single clade; *C. caspica* and *C. sibirica* formed well-supported monophyletic groups and were positioned between numerous *C. suaveolens* s. str. branches, which lacked geographical associations. The grouping of *C. suaveolens* s. str. + *C. caspica* + *C. sibirica* had only minor support in the BI analysis and totally lacked support in the ML analysis (0.74/27).

DISCUSSION

Several discrete forms occur within the *C. suaveolens* s. l. species complex according to the multilocus phylogenetic tree. While the order of their divergence remained unclear, a distinct nature is evident for the majority of the forms.

Crocidura mimula is an isolated monophyletic form, which was represented by few shrews from different parts of the range in our study. The species inhabits Ukrainian Transcarpathia according to our data. Signs of hybridization between *C. mimula* and *C. suaveolens* s. str. were not detected.

Two sister lineages were found in *C. güeldenstaedtii* according to the nuclear data, one inhabiting the north-western and the other, the eastern parts of the range. Shrews of the north-western lineage from the Black Sea coast of the Caucasus (Tuapse region) carry mtDNA introgressed from *C. suaveolens* s. str. However, no sign of introgression was detected in the nuclear genes. This might be explained by recent hybridization with mtDNA introgression, as is evident from the finding that mitochondrial haplotypes of *C. güeldenstaedtii* from the Black Sea coast of the Caucasus are fully identical to *C. suaveolens* s. str. haplotypes.

Crocidura suaveolens s. str. is paraphyletic to *C. sibirica* and is extremely poorly structured, which is in line with the mitochondrial data [7, 12]. *Crocidura caspica* is monophyletic according to both mitochondrial and nuclear data, but the latter are insufficient for reconstructing its phylogenetic relationships with other species of the *C. suaveolens* s. l. species complex.

Well-supported monophyly of *C. sibirica* is a substantial difference from the mitochondrial tree. We have previously assumed from mitochondrial data that

C. sibirica formed in a period of its isolation from *C. suaveolens* s. str. approximately 20 thousand years ago [12]. Our nuclear gene analysis generally supports the hypothesis of a recent origin of *C. sibirica* and indicates that one of the *C. sibirica* mitochondrial lineages might be authentic. Lack of monophyly is observed for *C. sibirica* by mtDNA data possibly because some of its mitotypes have been acquired as a result of a secondary contact with *C. suaveolens* s. str. Thus, *C. sibirica* is very closely related to *C. suaveolens* s. str., although having some specifics of the nuclear genome. The level of its genetic divergence does not correspond to a species level. Genetic data do not give grounds to assign the status of a full species to *C. sibirica*.

The most intricate problem is how *C. aff. suaveolens* is related to other groups of the species complex. In the mitochondrial tree, this form includes only samples from southern Gansu and Sichuan (China) and is a sister group to *C. suaveolens* s. str./*C. sibirica*. A similar trend is seen in the nuclear tree, but the respective group additionally includes the Buryatia and Khentei samples, which form a haplogroup within *C. shantungensis* in the mitochondrial tree. Samples from southern Primorye and Khingan have a true *C. shantungensis* mitotype and form a separate clade corresponding to *C. shantungensis* in the nuclear tree as well. Thus, shrews from Buryatia and Khentei belong to *C. aff. suaveolens*, but their mtDNA seems to be introgressed from *C. shantungensis* in the past. Interestingly, an opposite situation also occurs: a shrew from a lower part of mountains of Lianhuashan (southern Gansu) had a common *C. suaveolens* mitotype, but grouped with *C. aff. suaveolens* from a subalpine mountain belt of norther Sichuan in the nuclear tree. The finding indicates that events of *C. aff. suaveolens* hybridization not only with *C. shantungensis,* but with *C. suaveolens* as well took place in various parts of the range at various times (and may still take place). Surprisingly, there is a vast gap in the *C. aff. suaveolens* range; i.e., only typical *C. suaveolens* s. str. is found in southern Mongolia. The introgression direction and the origin and status of *C. aff. suaveolens* need further detailed investigation.

Thus, given that *C. suaveolens* s. str. and *C. shantungensis* mtDNAs had probably introgressed in the *C. aff. suaveolens* genome and that hybridization between *C. suaveolens* s. str. and *C. güeldenstaedtii* recently occurred in the eastern part of the Black Sea coast, the nuclear phylogeny is generally consistent with the mitochondrial phylogeny. However, the innermost nodes have low support in the mitochondrial tree and are absent in the nuclear tree, making it impossible to reliably reconstruct the sequence of cladogenetic events in the evolutionary history of *C. suaveolens* s. l.

To summarize, the *C. suaveolens* s. l. species complex provides an illustrative example of a recent series of speciation events, in which important roles were played by both divergent and reticular processes. Divergence of the unquestionable species of the complex occurred no more than 1.3 million years ago (*C. shantungensis*) and no less than 680 thousand years ago (*C. caspica*/(*C. suaveolens* s. str. + *C. sibirica*)) according to mitochondrial estimates [8], and extremely recent divergence of the C. *suaveolens* s. str. + *C. sibirica* lineages was mentioned above. To resolve phylogenetic tree branches for a group that has recently underwent rapid diversification accompanied by reticular events, it is necessary to greatly increase the number of informative genetic loci involved in the analysis. We intend to develop such markers in our further research.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflicts of interest.

Statement on the welfare of animals. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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