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CYTOGENETIC AND MOLECULAR RELATIONSHIPS BETWEEN ZARUDNY'S ROCK SHREW (*CROCIDURA ZARUDNYI*; MAMMALIA: SORICOMORPHA) AND EURASIAN TAXA

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We karyotyped and sequenced 1,140 base pairs of the mitochondrial DNA cytochrome *b* of a specimen of Zarudny's rock shrew (*Crocidura zarudnyi*) from Baluchestan, southeastern Iran, to clarify its cytogenetic and molecular relationships with other Eurasian species of *Crocidura*. According to the karyotype (2N = 40, FN = 50), Zarudny's rock shrew belongs to the group of the lesser white-toothed shrew (*C. suaveolens*), which is different from other known crocidurine karyotypes, considering the combination of the diploid and fundamental number of chromosomes. Molecular results revealed that *C. zarudnyi* is included in a monophyletic clade with the *C. suaveolens* group, where it is a sister taxon to the others (mean Kimura 2-parameter distance = 9.7%).

Key words: *Crocidura*, cytogenetics, Iran, mitochondrial DNA, systematics

Within the family Soricidae (order Soricomorpha), *Crocidura* is the largest genus, represented by about 170 species distributed throughout Eurasia and Africa. Not surprisingly, the taxonomic status of many rare species commonly remains uncertain (Hutterer 2005). This is also the case for some shrews in the Middle East, for example, Zarudny's rock shrew (*Crocidura zarudnyi* Ognev, 1928), whose original description was based on a single specimen collected from the modern-day Iranian province of Baluchestan, on the Pakistan border. Ellerman and Morrison-Scott (1951) and Lay (1967) considered Zarudny's rock shrew to be a subspecies of the pale gray shrew (*C. pergrisea* Miller, 1913). However, later authors who studied additional specimens of Middle Eastern white-toothed shrews that also included specimens of *C. zarudnyi* from Iran, Afganistan, and Pakistan considered *C. zarudnyi* to be a valid species (e.g., Hassinger 1970, 1973; Jenkins 1976; Kryštufek and Vohralík 2001; Spitzenberger 1971). This view also was adopted by Hutterer (1993, 2005) and Wolsan and Hutterer (1998). The known distribution of *C. zarudnyi* includes southeastern Iran, southwestern Pakistan, and Afghanistan (Hutterer 2005). So far, fewer than 20 specimens of the species have been collected. During a field trip to Iran, PN and VV collected a very small shrew identified as *C. zarudnyi*; in this paper we describe its karyotype and phylogenetic position within Eurasian crocidurine shrews.

MATERIALS AND METHODS

On 12 April 2000, we collected a specimen of *Crocidura* in Pir Sohrab, Baluchestan, southeastern Iran (25°45′N, 60°50′E, elevation 130 m). The shrew was trapped in an abandoned 2-m-deep lair dug by the crocodile *Crocodylus palustris* in the sheer bank of a temporarily dry riverbed. The habitat was a sandy-loess riverbank covered by dense reeds. We used the direct treatment of bone marrow cells for karyotype analysis, following the standard method modified after Ford and Hamerton (1956). The slides were conventionally stained by 5% Giemsa, and 50 metaphase chromosome preparations were analyzed. We used the chromosome nomenclature of Zima and Král (1984). The voucher specimen and microscopic slides are deposited in the Department of Zoology, Charles University, Prague, Czech Republic, under no. I-89.

DNA was extracted from muscle tissue using a Qiagen kit (QIA Amp DNA Mini Kit; Qiagen, Valencia, California). Double-stranded DNA amplifications of the mitochondrial cytochrome-*b* (*Cytb*) gene were performed with the primers Cytb3 (5′-TAT TCT CCC CAG ACA TAT TAG G-3′), Cytb6 (5′-CTT GAA ACA TGA AAC ATT GG-3′), and Cytb7 (5′-AAT AGA AAA TAT CAT TCT GG-3′), which are specific to the genus *Crocidura* and were designed in our laboratory, and

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L14724, H15149, and H15915 (Irwin et al. 1991). Amplification conditions consisted of 35 thermal cycles with the primers L14724/H15149, Cytb6/Cytb7, and Cytb3/H15915 under the following conditions: 30 s denaturation at 94°C, 45 s annealing at 50°C, and 60 s extension at 72°C. Polymerase chain reaction products were then electrophoresed on a 1% agarose gel, visualized with ethidium bromide staining to verify polymerase chain reaction quality, and purified by centrifugal dialysis using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. Cycle sequencing was performed on a 10-μl sample of 1–3 μl of amplified DNA, 1 μl of 10 μM primer, and 4 μl of ABI PRISM Dye Terminator 1 (Perkin Elmer, Boston, Massachusetts); and water was added to bring the reaction volume to 10 μl. Reaction sequences were visualized on an ABI 3100 genetic analyzer (Applied Biosystems, Foster City, California).

To clarify the genetic relationship between *C. zarudnyi* (AY925211) and other Eurasian crocidurine shrews, additional DNA sequences were obtained from GenBank (Appendix I). Nucleotide sequences were aligned by eye; no insertions or deletions were observed.

Two methods of phylogenetic analyses were carried out using PAUP version 4.0b10 PPC (Swofford 1998). Maximum-parsimony analyses were performed with the following options: heuristic search, stepwise addition of sequences, 10 replicates of random addition of taxa, and tree-bisection-reconnection branch swapping (Swofford 1998). Support values were estimated using 1,000 bootstrap resamples using the same heuristic settings. All codon positions were equally weighted. The tree was rooted using sequences of the Eurasian water shrew (*Neomys fodiens*; DQ065611) and the Caucasian pygmy shrew (*Sorex volnuchini*; DQ065610).

For maximum-likelihood analysis, we 1st used likelihood ratio tests implemented via the computer program MODELTEST 3.06 to choose the mutation model that best fits the data according to the protocol of Posada and Crandall (1998). Tests were conducted on the total fragment of 1,140 base pairs (bp). The TRn+I+G model was selected. Maximum-likelihood analyses were then performed, assuming this model. Estimated parameters from the data were as follows: the proportion of invariable sites is 0.5435, the unequal distribution of rates at variable sites (γ) is 1.5224, and 4 different substitution types (rate [A–C] = 0.9483, rate [A–G] = 9.9527, rate [A–T] = 1.2830, rate [C–G] = 0.3757, rate [G–T] = 1.0000, rate [C–T] = 20.6991). Maximum-likelihood bootstrap analyses (1,000 replicates) were performed using the software phyML version 2.4.4. (Guindon and Gascuel 2003).

Bayesian analysis was conducted using MrBayes version 3.0 b4 (Huelsenbeck and Ronquist 2001), which performs Metropolis-coupled Markov chain Monte Carlo analysis. A GTR model was used with an among-site rate variation following a gamma distribution. The Markov chain was run for 1,000,000 generations and sampled once every 100 generations; burn-in was set at 100,000 generations. To assure convergence in the Bayesian analyses, 2 independent runs were performed and compared.

The molecular clock hypothesis was tested following Posada and Crandall (1998). Estimation of divergence time from the molecular data was performed according to the calibration developed for the Soricidae by Fumagalli et al. (1999).

Results

Morphological identification.—Our specimen was a tiny, long-tailed, and gracile shrew. Its mass was 5 g, head and body length was 68 mm, tail length (taken from the anus, without terminal hairs) was 47 mm, hind-foot length (without claws) was 12.0 mm, and ear length was 9 mm. The ratio of tail length to head and body length was therefore 69%. The specimen was an adult female with signs of previous lactation but only slightly worn teeth. Principal skull measurements were as follows: condylobasal length = 16.9 mm, zygomatic breadth = 5.5 mm, maximum braincase breadth = 7.7 mm, P4-M3 length = 4.5 mm, height of the mandible taken across the coronoid process = 4.4 mm. The back was pale brown with reddish shades and the belly was whitish; there was no clear demarcation line. The tail was bicolored, with the upper part being pale brown and the under part whitish gray. White hairs covered fairly conspicuous ears.

Species identification was based mostly on comparison with the very detailed description of the type specimen by Ognev (1928). The coloration, body and skull measurements, and shape of the skull of our specimen fit the type description well. Measurements of our specimen were also consistent with those given by Hassinger (1970) for the series of *C. zarudnyi* from Iran, West Pakistan, and Afganistan (Table 1).

Other species reported from the Middle East, such as the lesser white-toothed shrew (*C. suaveolens* (Pallas, 1811)), the pale gray shrew (*C. pergrisea* Miller, 1913), the lesser rock shrew (*C. serezkyensis* Laptev, 1929), and the Iranian shrew (*C. susiana* Redding and Lay, 1978), were excluded for the following reasons. *C. pergrisea* (type locality in Kashmir) is larger, with its condylobasal length being at least 19.0 mm (Hassinger 1973; Jenkins 1976; see Table 1). *C. susiana* (type locality in Khuzestan, northwestern Iran) is rather large, its condylobasal length exceeds 20.5 mm, and the tail is not bicolored (Redding and Lay 1978). In Table 1 it is clearly demonstrated that there is no overlap between available specimens of *C. zarudnyi* and the type series of *C. pergrisea* and *C. susiana* in any measurement. *C. serezkyensis* (type locality in the Pamir Mountains, Tajikistan) has a deeply grooved posterior edge on the 3rd upper unicuspid (Zaitsev 1993). The 3rd upper unicuspid of our specimen was grooved only very slightly as is common, for example, in *C. suaveolens*. Further, *C. suaveolens* (type locality Crimea, Ukraine) can be excluded because the divergence in C*ytb* sequence observed between our specimen of *C. zarudnyi* and *C. suaveolens* from Crimea (Kimura 2-parameter distance = 9.75 %) clearly points out that they belong to different species.

Karyological result.—In the examined specimen, the karyotype diploid number (2N) = 40 chromosomes, fundamental number (FN) = 50 was found. Fifteen autosomal pairs were gradually smaller acrocentrics and 4 pairs of autosomes were biarmed (Fig. 1). One pair of biarmed autosomes was subtelocentric, and was the largest of the entire group. The

Table 1.—Measurements (mm) of *Crocidura zarudnyi*, *C. pergrisea*, and *C. susiana* from the Middle East. Means are given with minimum and maximum values in parentheses.

	C. zarudnyi				
	Holotype[a]	Our specimen[b]	Series, $n = 11–15$[c]	*C. pergrisea*, type series, $n = 3$[d]	*C. susiana*, type series, $n = 4–7$[e]
Total length	108.3	115	104.7 (92–116)	127.3 (125–129)	138.3 (134–148)
Tail length	47.7	47	—	53.3 (53–54)	58.6 (56–63)
Condyloincisive length	18.1	17.5	18.1 (16.9–19.0)	—	20.7 (20.6–21.0)
Condylobasal length	—	16.9	— (16.5–18.2)	19.1 (19.0–19.2)	—
Maximum braincase breadth	8.3	7.7	7.9 (7.5–8.3)	8.8 (8.8–8.8)	9.3 (9.0–9.4)
Zygomatic breadth	—	5.5	—	5.9 ($n = 1$)	5.9 (5.7–6.4)

[a] Ognev 1928.
[b] This study.
[c] Hassinger 1970 (specimens from Iran, West Pakistan, and Afganistan).
[d] Spitzenberger 1971.
[e] Redding and Lay 1978.

other 3 pairs were medium sized, with various positions of the centromere: 1 pair was subtelocentric, 1 submetacentric, and 1 metacentric. The X chromosome was a large submetacentric.

Phylogenetic analyses.—The 37 sequences of 1,140 bp used in this study showed 490 variable sites of which 417 were parsimony-informative; we found 1 most-parsimonious tree with a length of 2,339 steps. Interspecific pairwise sequence divergences between Crocidurinae species (Kimura 2-parameter distances) varied from 0.032 (savanna path shrew [*C. viaria*] versus African giant shrew [*C. olivieri*]) to 0.205 (Canarian shrew [*C. canariensis*] versus Dsinezumi shrew [*C. dsinezumi*]). Pairwise sequence divergences between *C. zarudnyi* and other species of *Crocidura* varied from 0.089 (*C. suaveolens* 8) to 0.182 (*C. olivieri*).

The phylogenetic relationship among haplotypes is given in Fig. 2. In all phylogenetic analyses, 6 well-defined clades were found. The 1st included the samples of the *C. suaveolens* group (Asian lesser white-toothed shrew [*C. shantungensis*] and *C. suaveolens*; for more details about the systematics of this group, see Dubey et al. [2006]) and *C. zarudnyi*, 2N = 40, FN = 50, which is the sister taxon to the latter species; this was supported by maximum-likelihood and maximum-parsimony bootstrap values of 100%, and Bayesian posterior probability of 1.0. The 2nd included samples with 2N = 40, FN = 56 chromosomes (*C. dsinezumi*, the Ussuri white-toothed shrew [*C. lasiura*], and Kuroda's shrew [*C. kurodai*]; bootstrap of 100% and 94%, respectively for maximum likelihood and maximum parsimony, and Bayesian posterior probability of 1.0). The 3rd included samples of Horsfield's shrew (*C. horsfieldii*; 2N = 40, FN = 54), and the lesser Ryukyu shrew (*C. watasei*; 2N = 26, FN = 52), and was supported by bootstrap values for maximum likelihood and maximum parsimony of 100%, and Bayesian posterior probability of 1.0. The 4th included the samples with 2N = 38, FN = 56 chromosomes (the black-footed shrew [*C. nigripes*] and the thick-tailed shrew [*C. brunnea*]; bootstrap values of 100% and 89% for maximum likelihood and maximum parsimony, and Bayesian posterior probability of 1.0). The 5th included the samples with 2N = 36, FN = 56 chromosomes (*C. canariensis* and the Sicilian shrew [*C. sicula*]; bootstrap values of 90% and 63% for maximum likelihood and maximum parsimony, and Bayesian posterior probablity of 0.8). The last clade included the samples of *C. olivieri* and *C. viaria* (2N = 50, FN = 66; maximum-likelihood and maximum-parsimony bootstrap values of 100%, and Bayesian posterior probability of 1.0). Nevertheless, the phylogenetic relationships between these 6 clades and the other samples (*C. theresae*, *C. russula*, *C. zimmermanni*, *C. fuliginosa*, and *C. orii*) remain uncertain.

A likelihood ratio test led to the acceptance of the molecular clock hypothesis for the whole sample ($\chi^2 = 44.52$, $d.f. = 35$, $P > 0.05$). Ln-likelihood values are 10,390.52 and 10,368.26, respectively, for the best trees, with and without the molecular clock assumption. On the basis of the calibration of Fumagalli et al. (1999), we estimate the divergence time between *C. zarudnyi* and the *C. suaveolens* group to be 2.68 million years, with the 95% confidence interval ranging from 2.1 to 3.38 million years.

Fig. 1.—Conventionally stained karyotype of *Crocidura zarudnyi*, collected 12 April 2000 in Baluchestan, Iran. The 2 large chromosomes at bottom right are the X chromosomes.

Discussion

Our specimen of *C. zarudnyi* had a karyotype that appears to be identical to that of the *C. suaveolens* group based on the combination of the diploid and fundamental number of

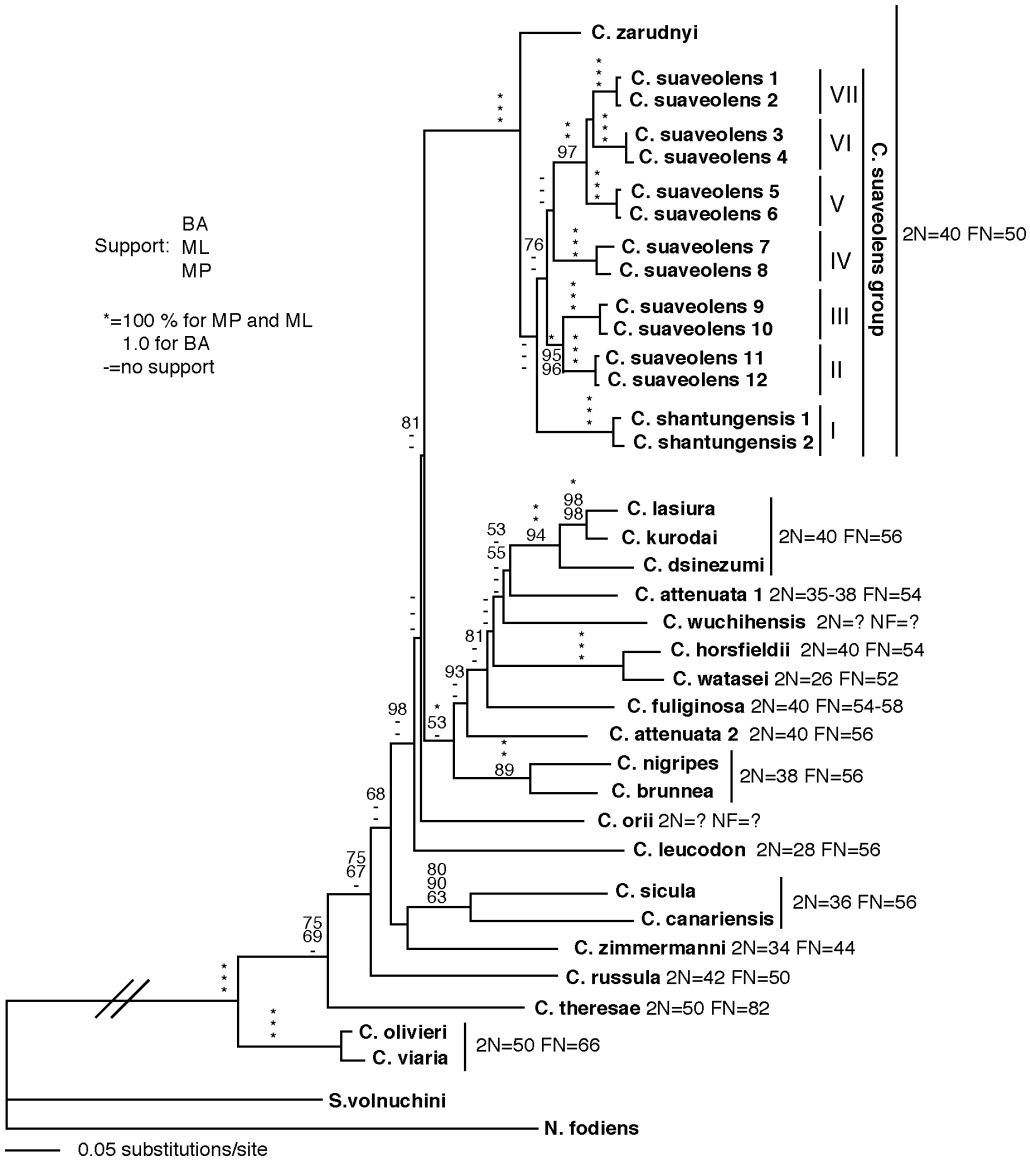

FIG. 2.—Phylogeny of the 1,140-bp C*ytb* fragment analyzed with maximum likelihood, using the GTR+I+G model of substitution and tree-bisection-reconnection branch swapping. Values in branches are bootstrap indices of support for maximum-parsimony (MP) and maximum-likelihood (ML) analyses (percentage of 1,000 replications for each of the 10 random orders of stepwise addition of sequences), and Bayesian posterior probabilities (BA). Clade numbers of *Crocidura suaveolens* group are those described in Dubey et al. (2006). Codes are as in Appendix I.

chromosomes (2N = 40, FN = 50). In fact, this karyotype is known not only from *C. suaveolens* (Catzeflis et al. 1985; Grafodatsky et al. 1988; Meylan 1966; Meylan and Hausser 1974; Reumer and Meylan 1986), but also from related forms such as Gueldenstaedt's shrew (*C. gueldenstaedtii*—Catzeflis et al. 1985; Grafodatsky et al. 1988) and *C. sibirica* (Grafodatsky et al. 1988) that may both be assigned to *C. suaveolens* (Dubey et al. 2006). Moreover, *C. shantungensis* has the same karyotype (Iwasa et al. 2001). In contrast, all other crocidurine shrews exhibit different combinations of diploid and fundamental numbers of chromosomes, including *C. serezkyensis* (assigned to *C. pergrisea*) reported from south-western Azerbaijan by Grafodatsky et al. (1988), which has a karyotype of 2N = 22 and FN = 34. Vogel et al. (2003) concluded that a shared karyotype in the genus *Crocidura* is a synapomorphy that signals a monophyletic relationship. If this is correct, then *C. zarudnyi* should theoretically cluster together with members of the *C. suaveolens* group in the phylogenetic analyses.

Our molecular results confirm this phylogenetic relationship. In fact, *C. zarudnyi* is included in a highly supported monophyletic clade (maximum-parsimony and maximum-likelihood bootstrap values of 100%, and Bayesian posterior probablity of 1.0), the *C. suaveolens* group, where it is placed as sister taxon to the other members of this clade (mean Kimura 2-parameter distance between *C. zarudnyi* and *C. suaveolens* group = 0.097). The separation between *C. zarudnyi* and its close relatives goes back to the Middle Pliocene (2.68 million years ago).

The other taxa analyzed, such as the Asian shrews, which show a similar diploid number of chromosomes but a different fundamental number, are clearly separated from this group (mean Kimura 2-parameter distance between *C. suaveolens* group and other 2N = 40 = 0.163)

In conclusion, the particular phylogenetic position of *C. zarudnyi* makes this a key species in understanding the evolution of the *C. suaveolens* group. However, its relationships with the other little-known Middle Eastern white-toothed shrews, *C. pergrisea* and *C. susiana*, remain obscure in the absence of further cytogenetic and molecular studies.

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APPENDIX I

Species, locations of samples, accession numbers, and collection numbers for our specimens included in our phylogenetic analyses, including citations. 1. *Crocidura zarudnyi*, Iran, Baluchestan, AY925211, I-89, our study. 2. *Crocidura suaveolens* 2, Hungary, Fülophasa, DQ242541, IZEA 6732, our study. 3. *Crocidura leucodon*, Turkey, Altindere, DQ065609, IZEA 6040, our study. 4. *Crocidura theresae*, Burkina Faso, Bobo Dioulasso, DQ521043, IZEA3092, our study. 5. *Crocidura olivieri*, Central African Republic, Bangui, DQ521045, IZEA 2821, our study. 6. *Crocidura viaria*, Burkina Faso, Oursi, DQ521044, IZEA 3108, our study. 7. *Sorex volnuchini*, Turkey, Altindere, DQ065610, IZEA 6079, our study. 8. *Neomys fodiens*, Italy, Reggio, DQ065611, IZEA 5643, our study. 9. *Crocidura sicula*, Italy, Sicily, DQ521040, IZEA 2943, Vogel et al. 2003. 10. *Crocidura canariensis*, Spain, Canary Is., DQ521042, IZEA 4285, Vogel et al. 2003. 11. *Crocidura zimmermanni*, Greece, Crete Is., DQ521041, IZEA 2053, Vogel et al. 2003. 12. *Crocidura suaveolens* 3, Greece, Lesvos Is., AY843460, Dubey et al. 2006. 13. *Crocidura suaveolens* 4, Turkey, Vukarikisilka, AY843461, Dubey et al. 2006. 14. *Crocidura suaveolens* 5, Turkey, Rize, AY843498, Dubey et al. 2006. 15. *Crocidura suaveolens* 6, Georgia, Shulaveri, AY843500, Dubey et al. 2006. 16. *Crocidura suaveolens* 7, Spain, Figueras, AY843491, Dubey et al. 2006. 17. *Crocidura suaveolens* 8, Spain, Candelario, AY843492, Dubey et al. 2006. 18. *Crocidura suaveolens* 9, Iran, Gilan, DQ059023, Dubey et al. 2006. 19. *Crocidura suaveolens* 10, Azebaijan, AY843487, Dubey et al. 2006. 20. *Crocidura russula*, Switzerland, La Côte, AY769264, Fontanillas et al. 2005. 21. *Crocidura shantungensis* 1, South Korea, Cheju Is., AB077077, Ohdachi et al. 2004. 22. *Crocidura shantungensis* 2, South Korea, Kyungju, AB077079, Ohdachi et al. 2004. 23. *Crocidura suaveolens* 1, Austria, Wien, AB077280, Ohdachi et al. 2004. 24. *Crocidura suaveolens* 11, China, Mosuowan, AB077087, Ohdachi et al. 2004. 25. *Crocidura suaveolens* 12, Ukraine, Sevastopol, AY843475, Dubey et al. 2006. 26. *Crocidura lasiura*, Russia, Ussuriisk, AB077071, Ohdachi et al. 2004. 27. *Crocidura dsinezumi*, Japan, Fukuoka, AB077275, Ohdachi et al. 2004. 28. *Crocidura attenuata* 1, Vietnam, Mt. Tay Con Linh, AB175082, Ohdachi et al. 2006. 29. *Crocidura wuchihensis*, Vietnam, Mt. Tay Con Linh, AB175084, Ohdachi et al. 2006. 30. *Crocidura horsfieldi*, Thailand, Kanchana Buri, AB175082, Ohdachi et al. 2006. 31. *Crocidura watasei*, Japan, Tokunoshima Is., AB077074, Ohdachi et al. 2006. 32. *Crocidura fuliginosa*, Vietnam, Mt. Tay Con Linh, AB175079, Ohdachi et al. 2006. 33. *Crocidura attenuata* 2, Taiwan, Nantou Co., AB175081, Ohdachi et al. 2006. 34. *Crocidura nigripes*, Indonesia, Sulawesi, DQ059024, Ohdachi et al. 2006. 35. *Crocidura brunnea*, Indonesia, Java, DQ059025, Ohdachi et al. 2006. 36. *Crocidura orii*, Japan, Amami-ohshima Is., AB175087, Ohdachi et al. 2006. 37. *Crocidura kurodai*, Taiwan, Nantou Co., AB175086, Ohdachi et al. 2006.