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A TAXONOMIC EVALUATION OF THE SHREW *SUNCUS MONTANUS* (SORICIDAE: CROCIDURINAE) OF SRI LANKA AND INDIA

Suyama Meegaskumbura[1,2,*] and Christopher J. Schneider[2]

[1]Department of Zoology, Faculty of Science, University of Peradeniya, Sri Lanka
[2]Department of Biology, 5 Cummington Street, Boston University, Boston, MA 02215, USA
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ABSTRACT

The Sri Lankan and Indian populations of the mountain shrew *Suncus montanus* have hitherto been recognized as a single species, due to their similarity in size and colour. Here we present mitochondrial DNA sequence data from the cytochrome-*b* and 16S genes that suggest these populations represent distinct species. Phylogenetic analyses further reveal that the Sri Lankan and Indian populations are not sister taxa: *S. montanus* sensu stricto from Sri Lanka is the sister species of *S. murinus*, while '*S. montanus*' from India is the sister species of *S. stoliczkanus*. The uncorrected genetic distance for cytochrome-*b* between Sri Lankan and Indian '*S. montanus*' is ca. 7.5%, which is substantially higher than genetic distances within other species of shrews, or within vertebrate species in general, again supporting their recognition as distinct species. The name *Suncus niger* is available for the Indian shrew hitherto referred to *S. montanus*. *Suncus montanus* and *S. niger* should now be considered as endemic to Sri Lanka and India, respectively.

Key words: Phylogenetic relationships, mitochondrial genes, genetic distance, cytochrome-*b*, 16S

INTRODUCTION

Shrews of the genus *Suncus* are widely distributed in Asia, Africa and Europe (Hutterer, 2005). Of the 18 currently recognized species of *Suncus*, five (*S. etruscus*, S. *fellowesgordoni*, *S. zeylanicus*, *S. murinus* and *S. montanus*) are native to Sri Lanka, of which two (*S. fellowesgordoni* and *S. zeylanicus*) are endemic to the island (Hutterer, 2005). *Suncus etruscus* and *S. fellowesgordoni* are pygmy shrews, with the former widely distributed in southern Europe, northern Africa and Asia, while the latter is confined to the highlands of Sri Lanka. *Suncus zeylanicus,* which is within the size range of *S. murinus* and *S. montanus*, was described from Kitulgala, and predicted to be distributed in the rainforests and hills in the Sabaragamuwa and Central Provinces at altitudes between 150 and 1070 meters (Phillips, 1980). It was later reported from Sinharaja rainforest in Sabaragamuwa province (Wijesinghe and Brooke, 2001). *Suncus murinus* lives in close contact with human dwellings and appears to have dispersed with humans. Populations of *Suncus murinus* in Sri Lanka have been classified as several subspecies: *S. m. montanus, S. m. murinus*, *S. m. kandianus* and *S. m. caerulescens* (Phillips, 1980). Ellerman and Morrison-Scott (1966), however, recognized only a single taxon, *S. murinus*, while other authors assigned the latter three taxa to *S. murinus* (Corbet and Hill, 1991, 1992; Nowak, 1999; Hutterer, 2005). *Suncus murinus*, which has a wide distribution in Asia, has been introduced in historical times to Africa, Madagascar and islands nearby (Hutterer, 2005). In Sri Lanka, *S. murinus* is distributed from the coastal plains to an elevation of 1220 m in the highlands. It is known to occupy grasslands, open scrub and edificarian habitats (Phillips, 1980). This species does not occur in the rainforests of the southwestern and southern part of the island. Corbet and Hill (1991, 1992) elevated *S. murinus montanus* to a species, *S. montanus*, based on its smaller size, blackish colour and its restriction to forest habitats. In Sri Lanka, *Suncus montanus* is recorded from the central highlands (910-2520 m) and above 150 m in the rainforests of Sabaragamuwa in the south-western and southern parts of the island (Phillips, 1980).

Suncus montanus has also been recorded from humid forests in the hills of southern India, mainly the Nilgiri and Palani hill regions

*Corresponding author's email: suyamam@pdn.ac.lk

(Shanker and Sukumar, 1998, 1999; Menon, 2003). The Indian mountain shrew originally described as *S. niger* Horsfield, 1851, was synonymized tentatively with *S. montanus* by Corbet and Hill (1992). Most subsequent authors uncritically adopted this classification and referred to the Indian population as *S. montanus* (Shanker and Sukumar, 1998, 1999; Querouil *et al.*, 2001; Ruedi *et al*., 1996; Menon, 2003; Dubey *et al.*, 2007; Dubey *et al.*, 2008). Hutterer (2005), however, regarded the Indian form as a subspecies, *S. montanus niger*.

To date, the taxonomic status of the Sri Lankan and Indian populations of *S. montanus* have remained unclear. Here, we present an analysis of mitochondrial DNA sequence data to determine the phylogenetic relationships of Sri Lankan and Indian populations of *S. montanus* and to clarify their taxonomy.

MATERIALS AND METHODS

Collections and field methods

Specimens of *S. montanus* (Fig.1) were collected from four sites in Sri Lanka: Agarapathana (06°50' N, 80°41' E, 1814 m), Kitulgala (06°59' N, 80°20' E, 64 m), Kudawa (06°25' N, 80°24' E, 464 m) and Morningside (06°24' N, 80°38' E, 1040 m). Shrews were collected using unbaited pitfall traps set between 45 cm- high drift fences approximately 30–50 m long and Sherman traps baited with pieces of roasted coconut. Animals were euthanized and tissue samples taken and preserved in 95% ethanol for genetic analysis. Capture and handling of specimens was carried out under Boston University's Institutional Animal Care and Use Protocol number 04-006.

DNA extraction, amplification, and sequencing

DNA was extracted from ethanol-preserved tissues using Qiagen tissue extraction kits following manufacturer's protocols. Two mitochondrial gene fragments, cytochrome-*b* and 16S rRNA, were sequenced for all the samples from Sri Lanka. These particular mitochondrial genes were selected to facilitate analysis of the new data in the context of previously published molecular data.

DNA was amplified by PCR in 25 µl reactions containing ca. 50 ng of DNA template, 1.25 µl of each primer (10 µM), 2.5 µl of 10 mM mixed dNTP, 2.5 µl of 25 mM $MgCl_2$, 2.5 µl of 10x PCR buffer, and 0.625 U of Taq DNA Polymerase. The thermal cycling for the cytochrome-*b* fragment was as follows: 35 cycles of denaturation at 94° C for 30 s, annealing at 45° C for 30 s, and extension at 72° C for 60 s, with a final extension of 72° C for 5 min. The same conditions were used to amplify the mitochondrial 16S rRNA gene fragment, except that the annealing temperature was 48° C. Primers were the same as in Meegaskumbura *et al.* (2007): MVZ 05 and MVZ 14 for cytochrome-*b* (primer sequences: MVZ 05 5' CGA AGC TTG ATA TGA AAA ACC ATC GTTG 3'; MVZ 14 5' GGT CTT CAT CTY HGG YTT ACA AGAC 3') and 16S ar and 16S br for 16S (primer sequences:16S ar 5' CGC CTG TTT ATC AAA AAC AT 3'; and 16S br 5' CCG GTC TGA ACT CAG ATC ACGT 3'). Original sequences of *S. montanus* and *S. etruscus* of Sri Lanka used in the phylogenetic analysis were submitted to GenBank under the accession numbers given in Table 1.

Phylogenetic analysis

A total of 1673 bp were sequenced, including 1140 bp of cytochrome-*b* (complete coding sequence) and 533 bp of 16S rRNA. Sequences of 16S rRNA were aligned using Clustal X (Jeanmougin *et al*., 1998) and adjusted by eye in Se-Al ver.2.0a9 (Rambaut, 1996). Hypervariable regions of ambiguous positional homology in the 16S rRNA sequences were excluded from the analysis. Sequences from the mitochondrial protein-coding gene cytochrome-*b* were aligned using the translated amino acid sequence. The final data set for phylogenetic analyses comprised 1625 bp including 485 bp of unambiguously aligned 16S rRNA sequence. We obtained a tissue sample of *S. montanus* of India from the Museum of Natural History (NMH), London but were unable to generate PCR products for any of the genes. Therefore, cytochrome-*b* and 16S rRNA sequences available for Indian *S. montanus* in GenBank (Table 1; Dubey *et al.*, 2007; and Dubey *et al.*, 2008) were incorporated into the analysis. The collection sites, voucher numbers, and GenBank accession numbers for each sample are given in Table 1.

For phylogenetic analysis, representatives of the genus *Crocidura* were used as outgroup to root the tree. *Suncus murinus, S. dayi*, *S. etruscus* and *S. stoliczkanus* were also included in the phylogeny as they are closely related to *S. montanus* (Querouil *et al.*, 2001; Dubey *et al.*, 2007; Dubey *et al.*, 2008). Bayesian inference as implemented in the program MrBayes v3.0b4

(Huelsenbeck and Ronquist, 2001) was used to estimate evolutionary relationships among taxa. We used the GTR+I+G model of sequence evolution, which was determined as the best-fit model of the 56 models examined by the hierarchical likelihood ratio test as implemented in Modeltest 3.06 (Posada and Crandall, 1998). To search parameter space and determine the posterior probabilities of phylogenetic trees, we ran four Metropolis-Coupled Markov Chain Monte Carlo (MCMCMC) chains for 1,000,000 generations. The posterior probabilities were calculated after excluding the first 250,000 generations as burn-in. The data were also analyzed under a maximum parsimony (MP) criterion in PAUP*v.4.0b10 (Swofford, 2000) with all characters unordered and weighted equally. Bootstrap values were also determined. The percent pair-wise uncorrected distances between the species were calculated using PAUP* v.4.0b10 for the cytochrome-*b* gene fragments. Because some of the hypervariable regions of the 16S gene fragment were removed from the analysis, only the cytochrome-*b* fragment was used to calculate genetic distance. Ten base pairs from the 5' and 13 base pairs from the 3' end of the cytochrome-*b* was excluded before calculating the genetic distance as these were missing for *S. montanus* of India.

Figure 1. *In-vivo* portrait of *Suncus montanus* from Sinharaja Rain Forest, WHT 6852, female, Head and Body Length 8.5 cm.

Table 1. Country, collection site, voucher number and GenBank accession number of sequences of *Suncus* and *Crocidura* species used in the study. (WHT – Wildlife Heritage Trust)

Species	Country (collection site)	Voucher number	GenBank accession numbers of Cytochrome–*b*/16S sequences
S. montanus			
	Sri Lanka (Agarapathana)	WHT 6814	FJ716837 /FJ716828
	Sri Lanka (Kitulgala)	WHT 6860	FJ716833 /FJ716829
	Sri Lanka (Kudawa)	WHT 6840	FJ716834 /FJ716830
	Sri Lanka (Morningside)	WHT 6852	FJ716835 /FJ716831
	India	–	EF524776/ EF524884 DQ630388/ DQ630304
S. murinus	Sri Lanka (Anuradhapura)	WHT 6906	EU122224/ EU122208
	India	–	EF524777/ EF524885
	Taiwan	–	AB175075/ –
	Japan	–	AB175074/ –
S. stoliczkanus	Nepal	–	AB175076/ – AB175077/ –
S. etruscus	Sri Lanka (Anuradhapura)	WHT 6936	FJ716836 /FJ716832
S. dayi	India	–	DQ630389/ DQ630305 DQ630432/ DQ630373
C. miya	Sri Lanka (Agarapathana)	WHT 6826	EU122216/ EU122215
C. horsfeildii	Sri Lanka (Peradeniya)	WHT 6869	EU122213 / EU122197
C. attenuata	Vietnam	MVZ 185237	EU122211/ EU122195
C. fuliginosa	Vietnam	MVZ 186404	EU122212/ EU122196

RESULTS

Phylogenetic trees obtained from both Bayesian and maximum parsimony analyses had the same topology and, for brevity, only the Bayesian phylogram is shown (Fig. 2). In this phylogeny Sri Lankan and Indian '*S. montanus*' do not form a monophyletic group. *Suncus montanus* of Sri Lanka is placed as a well-resolved sister group to *S. murinus*, with 97% bootstrap and 100% posterior probability support, whereas '*S. montanus*' of India was placed as the sister group to *S. stoliczkanus* from Nepal with 68% bootstrap and 100% posterior probability support. These two clades are sister groups with 100% bootstrap and posterior probability support. *Suncus dayi* of India is the sister group of the clade including the above taxa (Fig. 2).

The non-monophyly of *S. montanus* supports the recognition of the Sri Lankan and Indian populations hitherto assigned to *S. montanus* as distinct species. This is supported by the high genetic distance between the two taxa. The percent pairwise, uncorrected sequence difference for cytochrome–*b* between *S. montanus* of Sri Lanka (SL) and that of India (I)

is 7.5-7.7% (Table 2). The distance between *S. montanus* (SL) and *S. murinus* (SL, Taiwan and Japan) is smaller than this (5.9-7.3%), while that between *S. montanus* (I) and *S. murinus* (SL, Taiwan and Japan) is 8.4-8.8% (Table 2). Though *S. stoliczkanus* of Nepal falls as a sister taxon to *S. montanus* of India, the genetic distance between the two is high (6.8-7.0%).

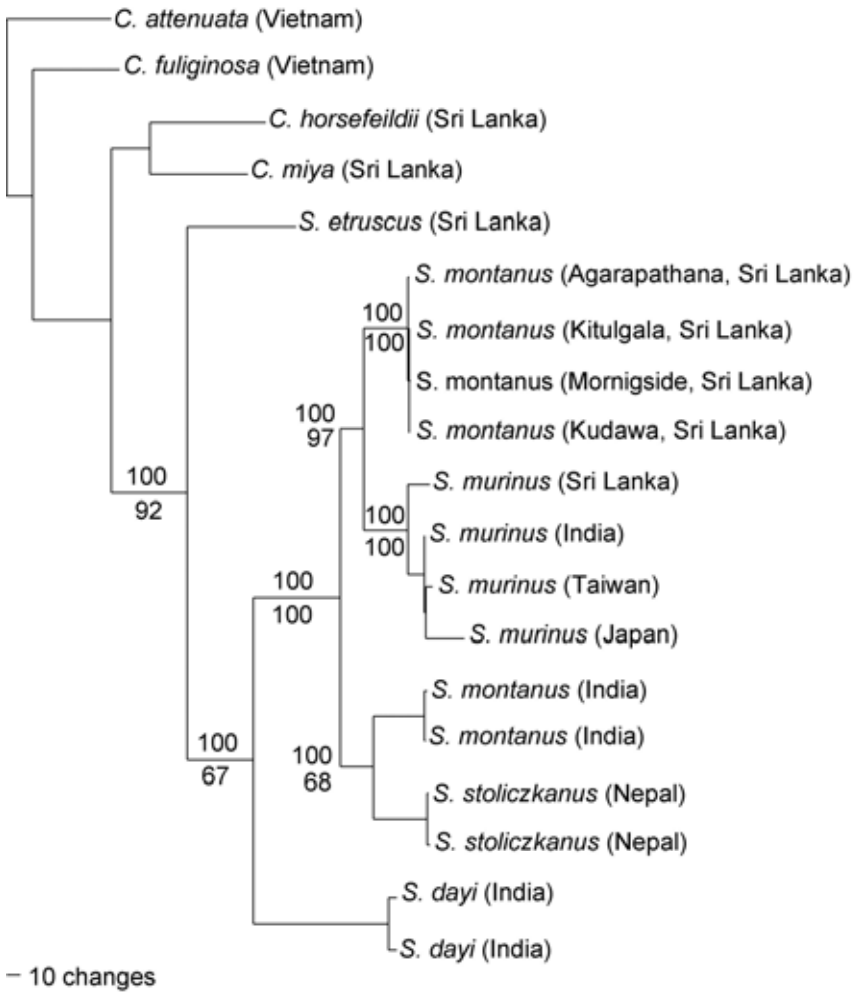

Figure 2. Phylogram inferred from Bayesian analysis of combined cytochrome–*b* + 16S data sets under model GTR+I+G. The maximum parsimony tree had the same topology. Posterior probability and bootstrap values (expressed as percentages) are given above and below branches, respectively.

Table 2. Percent pairwise uncorrected genetic distance for cytochrome-*b* among *S. montanus*, *S. murinus* and *S. stoliczkanus*. SL – Sri Lanka, I – India, T – Taiwan, J – Japan and N – Nepal

	S. montanus (SL)	*S. montanus* (I)	*S. murinus* (SL)	*S. murinus* (T & J)
S. montanus (I)	7.5 – 7.7	–	–	–
S. murinus (SL)	5.9 – 6.5	8.8	–	–
S. murinus (T & J)	6.2 – 7.3	8.4 – 8.8	2.3 – 4.2	–
S. stoliczkanus (N)	8.0 – 8.6	6.8 – 7.0	8.7 – 8.8	8.5 – 9.0

DISCUSSION

The phylogenetic relationships of mitochondrial genes (Fig. 2) and the genetic distance of the cytochrome-*b* gene (Table 2) show *S. montanus* of Sri Lanka to be genetically more distant from *S. montanus* of India than it is from *S. murinus* of Asia. In the phylogeny, Sri Lankan and Indian *S. montanus* do not form a monophyletic group. Genetic distances estimated from the mitochondrial cytochrome-*b* gene have been used in many instances to validate vertebrate species (Bradley and Baker, 2001; Johns and Avise, 1998). Johns and Avise (1998) noted that 90% of vertebrate sister species show more than 2% genetic divergence in cytochrome-*b* sequence and suggested that this level of divergence may indicate species-level divergence. Similarly, Bradley and Baker (2001) surveyed mammal species and suggested that greater than 2% divergence of cytochrome-*b* sequences may indicate a species-level divergence. While defining species solely on the basis of mtDNA sequence-difference is problematic, we consider the 7.5–7.7% genetic divergence between Sri Lankan and Indian '*S. montanus*' to be suggestive of an independent evolutionary history of mtDNA lineages for a prolonged period.

The placement of Indian '*S. montanus*' as sister to *S. stoliczkanus* (collected from Nepal) and not to *S. murinus* or *S. montanus* of Sri Lanka in the phylogeny again supports their independent evolution. The same relationship among Indian *S. montanus*, *S. stoliczkanus* and *S. murinus* is reported in Dubey *et al.*, 2008. Previous phylogenetic studies that included *S. montanus* from India and *S. murinus* from Asia, but did not include *S. montanus* from Sri Lanka, suggested that the Indian '*S. montanus*' and *S. murinus* are sister species (Querouil *et al.*, 2001; Dubey *et al.*, 2007). However, the addition of *S. montanus* from Sri Lanka to our study resolves this pseudo-relationship. Ruedi *et al*. (1996) also reported that *S. montanus* of India is more closely related to *S. murinus* collected from Nepal than to those of other places (India, Java, Philippines and Japan). This close relationship of '*S. montanus*' (India) and *S. murinus* (Nepal) is explained as retention of ancestral polymorphism or a result of hybridization. In any event this close relationship of the '*S. montanus*' of India to a *Suncus* species from Nepal and not to the Sri Lankan *S. montanus* is evidence for their independent evolution.

The tentative placement of *S. niger* in *S. montanus* by Corbet and Hill (1992) was due to similarities in external morphology and habitat. However, differences in size between the population in Sri Lanka (Phillips, 1928, 1980; personal data) and India (Menon, 2003) are noted: 7.8 – 11.5 cm body length of Sri Lankan *S. montanus* (*vs.* 8.0 – 10.5 cm in Indian *S. montanus*) and 5.6 – 7.5 cm tail length of Sri Lankan *S. montanus* (*vs.* 4.5 – 6.5 cm in Indian *S. montanus*). Though the body length and tail length overlap to some degree in the two populations the Sri Lankan *S. montanus* is generally larger than the Indian '*S. montanus*' and have a longer tail. Body size and tail length are considered to be important distinguishing characters among species of shrews; hence, the larger size and longer tail of *S. montanus* sensu stricto may be diagnostic, but a rigorous comparison of the morphology of *S. montanus* and *S. niger* must await the availability of a larger series of specimens.

The present study suggests that the Sri Lankan and the Indian shrews previously referred to *S. montanus* are indeed distinct species. We recommend that the Indian population previously referred to *S. montanus* (Kelaart, 1850; type locality Pidurutalagala, near Nuwara Eliya, Sri Lanka) be placed in *S. niger* (Horsfield, 1851: type locality Madras [Presidency, now Tamil Nadu State], southern India).

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