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PHYLOGENETIC RELATIONSHIPS OF MALAYAN AND MALAGASY PYGMY SHREWS OF THE GENUS *SUNCUS* (SORICOMORPHA: SORICIDAE) INFERRED FROM MITOCHONDRIAL CYTOCHROME *B* GENE SEQUENCES

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ABSTRACT. – This study used a portion of the mitochondrial cytochrome *b* gene to investigate the phylogenetic relationships of *Suncus* pygmy shrews and to help clarify the taxonomic status of the Malayan pygmy shrew (*S. malayanus*) and the Malagasy pygmy shrew (*S. madagascariensis*). Phylogenetic reconstructions were performed using neighbour-joining and Bayesian analysis methods and revealed that *S. malayanus* is related to, but distinct from *S. etruscus*. The K2P-corrected genetic distance among the Malaysian taxon and other pygmy shrews for the cytochrome *b* gene was between 6.1 and 8.5%, supporting recognition that *S. malayanus* represents a distinct species from the geographically widespread *S. etruscus* species complex. A close (1.5% K2P distance) sister-group relationship was revealed between *S. etruscus* from Sri Lanka and *S. madagascariensis* from Madagascar, which has been considered an island endemic, and suggests that these animals are not specifically distinct. The Malagasy population of this shrew most probably was translocated to the island by human intervention, with the lineage originating from Southeast Asia or the Indian subcontinent.

KEY WORDS. – Crocidurinae, cytochrome *b*, genetic distance, mitochondrial DNA, phylogenetic relationships, *Suncus*.

INTRODUCTION

Due to their extremely small size (about 2 g), pygmy shrews of the genus *Suncus* (Family Soricidae) are difficult to trap and are therefore poorly represented in reference collections, which in turn has added to ongoing taxonomic confusion regarding species limits within the genus. For instance, *S. malayanus* (Fig. 1), the principal focus of this study, was initially named *Pachyura malayana* by Kloss (1917) and subsequently considered a subspecies of the Etruscan shrew *S. etruscus* (Savi, 1822) under the name *S. etruscus malayanus* (see e.g. Medway, 1978). More generally, Ellerman &

Morrison-Scott (1966) and Corbet & Hill (1992) considered, with some reservation, that all pygmy shrews occurring in the Indomalayan region were part of a single widespread species, *S. etruscus*, originally described from specimens collected in Italy. Numerous authors, including recent overviews of Southeast Asian mammals (Francis, 2008), adopted this view. However, in his revision of the Soricidae, Hutterer (2005) recognised four additional, geographically restricted species in the *etruscus* group: *S. fellowesgordoni* (Phillips, 1932) from the highlands of Sri Lanka, *S. malayanus* from the Thai-Malay peninsula, *S. hosei* (Thomas, 1893) from Borneo and *S. madagascariensis* (Coquerel, 1848) from Madagascar. These various forms differ slightly in morphology from European *S. etruscus* but live in very different habitats, and were considered endemic to their specific regions (Hutterer, 2005).

Molecular phylogenetic techniques have been employed to clarify aspects of the systematic relationships among other groups within the family Soricidae and subfamily Crocidurinae (e.g. Jenkins et al., 1998; Ruedi et al., 1998; Motokawa et al., 2000; Quérouil et al., 2001; Ohdachi et al., 2004; Dubey et al., 2007; Meegaskumbura & Schneider, 2008; Meegaskumbura et al., 2010). These studies have shown that within the Crocidurinae the genus *Suncus* is paraphyletic with the genera *Sylvisorex* and *Crocidura* (Quérouil et al., 2001; Dubey et al., 2007), with *Suncus etruscus* basal to other Asian *Suncus* and the remaining members of the Crocidurini tribe (Dubey et al., 2008). Large divergence among populations of this genus formally classified as a single taxon (e.g. *S. murinus*; Meegaskumbura & Schneider, 2008), suggests that additional cryptic taxonomic diversity exists. Although adding to systematic knowledge of the group, these studies have generally not addressed the relationships among certain Asian members of pygmy shrews including *S. malayanus*.

In this study, we present an analysis of mitochondrial DNA sequence data to determine the phylogenetic relationships and genetic distances between *S. malayanus*, *S. madagascariensis* and *S. etruscus*. The results help to clarify their taxonomic status and document new occurrences of *S. malayanus* at three sites across the Thai-Malay peninsula, including one from a montane location.

MATERIAL AND METHODS

***Sample collection.* –** Samples of *Suncus malayanus* (Fig. 1), were collected from three locations in Peninsular Malaysia: Ulu Gombak (N03°60.170' E101°60.46', altitude 200 m), Bukit Rengit [=Rengit Hill] (N03°35.987' E102°10.758', altitude 132 m) and Cameron Highlands (N04°28.460' E101°23.05', altitude 1465 m). These locations are covered by extensive secondary lowland evergreen forests (Ulu Gombak and Bukit Rengit) or by montane forests surrounding tea plantations (Cameron Highlands). At each site shrews were captured in 50–100 unbaited pitfall traps (buckets dug into earth) set within a 45 × 45 m grid. All individuals were euthanized immediately after capture using chloroform. Sex and external measurements were recorded, and the specimens were either prepared as flat skins or fixed in 5% formalin and subsequently transferred to 70% ethanol. Kidney samples were removed soon after death and stored at –80°C or preserved in 95% ethanol for genetic analysis. The voucher specimens are housed at the Zoological Department, University of Malaya. The specimens of *S. madagascariensis* were collected in the dry deciduous forest of Beanka, near Maintirano (S18°03.75' E44°31.5'E, altitude 320 m) with the use of pitfall traps. Muscle tissue samples from these animals were preserved in EDTA. Other *Suncus* samples were also employed in this study and the genus *Myosorex* was employed as the outgroup (see below and Table 1).

***DNA extraction, PCR amplification and sequencing.* –** DNA was extracted from preserved tissue samples using Promega Wizard extraction kits following the manufacturer's protocol (Promega Co.). Polymerase chain reaction (PCR) amplification of the target fragment of the mitochondrial cytochrome *b* gene was performed using primers L14724 and H15915 (Irwin et al., 1991). 25 µl PCR reactions contained 5 µl of DNA template (~20 ng/µl), 2.5 µl of each primer (10 mM), 0.5 µl of dNTP (10 mM), 2.0 µl of $MgCl_2$ of (25 mM), 2.5 µl of 5x colorless GoTaq ® Flexi buffer and 0.2 µl of GoTaq DNA Polymerase (5 u/µl). PCR conditions were as follows: 40 cycles of denaturation at 93°C for 45 s, annealing at 45.9°C for 45 s, extension at 72°C for 1 min, with a final extension at 72°C for 5 min. PCR products were electrophoresed on a 1% agarose gel then visualised with ethidium bromide staining to check success of PCR amplification. PCR products were gel purified using Promega Wizard SV Gel and PCR Clean up System with microcentrifudge (Promega Co.). Cleaned amplicons were sequenced in both directions using the same primers by a commercial laboratory (First Base Co., Selangor, Malaysia) using a BigDye ® Terminator v3.0 Cycle Sequencing Kit with products run on an ABI 3730 automatic sequencer.

***Sequence alignment and phylogenetic analyses.* –** New sequence data from the cytochrome *b* gene of three *S. malayanus* from Malaysia, two *S. madagascariensis* from Madagascar and one *S. etruscus* from France was obtained in the current study. The sequences were edited using Chromas version 1.45 (MacCarthy, 1996). Another 13 *Suncus* cytochrome *b* sequences, representing additional samples of

Fig. 1. Male Malayan pygmy shrew (*Suncus malayanus*) captured in the Cameron Highlands, Pahang, Peninsular Malaysia, in a pitfall trap set on the forest floor. Notice the characteristic large ears and dark fine pelage.

Table 1. List of all *Suncus* and *Myosorex* (outgroup) specimens that were used in this study with information on collection site, country and GenBank accession number. The numbers given after the species names are associated with the sample numbers of the different taxa and are used in Table 2.

Species	Collection site (Locality)	Country	GenBank accession number (cytochrome *b*)	Reference
*Suncus malayanus*1	Ulu Gombak (Selangor)	Malaysia	JF817391	This study
*S. malayanus*2	Cameron Highlands (Pahang)	Malaysia	JF817392	This study
*S. malayanus*3	Bukit Rengit (Pahang)	Malaysia	JF817393	This study
*S. madagascariensis*1	Beanka Forest	Madagascar	JF817394	This study
*S. madagascariensis*2	Beanka Forest	Madagascar	JF817395	This study
*S. etruscus*1	Anuradhapura	Sri Lanka	FJ716836	Meegaskumbura & Schneider (2008)
*S. etruscus*2	Fivizzano	Italy	DQ630397	Dubey et al. (2007)
*S. etruscus*3	Camargue	France	DQ630396	Dubey et al. (2007)
S. etruscus4	Gard	France	JF817396	This study
*S. montanus*1	Agarapathana	Sri Lanka	GQ290378	Meegaskumbura & Schneider (2008)
*S. montanus*2	Morningside	Sri Lanka	FJ716835	Meegaskumbura & Schneider (2008)
S. niger (previously analysed as *montanus*)	Nilgiri Hills	India	DQ630388	Dubey et al. (2007)
*S. murinus*1	Negros Island	Philippine	FJ813963	Esselstyn et al. (2009)
*S. murinus*2	–	India	EU122224	Meegaskumbura & Schneider (2008)
S. remyi	Moueva	Gabon	DQ630399	Dubey et al. (2007)
S. dayi	Nilgiri Hills	India	DQ630432	Dubey et al. (2007)
*S. stoliczkanus*1	–	Nepal	AB175077	Ohdachi et al. (2004)
*S. stoliczkanus*2	–	Nepal	AB175076	Ohdachi et al. (2004)
S. varilla	–	South Africa	DQ630434	Dubey et al. (2007)
Myosorex cafer	Serola	South Africa	FJ814024	Dubey et al. (2007)
M. sclateri	Natal	South Africa	FJ814043	Dubey et al. (2007)

S. etruscus from France and Italy and a further six *Suncus* species were compiled from GenBank records, along with cytochrome *b* sequences for two outgroup taxa, *Myosorex cafer* and *M. sclateri* (Table 1). The initial 270 base-pairs of the sequences of *S. etruscus* from Italy deposited by Dubey et al. (2007) under GenBank number DQ630397 were omitted because of unreliable readings. Multiple alignments of all sequences were performed with ClustalX 1.81 (Thompson et al., 1997). Missing nucleotides in partial sequences were replaced by N in the final alignment. Estimates of pairwise genetic distance among cytochrome *b* gene fragments from the different forms of pygmy shrews were calculated using the Kimura two-parameter (K2P) model (Kimura, 1980) implemented in MEGA 4 software (Tamura et al., 2007).

We conducted phylogenetic analyses to illustrate the level of divergence and relationships among all sampled taxa. Trees were constructed by using neighbour-joining (NJ) and Bayesian analysis (BA) methods. All trees were rooted with *Myosorex* spp. as the outgroup. The NJ analysis was conducted using MEGA 4 (Tamura et al., 2007) with the K2P distance matrix and uniform rates across sites. Bootstrap analysis used 1000 replications to compute 50% majority rule consensus trees for node confidence limits (Felstenstein, 1985). For BA phylogenetic analysis, trees were constructed using the TrN + I + G model of DNA evolution that best fits data according to the protocol of Posada & Crandall (1998) implemented in Modeltest version 3.7. BA was performed in MrBayes version 3.0 (Huelsenbeck & Ronquist, 2001) with one million generations implementing Metropolis-Coupled Markov Chain Monte Carlo (MCMCMC) with trees sampled every 1000 generations. Tree parameters reached stationary distributions after a burn-in period of 100,000 generations. Posterior probabilities (PP) were calculated for each node as the percentage of sampled trees containing the node in question.

RESULTS

We obtained between 1013 (from the three Malayan samples) and 1140 base-pairs (from the single French and two Malagasy samples) of the cytochrome *b* gene, which are deposited in GenBank under numbers JF817391 – JF817396. All 21 sequences used in the phylogenetic reconstructions and distance calculations represented 10 putative *Suncus* and two *Myosorex* species (Table 1). The final alignment represented 704 conserved sites, and 366 of the 436 variable sites proved to be parsimony-informative. Corrected (K2P) pairwise genetic distances among the pygmy *Suncus* are shown in Table 2, with the African pygmy shrew, *S. remyi*, also included for comparison as a distant relative (Dubey et al., 2008). They reveal relatively high inter-specific genetic distances (6.1–8.5%) between *S. malayanus* and *S. madagascariensis* or *S. etruscus*, but much greater distances when compared to *S. remyi* (23.6–24.0%). Among the three

Table 2. Percent pairwise corrected (K2P) genetic distance among the pygmy shrews *Suncus malayanus*, *S. madagascariensis*, *S. etruscus* and *S. remyi* cytochrome *b* sequences.

	Sample	1	2	3	4	5	6	7	8	9
1	*S. malayanus*1 Malaysia	–								
2	*S. malayanus*2 Malaysia	1.4	–							
3	*S. malayanus*3 Malaysia	0.3	1.1	–						
4	*S. madagascariensis*1 Madagascar	8.0	8.5	7.7	–					
5	*S. madagascariensis*2 Madagascar	8.0	8.5	7.7	0.1	–				
6	*S. etruscus*1 Sri Lanka	7.6	8.0	7.3	1.5	1.4	–			
7	*S. etruscus*2 Italy	6.6	6.9	6.1	3.3	3.3	3.4	–		
8	*S. etruscus*3 France	6.9	7.3	6.4	3.1	3.1	3.3	0.4	–	
9	*S. etruscus*4 France	7.1	7.6	6.8	3.1	3.0	3.6	0.3	0.1	–
10	*S. remyi* Gabon	24.0	23.9	23.6	23.5	23.5	23.3	21.7	21.1	21.8

S. malayanus sequenced, the two lowland individuals from Bukit Rengit and Ulu Gombak were more closely related to each other (0.3% K2P divergence), than they were to the individual from the Cameron Highlands (1.1 and 1.4% divergence). Intra-specific divergences among *S. etruscus* individuals collected in Europe were minimal (0.1–0.4% which corresponds to one to three point mutations), but higher (up to 3.6% or 39 point mutations) when the sample from Sri Lanka was included. This distance is comparable to the divergences measured between European samples of *S. etruscus* and *S. madagascariensis* (3.0–3.3%). The lowest inter-specific divergence (1.4–1.5%) was observed between the Sri Lankan *S. etruscus* and Malagasy *S. madagascariensis*, identifying a close genetic distance between these individuals.

The phylogenetic trees constructed using the two methods (NJ and BA) were largely congruent (Fig. 2). Both analyses identified a major clade among *Suncus* corresponding to all members of this genus sampled with Eurasian/Madagascar distributions, and excluding the African species *S. remyi* and *S. varilla*. Within the Asian clade, two groups were resolved, corresponding to the pygmy and the larger *Suncus* species. The monophyletic pygmy shrew clade contained *S. madagascariensis*, *S. etruscus* and *S. malayanus* and was well supported, with a BA posterior probability of 1.0 and 100% bootstrap support in the NJ analysis. Within this clade, the monophyly of *S. malayanus* was well supported. Both analyses indicated that *S. etruscus* is paraphyletic, with the Sri Lankan *S. etruscus* consistently sister to *S. madagascariensis*, reflecting the low divergence observed between these populations in pairwise estimates.

DISCUSSION

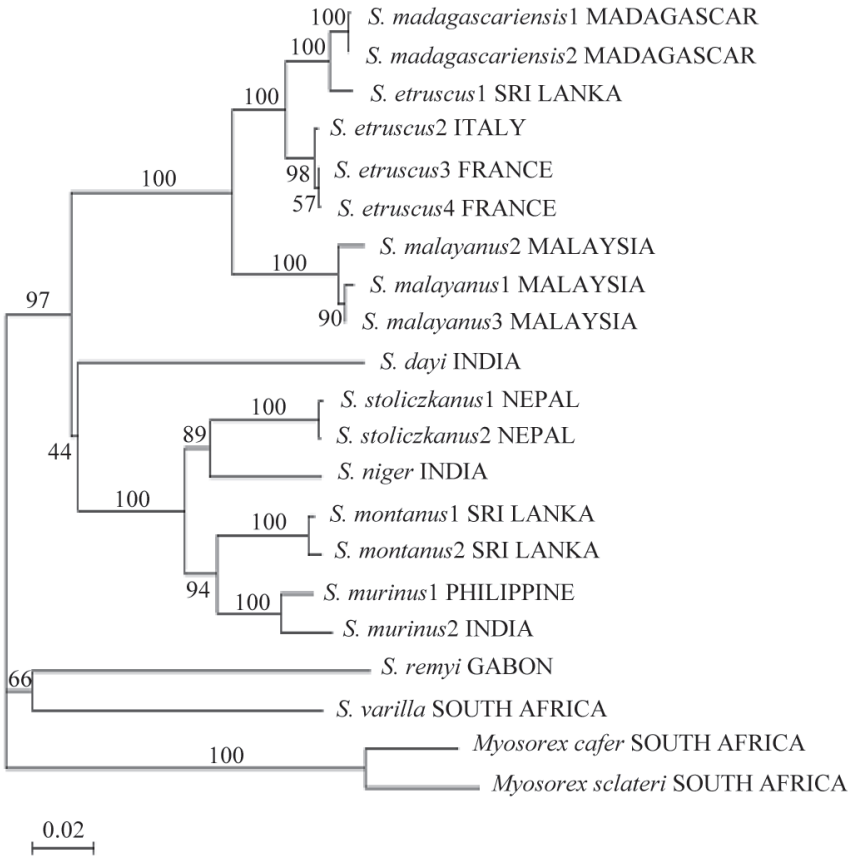
The phylogenetic reconstruction presented here demonstrates that pygmy shrews from Italy, France, Sri Lanka, Madagascar and Malaysia form a monophyletic clade to the exclusion of other Asian and African shrews of the genus *Suncus*. Although the taxon sampling in southeastern Asia is still incomplete, we confirm that the morphological resemblance among Eurasian taxa included in the *S. etruscus* species complex (sensu Corbet & Hill, 1992) is also found at the molecular level. Our results show that Eurasian *S. etruscus* are closely related genetically to *S. madagascariensis*, but not to *S. malayanus*. Aside from the pygmy shrews, the other relationships identified herein among *Suncus* species are in agreement with earlier findings, such as close relationships between *S. murinus* and *S. montanus* (Meegaskumbura & Schneider, 2008), and the distinct position of *S. niger* relative to other large *Suncus* (Meegaskumbura et al., 2010). In addition, the separation of the African and Eurasian taxa into two clades (Fig. 2A) mirrors the relationships observed by Dubey et al. (2008) based on a more comprehensive taxonomic and larger character sampling of Crocidurinae shrews.

Previous studies of divergence in the cytochrome *b* gene of mammals in general (Bradley & Baker, 2001) or Crocidurinae in particular (Ohdachi et al., 2004; Dubey et al., 2006) have found that divergences of between 1.5 and 2.4% indicate intra-specific variation, while divergences of 7.5% or greater are present at the inter-specific level (see also Ruedi et al., 1998; Meegaskumbura & Schneider, 2008). Although relying on a genetic species concept based solely on mitochondrial DNA to classify samples of uncertain taxonomic rank can be problematic (Ferguson, 2002), this approach is still useful when the morphology of the taxa being compared is quite conserved, as is the case in pygmy shrews.

The notably low "inter-specific" divergence of 1.4% between *S. madagascariensis* and Sri Lankan *S. etruscus* was significantly less than the intra-specific divergence present among the most distinct *S. etruscus* individuals (up to 3.6% K2P distance; Table 2). This indicates that *S. madagascariensis*, that is treated as endemic to Madagascar (Hutterer, 2005), and *S. etruscus* should not be considered separate species and that the former is a junior synonym of the latter. Furthermore, animals originally identified as *S. madagascariensis* are more closely related to Asian *S. etruscus* than those from Europe.

We hypothesize that the Madagascar population recently colonized the island with founding animals originating from the Indian Subcontinent or Southeast Asia, presumably

A)



B)

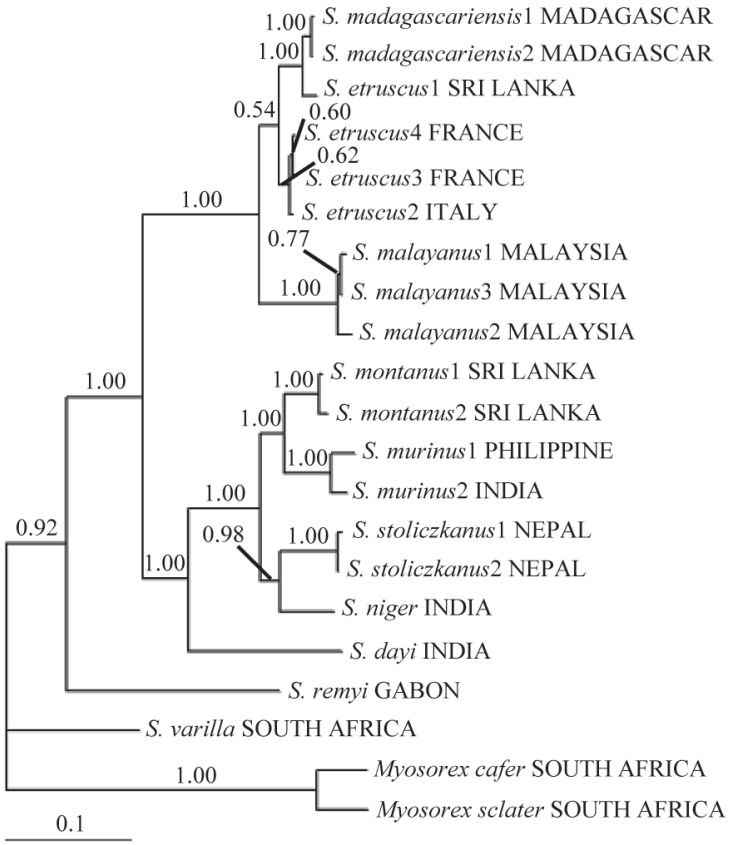


Fig. 2. The neighbour-joining (A) and Bayesian (B) trees for *Suncus* inferred from 1140 base-pairs of cytochrome *b* gene sequence. Bootstrap and posterior probability values are given above branches.

transported by maritime travellers. The earliest evidence of humans on Madagascar is slightly more than 2,000 years ago and during the 11th–14th centuries there was extensive maritime movement between portions of Asia and Madagascar (Burney et al., 2004). This situation is similar to that of the large, commensal musk shrew (*S. murinus*), where all African and Malagasy populations are considered as recent introductions due to human trade (Hutterer & Tranier, 1990). Unlike the relatively robust musk shrew, however, *S. etruscus* is a notably small species, but given its broad geographical range, it appears to be adapted to a wide range of environmental conditions (see below), and might have survived translocation in, for example, soil associated with the shipment of live commercial plants (tea or coffee) or in boat holds containing invertebrate populations. Elsewhere, in the Mediterranean region, *S. etruscus* is also believed to have reached remote islands though passive human transport (Dobson, 1998). Another potential species of pygmy shrew, *S. coquereli*, is named from Mayotte in the Comores Archipelago in close proximity to Madagascar. Although we lack tissue samples of this population, Hutterer (2005) indicated that close genetic similarity exists between this taxon and Malagasy individuals. We strongly suspect that its status is similar to *S.* "*madagascariensis*" and therefore, that *S. coquereli* actually represents another introduced form of *S. etruscus*.

In contrast, the level of genetic divergence present between *S. malayanus* and the other Eurasian pygmy shrews (6.1–8.5%) suggests that Malayan populations have been evolving independently for a relatively long period and are best considered as a distinct species. In addition, *S. malayanus* is ecologically different from its European relative, as it lives in tropical humid forests, including montane habitats (see Fig. 1 and trapping sites in Materials & Method section), whereas Eurasian *S. etruscus* is adapted to much more xeric and lowland habitats (Spitzenberger, 1990). The Malagasy population of *S. etruscus* occurs in different biomes on Madagascar, ranging from notably dry habitats to tropical humid forests across an elevational gradient from near sea level to 1200 m (Soarimalala & Goodman, 2011). Hutterer (2005) mentioned possible morphologic differences between *S. malayanus* and *S. etruscus*, but besides darker pelage coloration in the former (Fig. 1), we did not investigate this aspect further.

The three *S. malayanus* individuals used in this study were collected from three different localities within Peninsular Malaysia. The Cameron Highlands site is situated at an elevation of about 1465 m, while the other two locations (Bukit Rengit and Ulu Gombak) are at lower elevations (<500 m) and within about 65 km distance from one another. Greater genetic similarity was observed between the lowland individuals than among lowland and highland individuals (0.3% vs 1.1–1.4% K2P genetic distance). As the highland location is also geographically more distant than the two lowland locations (>150 km), the greater genetic distance may be associated with isolation by distance (Wright, 1943). Alternatively, the divergence between the Cameron Highlands sample and lowland individuals may indicate that historically, elevation has been an important factor in limiting gene flow among *S. malayanus* populations in this region. More geographically extensive sampling across the Thai-Malay peninsula will be necessary to ascertain whether either of these hypotheses is true for *S. malayanus* and to unravel its northern and western range limits. Further, samples from northern and central Thailand also need to be included in subsequent studies to investigate the level of divergence between *S. malayanus* and the endemic Borneo pygmy shrew *S. hosei.*

In conclusion, this study demonstrates that *S. malayanus*, which is endemic to the Thai-Malay peninsula, represents a unique lineage among *Suncus* pygmy shrews distinct from the *S. etruscus* species complex. It also provides information on the extent of genetic variation present among *S. etruscus* individuals, and shows that animals previously considered *S. madagascariensis* are taxonomically synonymous with *S. etruscus*. The Malagasy animals were most likely introduced to Madagascar via human intervention from a Indian Subcontinent or Southeast Asia source population. For a better understanding of the evolutionary history of different forms in the *etruscus* group, other taxa recognised by Hutterer (2005) as distinct species, notably *S. hosei* and *S. fellowesgordoni*, also need to be included in subsequent studies to investigate their level of genetic divergence.

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