

Phylogenetic position of the Malaysian mole, *Euroscaptor micrura* (Mammalia: Eulipotyphla), inferred from three gene sequences

Akio Shinohara^{1,*}, Shin-ichiro Kawada², Masatoshi Yasuda³ and Lim Boo Liat⁴

¹ Department of Bio-resources, Division of Biotechnology, Frontier Science Research Center, University of Miyazaki, 5200 Kihara, Kiyotake, Miyazaki 889-1692, Japan

² Laboratory of Animal Management and Resources, Graduate School of Bio-Agricultural Sciences, Nagoya University, Nagoya, Aichi 464-8601, Japan

³ Wildlife Ecology Laboratory, Forestry and Forest Products Research Institute, Tsukuba, Ibaraki 305-8687, Japan

⁴ Department of Wildlife and National Parks, 43200 Kuala Lumpur, Malaysia

East Asia is an area of species richness for the family Talpidae, especially the highly fossorial genera *Mogera* and *Euroscaptor*, which possess seven and six species, respectively (Hutterer 1993). Although the phylogenetic relationships within the genus *Mogera* have been well established on the basis of morphology (Motokawa 2004), chromosome analysis (Kawada et al. 2001), and DNA sequences (Okamoto 1999; Tsuchiya et al. 2000; Shinohara et al. 2003), little is known about the evolution and radiation of the genus *Euroscaptor*. In fact, the Japanese mountain mole *Euroscaptor mizura* is the only member of this genus studied in detail (Tsuchiya et al. 2000; Kawada et al. 2001; Shinohara et al. 2003). These studies suggest that *Euroscaptor* is a relic East Asian species group closely aligned with the genus *Mogera*. The genus *Euroscaptor* was first described by Miller (1940), but the characteristics of the genus were indistinct and its taxonomic status uncertain. Motokawa (2004) carried out a cladistic study of the skull characters and concluded that *Euroscaptor* is likely a paraphyletic group, and noted that systematic revision of *Euroscaptor* is required. To better understand the evolutionary history of East Asian talpids, including the genera *Talpa*, *Euroscaptor* and *Mogera*, it is necessary to examine the phylogenetic and systematic status of the genus *Euroscaptor*. In particular, comparative studies of *Euroscaptor* representatives from both Japan and Southeast Asia are required to provide further insight into the evolution of this group.

Peninsular Malaysia is the southernmost region of the world's talpid distribution (Corbet and Hill 1992). Malaysian moles inhabiting the main range of the

Malay Peninsula (1000 to 2000 m above sea level) were first described by Chasen (1940) from the Cameron Highlands, Pahang State. However, the systematic position and ecology of this taxon have remained unclear, because only six specimens have been collected in the past 50 years. In January 2002, we conducted a field survey of the Malaysian mole on the BOH Estate, Cameron Highlands, Pahang, Malaysia, and successfully captured 10 moles (Kawada et al. 2003) — 40 years after the species was last recorded (Cranbrook 1962). Cranbrook (1962) identified the Malaysian mole as the southernmost population of the Himalayan mole, *Talpa* (*Euroscaptor*) *micrura*, and our preliminary results, based on the morphological traits of the skull, supported his classification at the species level (Kawada et al. 2003). Although the genus *Euroscaptor* ranges from south China to northern Thailand, the core range of distribution of *E. micrura* is in the eastern Himalayas (Hutterer 1993), far away from Peninsular Malaysia. Thus, members of the Malaysian *E. micrura* population we collected are peripheral to the core range of the species. The question remains as to how *E. micrura* became dispersed into Peninsular Malaysia. As the distribution of small mammals is generally determined by migration events and past ecological changes, phylogenetic study of the genus *Euroscaptor* will give us valuable information on the paleoecology of Southeast Asia as well as on the evolutionary history of talpids endemic to this region.

To clarify the phylogenetic position of *E. micrura* and the taxonomic status of the genus *Euroscaptor*, we first determined the sequences of the mitochondrial cytochrome *b* (cyt *b*; 1140 bp) gene, the 12S rRNA gene

*To whom correspondence should be addressed. E-mail: akioshi@med.miyazaki-u.ac.jp

(12S; about 840 bp), and the nuclear recombination activating gene-1 (RAG1; 1010 bp) from two Malaysian mole specimens. We then performed comparative molecular phylogenetic analyses using this data together with published sequences of these genes from nine additional Eurasian talpid species (Tsuchiya et al. 2000; Shinohara et al. 2003).

Materials and methods

The Malaysian moles used for the study were captured in the Cameron Highlands, Pahang, Malaysia, in January 2002. Morphological measurements and several ecological notes have been published elsewhere (Kawada et al. 2003). Genomic DNA from the ethanol-preserved livers of two specimens (SIK0550 and SIK0557, Kawada et al. 2003) was extracted by proteinase K digestion and phenol–chloroform–isoamyl alcohol extraction procedures. The complete mitochondrial *cyt b* gene was first amplified using the universal primer pair L-14724 and H-15915 (Irwin et al. 1991). Secondary nested PCR amplification from this product was carried out with two primer pairs: (1) L-14724 (Suzuki et al. 1997) and H-15401 (Shinohara et al. 2004) and (2) H-15916 (Suzuki et al. 2000) and L-15423 (Shinohara et al. 2004). Amplification of a fragment of the mitochondrial 12S rRNA gene (ca. 900 bp) was obtained using the universal primer pair L-613 (Mindell et al. 1991) and H-1478 (Kocher et al. 1989). Nested PCR was carried out with two primer pair sets: (1) R-L613 (Yamada et al. 2002) and U-H1066 (Suzuki et al. 1997) and (2) R-L 946 (Shinohara et al. 2004) and U-H1478 (Suzuki et al. 1997). A partial exon sequence of the RAG-1 gene was amplified using the primer pair RAG1-F1851 (Sato et al. 2004) and RAG1-R2951 (5'-GAGCCATCCCTCTC-AATAATTCAGG-3'; = RAG1-R2864, Teeling et al. 2000). The PCR product was then re-amplified with the following primer sets: RAG1-F1851 and RAG1-R2486 (Sato et al. 2004) and RAG1-F2401 (Shinohara et al. 2004) and RAG1-R2951. The PCR products of the secondary reaction were primed using the Big-Dye Terminator Cycle Sequencing Kit (ABI, Foster City, CA) and both strands were sequenced directly (Model 310; ABI, Foster City, CA).

The six new DNA sequences determined in this study were deposited in the GenBank/EMBL/DDBJ nucleotide sequence database under accession numbers AB185151–AB185156. Corresponding gene sequences from *Mogera wogura*, *M. imaizumii*, *M. tokudae*, *M. insularis*, *E.*

mizura, *Talpa altaica*, *T. europaea*, *Urotrichus talpoides* and *Uropsilus gracilis* (Appendix I) were downloaded from the database for use in subsequent phylogenetic analyses. Because the sequence lengths of the 12S rRNA products varied among species, they were first aligned together using ClustalX (Thompson et al. 1997) with default options, then manually adjusted by eye. To evaluate the congruency of the three gene sequences, we conducted the incongruence length difference test (ILD test; Farris et al. 1994). Whenever the ILD test found a *P* value greater than 0.01, combining the data improved or did not reduce phylogenetic accuracy (Cunningham 1999). A maximum likelihood (ML; Felsenstein 1981) phylogenetic tree was constructed using a heuristic search with the tree bisection-reconnection (TBR) swapping algorithm. The nucleotide substitution model for ML criteria was selected by use of Modeltest 3.06 (Posada and Crandall 1998). To assess the phylogenetic topology, we also constructed a neighbor-joining (NJ; Saitou and Nei 1987) tree employing the Kimura-2-parameter model (Kimura 1980), and a maximum parsimony (MP; Swofford and Olsen 1990) tree using heuristic searches with 100 random addition replicates utilizing the TBR swapping algorithm. The statistical confidence of branching patterns was evaluated by 1000 bootstrap replications (Felsenstein 1985). All phylogenetic analyses were carried out with the computer software program package PAUP*4.0 (Swofford 2001).

Results and discussion

We determined the *cyt b* (1140 bp), 12S (842 bp), and RAG1 (1010 bp) gene sequences from two specimens of *E. micrura*. Between the two specimens, 10, three and zero nucleotide differences were noted for the *cyt b*, 12S and RAG1 gene sequences, respectively. All of the nucleotide changes were caused by pyrimidine (A–G) or purine (T–C) transition transfers. Because some insertions and deletions were detected among the 12S gene sequences of *E. micrura* and other Eurasian talpids, the final alignment for this gene was 870 bp, and the final concatenated data set 3020 bp. The degree of phylogenetic congruency between the three genes was high ($P = 0.87$), so we constructed phylogenetic trees from the concatenated data set only. When constructing the ML tree (Fig. 1), the general time reversible (GTR; Rodríguez et al. 1990) model of nucleotide substitutions was selected with the following parameters: proportion of invariable sites (= 0.55) and gamma distribution (shape parameter =

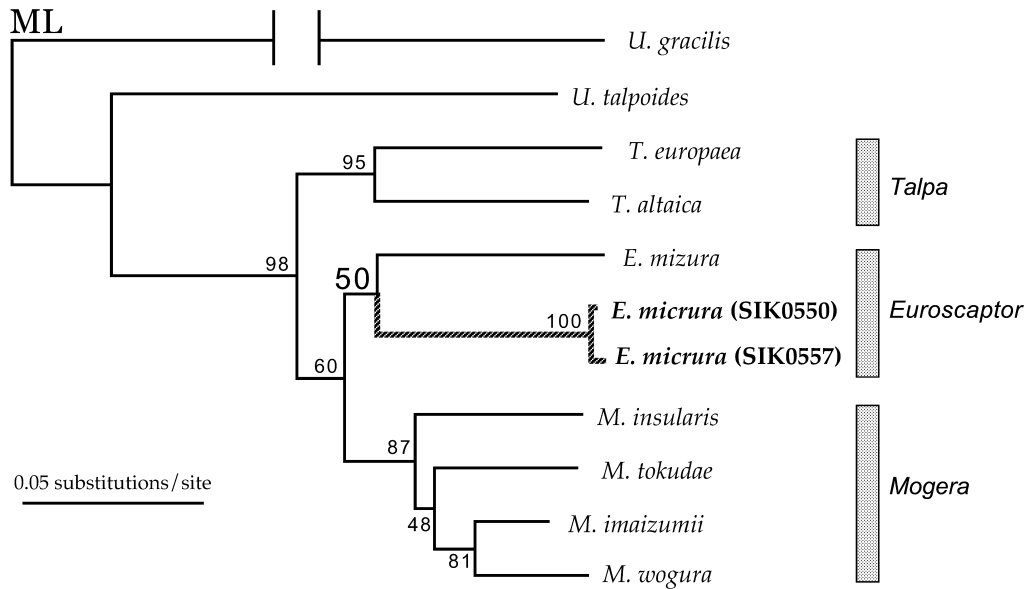


Fig. 1. Maximum likelihood tree of the Malaysian mole and its allies based on a concatenated data set utilizing the *cyt b*, 12S rRNA and RAG1 gene sequences. Bootstrap values (percentages of 1000 replications) are shown at each node.

0.536 and rate categories = 4). Our results suggest that the Malaysian mole (*E. micrura*) forms a monophyletic clade with the Japanese mountain mole (*E. mizura*), however this relationship was associated with a low bootstrap score (50%; Fig. 1). Slightly higher support values for this grouping were found for both the NJ and MP trees 68% and 67%, respectively; data not shown. Phylogenetic relationships among the other Eurasian talpids in the ML tree were identical to those described in previous studies (Okamoto 1999; Tsuchiya et al. 2000; Shinohara et al. 2003).

Historically, classification of the Malaysian mole had been equivocal, even at the genus level (Ellerman and Morrison-Scott 1966; Corbet 1978; Corbet and Hill 1992). For instance, morphological evidence suggested that *Euroscaptor* is a subgenus or synonym of *Talpa* based on the observation that they share the same dental formula – I3/3 + C1/1 + P4/4 + M3/3 = 44 (Schwarz 1947; Motokawa 2004). However, recent systematic and phylogenetic works recognize *Euroscaptor* as a valid genera (Yates and Moore 1990; Nowak 1999). Indeed, molecular studies (Tsuchiya et al. 2000; Shinohara et al. 2003; this study) support a sister-taxa relationship between *Euroscaptor* and *Mogera* (which possesses a different dental formula – I3/2 + C1/1 + P4/4 + M3/3 = 42). These conflicting data can be explained by hypothesizing that the common dental formula of *Talpa* and *Euroscaptor* is an ancestral characteristic (Motokawa 2004). The shared dental formula of *Talpa* and *Euroscaptor*

is also the same as the fundamental eutherian formula; therefore, we consider it to be a symplesiomorphic character that has no meaning in the definition of the genus.

Based on the skull morphology of *E. micrura* and *E. longirostris*, Motokawa (2004) suggested that this genus is paraphyletic. While we recovered *Euroscaptor* as a monophyletic group, bootstrap support for this association was not high (Fig. 1). However, this finding may have arisen from the small number of taxa sampled in this study, and the extensive period of isolation separating these two species (see below). Further studies are needed, including samples from other closely related genera (i.e. *Scaptochirus* and *Parascaptor*), to address this issue.

The most likely scenario that explains the present distribution of Japanese fossorial moles, including *E. mizura* and three species of *Mogera*, is as follows (Tsuchiya et al. 2000): First, *E. mizura*, whose current distribution is restricted to scattered mountainous regions of Honshu (the main island of Japan), was the first and the most primitive fossorial mole species that migrated into Japan, and it once was distributed widely. Second, modern fossorial moles of the genus *Mogera* probably evolved from an ancestral species of *Euroscaptor* on the Asian Continent and migrated into Japan, presumably displacing *E. mizura* from the lowland habitat. The Malaysian *E. micrura* represents the southernmost population of the family Talpidae and presum-

Table 1. Distance of sequence divergence of the three gene sequences between fossorial moles*.

	<i>T. europaea</i>	<i>T. altaica</i>	<i>E. mizura</i>	<i>E. micrura</i>	<i>M. insularis</i>	<i>M. tokudae</i>	<i>M. imaizumii</i>	<i>M. wogura</i>
<i>T. europaea</i>	–							
<i>T. altaica</i>	0.077	–						
<i>E. mizura</i>	0.091	0.087	–					
<i>E. micrura</i>	0.089	0.094	0.078	–				
<i>M. insularis</i>	0.090	0.085	0.079	0.081	–			
<i>M. tokudae</i>	0.087	0.087	0.077	0.077	0.063	–		
<i>M. imaizumii</i>	0.085	0.083	0.074	0.076	0.056	0.052	–	
<i>M. wogura</i>	0.088	0.092	0.080	0.076	0.064	0.057	0.042	–

*Sequence divergence was computed by Kimura (1980) with all substitutions at all codons positions.

ably has been isolated from its core population since the early stages of mole evolution in Southeast Asia, perhaps by changes in climate and sea level. Consequently, *E. mizura* and *E. micrura* have presumably been isolated for a long period of time. To examine this hypothesis, we used the Kimura two-parameter model (Kimura 1980) to calculate the genetic distances between Eurasian and Japanese fossorial moles (Table 1). The distance between *E. micrura* and *E. mizura* (= 0.078) was much larger than the maximum distance found between members of the genus *Mogera* (= 0.064), and nearly equal to that between *T. europaea* and *T. altaica* (= 0.077). Thus our data supports the contention that *E. micrura* and *E. mizura* have undergone long period of geographic isolation.

Finally, Tsuchiya et al. (2000) estimate divergence between *Mogera* and *Euroscaptor* to have occurred about 15 Mya (in the middle Miocene), which corresponds with the onset of the spread of the Sea of Japan and with the time when the climate of the islands of Japan changed dramatically from tropical and subtropical to temperate (Chinzei 1991). After these climate changes, the genus *Euroscaptor* may have shifted its habitat from the lowlands to the highlands in both tropical and temperate regions. This hypothesis needs to be examined by further studies that sample a much greater number of taxa from this genus, including Himalayan populations of *E. micrura*.

Acknowledgments: The authors thank Sahir Bin Othman (Director of the Research Division of the Department of Wildlife and National Parks, Malaysia) and Lim Cheng Hoon (Environmental Supplies and Services, Malaysia) for their kind assistance with the field survey in the Cameron Highlands. We are also grateful to Dr. Kimiyuki Tsuchiya (Tokyo University of Agriculture) for providing precious reference books and valuable sug-

gestions. We also thank Dr. Hitoshi Suzuki (Hokkaido University) and the staff of the Department of Bio-resources (Frontier Science Research Center, University of Miyazaki) for their help and encouragement in this study. We thank Dr. Kevin L. Campbell for commenting on the manuscript. This study was supported in part by Grant-in-Aid for Young Scientists (A) no. 15770060 from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

- Chasen, F. N. 1940. A handlist of Malaysian mammals. Bulletin of the Raffles Museum 15: 13–14.
- Chinzei, K. 1991. Late Cenozoic zoogeography of the Sea of Japan area. Episodes 14: 231–235.
- Corbet, G. B. 1978. The Mammals of the Palaearctic Region: A Taxonomic Review. British Museum, London, 314 pp.
- Corbet, G. B. and Hill, J. E. 1992. The Mammals of the Indomalayan Region: A Systematic Review. Oxford University Press, New York, 488 pp.
- Cranbrook, Earl of. 1962. The identity of the Malayan mole. Journal of the Bombay Natural History Society 59: 942–945.
- Cunningham, C. W. 1999. Can three incongruence tests predict when data should be combined? Molecular Biology and Evolution 14: 733–740.
- Ellerman, J. R. and Morrison-Scott, T. C. S. 1966. Checklist of Palaearctic and Indian Mammals. British Museum (Natural History), London, 810 pp.
- Farris, J. S., Källersjö, M., Kluge, A. G. and Bult, C. 1994. Testing significance of incongruence. Cladistics 10: 315–319.
- Felsenstein, J. 1981. Evolutionary trees from DNA sequences: A maximum likelihood approach. Journal of Molecular Evolution 17: 368–374.
- Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 36: 783–791.
- Hutterer, R. 1993. Insectivora. In (D. E. Wilson and D. M. Reeder, eds.) Mammal Species of the World. 2nd ed. Pp. 69–130. Smithsonian Institution Press, Washington and London.
- Irwin, D. M., Kocher, T. D. and Wilson, A. C. 1991. Evolution of the cytochrome *b* gene of mammals. Journal of Molecular Evolution 32: 128–144.
- Kawada, S., Harada, M., Obara, Y., Kobayashi, S., Koyasu, K. and Oda, S. 2001. Karyosystematic analysis of Japanese talpine

- moles in the genera *Euroscaptor* and *Mogera* (Insectivora, Talpidae). *Zoological Science* 18: 1003–1010.
- Kawada, S., Shinohara, A., Yasuda, M., Oda, S. and Lim, B. L. 2003. The mole of Peninsular Malaysia: notes on its identification and ecology. *Mammal Study* 28: 73–77.
- Kimura, M. 1980. A simple method for estimating evolutionary rate of base substitution through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* 16: 111–120.
- Kocher, T. D., Thomas, W. K., Meyer, A., Edwards, S. V., Pääbo, S., Villablanca, F. X. and Wilson, A. C. 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Science of the United States of America* 86: 6196–6200.
- Miller Jr., G. S. 1940. Notes on some moles from southeastern Asia. *Journal of Mammalogy* 21: 442–444.
- Mindell, D. P., Dick, C. W. and Baker, R. J. 1991. Phylogenetic relationships among megabats, microbats, and primates. *Proceedings of the National Academy of Science of the United States of America* 88: 10322–10326.
- Motokawa, M. 2004. Phylogenetic relationships within the family talpidae (Mammalia: Insectivora). *Journal of Zoology* 263: 147–157.
- Mouchaty, S. K., Gullberg, A., Janke, A. and Arnason, U. 2000. The phylogenetic position of the talpidae within Eutheria based on analysis of complete mitochondrial sequences. *Molecular Biology and Evolution* 17: 60–67.
- Murphy, W. J., Eizirik, E., Johnson, W. E., Zhang, Y. P., Ryder, O. A. and O'Brien, S. J. 2001. Molecular phylogenetics and the origins of placental mammals. *Nature* 409: 614–618.
- Nowak, R. M. 1999. *Walker's Mammals of the World*, 6th ed. Johns Hopkins University Press, Baltimore and London, 1936 pp.
- Okamoto, M. 1999. Phylogeny of Japanese moles inferred from mitochondrial CO1 gene sequences. In (Y. Yokohata and S. Nakamura, eds.) *Recent Advances in the Biology of Japanese Insectivora*. Hiba Society of Natural History, Shobara, pp. 21–27.
- Posada, D. and Crandall, K. A. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* 14: 817–818.
- Rodríguez, F., Oliver, J. F., Marín, A. and Medina, J. R. 1990. The general stochastic model of nucleotide substitution. *Journal of Theoretical Biology* 142: 485–501.
- Saitou, N. and Nei, M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4: 406–425.
- Sato, J. J., Hosoda, T., Wolsan, M. and Suzuki, H. 2004. Molecular phylogeny of Arctoids (Mammalia: Carnivora) with emphasis on phylogenetic and taxonomic positions of the ferret-badgers and skunks. *Zoological Science* 21: 111–118.
- Schwarz, E. 1947. Revision of the old world moles of the genus *Talpa* Linnaeus. *Proceedings of the Zoological Society of London* 118: 36–48.
- Shinohara, A., Campbell, K. L. and Suzuki, H. 2003. Molecular phylogenetic relationships of moles, shrew-moles and desmans from the New and Old Worlds. *Molecular Phylogenetics and Evolution* 26: 247–258.
- Shinohara, A., Suzuki, H., Tsuchiya, K., Zhang, Y-P., Luo, J., Jiang, X-L., Wang, Y-X. and Campbell, K. L. 2004. Evolution and biogeography of talpid moles from continental East Asia and the Japanese Islands inferred from mitochondrial and nuclear gene sequences. *Zoological Science* (in press).
- Suzuki, H., Minato, S., Sakurai, S., Tsuchiya, K. and Fokin, I. M. 1997. Phylogenetic position and geographic differentiation of the Japanese dormouse, *Glirulus japonicus*, revealed by variations among rDNA, mtDNA, and the *Sry* gene. *Zoological Science* 14: 167–173.
- Suzuki, H., Tsuchiya, K. and Takezaki, N. 2000. A molecular phylogenetic framework for the Ryukyu endemic rodents *Tokudaia osimensis* and *Diplothrix legata* (Rodentia, Mammalia). *Molecular Phylogenetics and Evolution* 15: 15–24.
- Swofford, D. L. 2001. "PAUP*: Phylogenetic Analysis Using Parsimony (*and other methods)," Version 4.0b8. Sinauer, Sunderland, MA.
- Swofford, D. L. and Olsen, G. J. 1990. Phylogenetic reconstruction. In (D. M. Hillis and C. Moritz, eds.) *Molecular Systematics*. Pp. 411–501. Sinauer, Sunderland, MA.
- Teeling, E. C., Madsen, O., Van Den Bussche, R. A., de Jong, W. W., Stanhope, M. J. and Springer, M. S. 2000. Microbat paraphyly and the convergent evolution of a key innovation in Old World rhinolophoid microbats. *Proceedings of the National Academy of Science of the United States of America* 99: 1431–1436.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. and Higgins, D. G. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 24: 4876–4882.
- Tsuchiya, K., Suzuki, H., Shinohara, A., Harada, M., Wakana, S., Sakaizumi, M., Han, S-H., Lin, L-K. and Kryukov, A. P. 2000. Molecular phylogeny of East Asian moles inferred from the sequence variation of the mitochondrial cytochrome *b* gene. *Genes & Genetic Systems* 75: 17–24.
- Yamada, F., Takaki, M. and Suzuki, H. 2002. Molecular phylogeny of Japanese Leporidae, the Amami rabbit *Pentalagus furnessi* of the Japanese hare *Lepus brachyurus*, and the mountain hare *Lepus timidus*, inferred from mitochondrial DNA sequences. *Genes & Genetic Systems* 77: 107–116.
- Yates, T. L. and Moore, D. W. 1990. Speciation and evolution in the family Talpidae (Mammalia: Insectivora). In (E. Nevo and O. A. Ring, eds.) *Evolution of Subterranean Mammals at the Organismal and Molecular Levels*. Pp. 1–22. Alan R. Liss, New York.

Received 30 July 2004. Accepted 30 September 2004.

Appendix I.

Accession numbers of additional gene sequences for phylogenetic analyses used in this study.

Sources of data: 1, Tsuchiya et al. (2000); 2, Shinohara et al. (2004); 3, Shinohara et al. (2003); 4, Murphy et al. (2001); 5, Mouchaty et al. (2000). *M. wogura* cyt *b*: AB037623 (ref. 1); 12S: AB106237 (ref. 2); RAG1: AB106244 (ref. 2). *M. imaizumii* cyt *b*: AB037609 (ref. 1); 12S: AB106236 (ref. 2); RAG1: AB106242 (ref. 2). *M. tokudae* cyt *b*: AB037607 (ref. 1); 12S: AB106235 (ref. 3); RAG1: AB106243 (ref. 2). *E. mizura* cyt *b*: AB037604 (ref. 1); 12S: AB106233 (ref. 2); RAG1: AB176543 (ref. 2). *U. talpoides* cyt *b*: AB076833 (ref. 3); 12S: AB106239 (ref. 2); RAG1: AB106245 (ref. 2). *T. altaica* cyt *b*: AB037602 (ref. 1); 12S: AY012100 (ref. 4); RAG1: AB176542 (ref. 2). *T. europaea* cyt *b*: AB037601 (ref. 1); 12S: AY19192 (ref. 5); RAG1: AB106246 (ref. 2). *U. gracilis* cyt *b*: AB076700 (ref. 2); 12S: AB106231 (ref. 2); RAG1: AB106240 (ref. 2).