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# Phylogenetic Positions of Insectivora in Eutheria Inferred from Mitochondrial Cytochrome *c* Oxidase Subunit II Gene

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**ABSTRACT**—For the elucidation of the phylogenetic position of insectivora in eutheria, we have sequenced the cytochrome *c* oxidase subunit II (COII) gene of mitochondria for three insectivoran species [musk shrew (*Suncus murinus*), shrew mole (*Urotrichus talpoides*), Japanese mole (*Mogera wogura*)] and analyzed these amino acid sequences with neighbor-joining (NJ) method and maximum likelihood (ML) method. NJ analysis shows polyphyly of Insectivora and Chiroptera. Assuming that each of Primates, Ferungulata, Chiroptera, Insectivora and Rodentia is a monophyletic group, ML analysis suggests that Chiroptera is a sister group of Insectivora and that Ferungulata is the closest outgroup to the (Insectivora and Chiroptera) clade.

## INTRODUCTION

The mammalian species observed on the earth have derived from the primitive shrew-like small beasts. Among their descendants there are four groups that are believed to share a close relationship each other among eutherians. They are the Insectivores, the Macroscelideans, the Scandentians, and the Chiropterans. Moreover, the Dermoptera and the Primates are said to be of insectivore origin (Colbert and Morales, 1991). However, it is very difficult to resolve their phylogenetic relationships only from the morphological characteristics and/or the fossil evidences, because of poor fossil record. In addition, although the insectivoran order is generally divided into three suborder, the relationships among these suborders remain uncertain due to inconsistencies in morphological traits.

In order to investigate the intraordinal relationships of Insectivora among primitive placental groups, we sequenced the cytochrome *c* oxidase subunit II (COII) genes of mtDNA at first, since this gene has been used extensively in the investigation for systematic relationships within and among mammalian orders. Although the nucleotide sequence of animal mtDNA is useful to investigate relationships among closely related species because of its high evolutionary rate, the amino acid sequence encoded by mtDNA can be also useful to probe deep branchings because of its slower rate than the rate at a nucleotide level. The other advantage of using mtDNA in a phylogenetic work is that we are relatively free from the danger of comparing paralogous genes, as may sometimes be the case in nuclear genes (Cao *et al.*, 1994).

In our study we used three insectivoran species, musk shrew (*Suncus murinus*), belonging to the family Soricidae,

and shrew mole (*Urotrichus talpoides*) and mole (*Mogera wogura*), belonging to the family Talpidae. Mole and shrew mole are specialized for underground life and they have the shovel-like limbs for digging soil and their visual organs are covered with the skin. On the other hand, musk shrew has slender limbs with five toes and small normal visual organs. Then we compared new amino acid sequences from these three insectivora with those of the other mammalian orders, with special attention to the other insectivoran species, the order Chiroptera and the order Primates.

## MATERIALS AND METHOD

### DNA sequencing

Total genomic DNA from frozen liver, kidney and heart was extracted with the standard techniques (Sambrook *et al.*, 1989). The mt DNA from frozen liver, kidney and heart was extracted with the alkaline lysis procedure (Tamura and Aotsuka, 1988). The complete COII gene was PCR-amplified with primers L7784, L7553, H8169 and H8320 (Adkins and Honeycutt, 1994). Each cycle of PCR consisted of denaturation at 95°C for 1 min, hybridization at 45°C for 1 min, and extension for 3 min at 72°C for 30 cycles. Double-strand PCR products of the COII gene were ligated into the plasmid pGEM-T vector. Cycle sequencing reactions were carried out according to the method described by Applied Biosystems, using a dye-labeled T7 and SP6 primers. Due to the inherent error rate of *Taq* polymerase (Saiki *et al.*, 1988; Tindall and Kunkel, 1988; Keohavang and Thilly, 1989), at least two independent clones were sequenced for each taxon. No sequence discrepancies were found between the clones.

### Data analysis

Raw sequence data were analyzed with the software DNASIS-Mac (Hitachi Software Engineering). We chose the human sequence (Horai *et al.*, 1992) as a reference sequence. Amino acid sequences encoded by mtDNA are particularly useful to probe deep branches, because selection (mainly negative selection in the framework of the neutral theory; Kimura, 1983) is likely to be operating stronger on an amino acid site than on a nucleotide site (Kocher *et al.*, 1989). There-

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**Table 1.** COII gene sequences included in this study

Class and order	Latin binomial and common name	Accession # and/or reference
Class Mammalia		
Monotremata	<i>Ornithorhynchus anatinus</i> (platypus)	X80903; Janke <i>et al.</i> (1996)
Marsupialia	<i>Didelphis virginiana</i> (opossum)	Z29573; Janke <i>et al.</i> (1994)
Marsupialia	<i>Macropus robustus</i> (wallaroo)	Y10524; Janke <i>et al.</i> (1997)
Rodentia	<i>Mus musculus</i> (mouse)	J01420; Bibb <i>et al.</i> (1981)
Rodentia	<i>Rattus norvegicus</i> (rat)	X14848; Gadaleta <i>et al.</i> (1989)
Edentata	<i>Dasypus novemcinctus</i> (nine-banded armadillo)	M80903; Adkins and Honeycutt (1991)
Primates	<i>Galago senegalensis</i> (lesser bushbaby)	M80905; Adkins and Honeycutt (1991)
Primates	<i>Nycticebus coucang</i> (slow loris)	L22781; Adkins and Honeycutt (1994)
Primates	<i>Lemur catta</i> (ring-tailed lemur)	L22780; Adkins and Honeycutt (1994)
Primates	<i>Cheirogaleus medius</i> (fat-tailed mouse lemur)	L22775; Adkins and Honeycutt (1994)
Primates	<i>Alouatta palliata</i> (mantled howler)	L22774; Adkins and Honeycutt (1994)
Primates	<i>Lagothrix lagotricha</i> (Humboldt's wooly monkey)	L22779; Adkins and Honeycutt (1994)
Primates	<i>Cercopithecus aethiops</i> (green monkey)	M58005; Ruvolo <i>et al.</i> (1991)
Primates	<i>Macaca mulatta</i> (rhesus monkey)	M74005; Disotell <i>et al.</i> (1992)
Primates	<i>Hylobates syndactylus</i> (siamang)	P25312; Horai <i>et al.</i> (1992)
Primates	<i>Pongo pygmaeus</i> (orangutan)	D38115; Horai <i>et al.</i> (1995)
Primates	<i>Homo sapiens</i> (human)	D38112; Horai <i>et al.</i> (1995)
Chiroptera	<i>Rousettus leschenaulti</i> (Leschenault's rousette)	M80908; Adkins and Honeycutt (1991)
Chiroptera	<i>Rhinolophus darlingi</i> (horse shoe bat)	U62580; Adkins and Honeycutt (1993)
Chiroptera	<i>Phyllostomus hastatus</i> (greater spear-nosed bat)	M80906; Adkins and Honeycutt (1991)
Insectivora	<i>Blarina brevicauda</i> (short-tailed shrew)	U62578; Adkins <i>et al.</i> unpublished
Insectivora	<i>Suncus murinus</i> (musk shrew)	this paper
Insectivora	<i>Urotrichus talpoides</i> (shrew mole)	this paper
Insectivora	<i>Mogera wogura</i> (mole)	this paper
Carnivora	<i>Felis catus</i> (cat)	U20753; Lopez <i>et al.</i> (1996)
Carnivora	<i>Canis simensis</i> (dog)	L29414; Gottelli <i>et al.</i> (1994)
Carnivora	<i>Phoca vitulina</i> (harbour seal)	X63726; Arnason and Johnsson (1992)
Carnivora	<i>Halichoerus grypus</i> (grey seal)	X72004; Arnason <i>et al.</i> (1993)
Artiodactyla	<i>Bos taurus</i> (cow)	J01394; Anderson <i>et al.</i> (1982)
Artiodactyla	<i>Capra hircus</i> (goat)	U62569; Janecek <i>et al.</i> unpublished
Cetacea	<i>Balaenoptera musculus</i> (blue whale)	X72204; Arnason <i>et al.</i> (1993)
Perissodactyla	<i>Equus caballus</i> (horse)	X79547; Xu and Arnason (1994)

fore, we used amino acid sequence of the COII gene for analysis, together with the published data (Table 1).

In analyzing the COII amino acid sequence data of protein, we used both neighbour-joining method (Saitou and Nei, 1987) and maximum likelihood method (Felsenstein, 1981). As for the case of the NJ method, we used the MEGA program (Kumar *et al.*, 1993) with a gamma distance  $\alpha = 0.7$  (Nei *et al.*, 1976). For maximum likelihood analysis, we used the PROTML program in MOLPHY package, ver 2.3 (Adachi and Hasegawa, 1996).

For the PROML analyses, the mt REV-F models for mtDNA-encoded protein (Adachi and Hasegawa, 1996) were used for the these data. Since it seems very likely that each of Primates, Ferungulata [Artiodactyla, Cetacea and Carnivora (Cao *et al.*, 1994)], Chiroptera, Insectivora and Rodentia forms a monophyletic group, we add armadillo into these 5 groups and examined all of the 945 possible trees among these 6 major lineage of eutheria. We estimated the bootstrap probability by the RELL (resampling of the estimated log-likelihood) method by Kishino *et al.* (1990). Although the sequence data of goldenmole and hedgehog, which are assumed to have been also the member of Insectivora, were available in a public data-base, both of them were excluded in our analyses from the following reasons. Goldenmole is suspected in a recent molecular study (Springer *et al.*, 1997) to be in a clade that contains members of presumed African origin and its phylogenetic position in Insectivora is also uncertain with morphological uncertainties (Van Valen, 1967; Eisenberg, 1981). On the other hand, hedgehog shows an higher levels of compositional bias in nucleotide bases of the overall mtDNA genes.

The branching orders within each of major lineage of eutherian

and within the outgroup were assumed as the consensus of previous works (Cao *et al.*, 1994; D' Erchita *et al.*, 1996; Bulmer *et al.*, 1991; Li *et al.*, 1990; Szalay, 1977; McKenna, 1975; Van Valen, 1967; Mindell *et al.*, 1991; Adkins and Honeycutt, 1991). The species and their assumed within-group relationships are Primates: (human, orangutan, siamang, rhesus monkey, green monkey, mantled howler, Humboldt's wooly monkey, ring-tailed lemur, fat-tailed mouse lemur, slow loris, bushbaby); Insectivora: ((short-tailed shrew, musk shrew), (shrew mole, Japanese mole)); Ferungulata: ((harbor seal, grey seal) dog, cat) ((goat, (cow, blue whale)), horse); Rodentia: (rat, mouse); Chiroptera: (Leschenault's rousette, greater spear-nosed, horse shoe); and outgroup: ((wallaroo, opossum), platypus).

## RESULTS AND DISCUSSION

The nucleotide sequences of cytochrome *c* oxidase sub-unit II gene and the amino acid sequences are shown in Fig. 1 and in Fig. 2, respectively, for three insectivoran species and human.

The phylogenetic tree based on NJ analysis is shown in Fig. 3 and the result is generally consistent with the morphological and molecular phylogenetic trees already reported (Honeycutt *et al.*, 1995; Johnson *et al.*, 1994), but shows some interesting differences.

As is expected, the close relationships between mole and

	M A H A A Q V G L Q D A T S P I M E E L I T F H D H A L M I I F L	100
human	ATGGCACATGCAGCGCAAGTAGGTCTACAAGACGCTACTTCCCCTATCATAGAAGAGCTTATCACCTTTTCATGATCAGCCCTCATAATCATTTCCTTA	
musk shrew	-----TT-CC--TTC---A-----T-----A--A-----T-----AT-A--A-AT-----C--T--AT-A-----TG-----T--A-	
shrew mole	-----T-CC--TTT---T-T--AT-T-----T--A--A--A-----T--G-----AT-AT-A-A-----A--A-----TGCC---T-A-	
mole	-----T--C--TTC---C-T---T-T-----A--A--A--A--T-----G--AT-AT-ACAT--C-----A--T-----GCC--TT-A-	
	7600	
	I C F L V L Y A L F L T L T T K L T N T N I S D A Q E M E T V W T I	200
human	TCTGCTTCCTAGTCTGTATGCCCCCTTTCTTAACACTCACAACAAACTAATACTAATCTCAGACGCTCAGGAAATAGAAACCGTCTGAACCTAT	
musk shrew	-TA-T-C-----TT-A---TTA---CTGCC-TGT-A-----T-----T--AC-----T-CAAT---T-----A-C-G-----AA-----	
shrew mole	--A---CAT-----T---ATTA---CA--T-TG-----T---T-----C---A-G--CAATG--T--A--A--G-----A--A-----G--	
mole	-TA---CTT---A--T--CATTA---CA--T-T---T-----T---AC---A-G--CAATG--T--A--A--G-G-----TA-T-----C--	
	7700	
	L P A I I L V L I A L P S L R I L Y M T D E V N D P S L T I K S I	300
human	CCTGCCCCCATCATCTAGTCTCATCGCCCTCCCATCCCTACGCATCCTTTACATAACAGACGAGGTCAACGATCCCTCCCTTACCATCAAAATCAATT	
musk shrew	T-----A-----T--T--A-TA-A--T--A--T-----AT---T--T--A--T-----T---T--AA-T--A---TA-TT-A--T--T--A--G-A	
shrew mole	-----T--T--T--TT--A-TA-A--T--TT-A-----A-----A--T--A--T-----TG--T--AA-T--A-C--A--T-G--AG-A-----G	
mole	---A--G--T--T-----A-TA-A--T--T--T-----AT---A--TT-A--T-----T---T--AA-T--TA-C--A-----A--AGGT---A---G	
	7800	
	G H Q W Y W T Y E Y T D Y G G L I F N S Y M L P P L F L E P G D L	400
human	GGCCACCAATGGTACTGAACCTACGAGTACACCGACTACGGCGGACTAATCTTCAACTCCTACATCTTCCCCATTATTCCTAGAACCGGCGACCTGC	
musk shrew	--A--T-----A-----G---T--A--T--A-----T-AT-A-----G-----T---A-----AG-T-CTGAA---A-----T--T--T--T--	
shrew mole	--T--T-----A--T---GT--T--A--T--G--T--T-AA-ACT---C---G---A--T---G-A--AA-CACTGATT--A-G-----G--AT-A-	
mole	--T-----A--T---G---T--A-----T--T--T-AA-AC---CA--TG---A-----G-A--AA-TACGGA---A-----T--A--G---	
	7900	
	R L L D V D N R V V L P I E A P I R M M I T S Q D V L H S W A V P T	500
human	GACTCCTTGACGTTGACATCGAGTAGTACTCCCGATTGAAGCCCCCATTCGTATAATAATACATCACAAGACGCTTGCACCTCATGAGCTGTCCTCCAC	
musk shrew	-TT-A-----A--A--T-----C-CG--C--A--A--A---ATAA-A-----AG-TT--G-A--C--CG---T--TC-T-----A--A--TT-	
shrew mole	-----TT-A--A-----T-----T--TT-A--A--A---ATAA-T-----A--GC-T---T---TG-----T--A-----C--A--GT-	
mole	--T-AT-A--A--C--T--C-----C---A--A--A---ATAA-----A--T-----T---TG---T--TC-A--TG-----C--A--GT-	
	8000	
	L G L K T D A I P G R L N Q T T F T A T R P G V Y Y G Q C S E I C	600
human	ATTAGGCTTAAAAACAGATGCAATTCCTGCGGACGCTCAAACCAACCACTTTTACCCTGCTACACGACCGGGGTATACTACGGTCAATGCTCTGAAATCTGT	
musk shrew	TC---A-----C-----T-----T-----A-CT-----T--AC--TTG--A-----A--T-----A-----T-----	
shrew mole	-C---G-----C-----C--A-----A-----T--G--A-----ACTAT-A--C-----T--AT---T-----A-----	
mole	-C---A-----C--C--C--A-----A-----T-----A--C--ACTAT-A--C-----C--CT---T--T--A-----T--A-----T---	
	8100	
	G A N H S F M P I V L E L I P L K I F E M G P V F T L B	684
human	GGAGCAAACACAGTTTCATGCCCCATCGTCTAGAAATTAATTCCTTAAATAATCTTGAATAGGGCCCGTATTACCTATAG	
musk shrew	--CT-T--T-----C--T--A-----T--A--T---C--G-----AT-----T-----A-T-AT-TTC--CA-TAA-C--A	
shrew mole	--CT-T--T--T--C-----A--T--T-----C---A--G-----A-----TAT-----A-T-AT-TTC--C--TG-----A	
mole	---T---T--T--C--T-----T--T-----A--G-A--T-----TAT--C---A-T-AT-TTC--CC-TA-----A	
	8200	

**Fig. 1.** Nucleotide sequence of the cytochrome *c* oxidase subunit II (COII) gene from the three insectivoran species along with the human sequence (Anderson *et al.*, 1981). Numbering is according to the human sequence. The predicted amino acid sequence of the human protein is shown above by one-letter code. Nucleotides identical to those of human are indicated by dashes. human = *Homo sapiens*, musk shrew = *Suncus murinus*, shrew mole = *Urotrichus talpoides*, mole = *Mogera wogura*.

shrew mole and between musk shrew and short-tailed shrew are observed with high bootstrap probabilities (97% in both cases) and the results are in accord with the traditional rela-

tionship by obtained morphological data (Butler, 1988; Szalay, 1977; McKenna, 1975; Van Valen, 1967). The NJ tree, however, have complicated the issue of fixing Insectivoran posi-

human	MAHAAQVGLQDATSPIMEELITFHDHALMIIFLICFLVLYALFLTTLTKLTNTNISDAQEMETVWTILPAIILVLIALPS	
musk shrew	MAYPFQMGLQDATSPIMEELMNFHDHALMIVFLISSLVLYVISAMLTTKLTHTNTMDAQAVETIWTILPAIILIMIALPS	
shrew mole	MAYPEQFGFQDATSPIMEELNLFHDHALMIAFLISSLVLYIISLMLTTKLTHSTMDAQEVETVWTILPAIILIMIALPS	
mole	MAYPFQLGFQDATSPIMEELLHFHDHALMIAFLISSLVLYIISLMLTTKLTHSTMDAQEVETIWTILPAIILIMIALPS	
160		
human	LRILYMTDEVNDPSLTIKSIHQWYWTYETDYGGFLIFNSYMLPPLFLEPGDLRLLDVNDNRVVLPIEAPIRMMITSQDVL	
musk shrew	LRILYMMDEINNPTLTIKTVGHQWYSYETDYDELNFDSYMIPASELKPGDLRLLEVDNRAVLPMENTIRVLVTSEDVL	
shrew mole	LRILYMMDEINNPSLTVKTMGHQWYSYETDYEDLTFDSYMPVPTDLKPGELRLLEVDNRRVLPMENTIRMLISSEDL	
mole	LRILYMMDEINNPSLTGKTMGHQWYSYETDYEDLTFDSYMPVPTDLKPGELRLLEVDNRRVLPMENTIRMLISSEDL	
227		
human	HSWAVPTLGLKTAIPGRNLNQTTFATRPGVYVYQCSEICGANHSFMPIVLELIPLKIFEMGPVFTL	
musk shrew	HSWAVPSLGLKTAIPGRPNQTTLLATRPGVYVYQCSEICGSNHSFMPIVLELVPLKIFEXWSSSMI	
shrew mole	HSWAVPSLGLKTAIPGRNLNQTTLSTRPGLYVYQCSEICGSNHSFMPIVLEMVPLKYFEKXSSSML	
mole	HAWAVPSLGLKTAIPGRNLNQTTLSTRPGLYVYQCSEICGSNHSFMPIVLEMVPLKYFEKXSSSML	

**Fig. 2.** Amino acid sequence of the cytochrome *c* oxidase subunit II gene along with the human sequence (Anderson *et al.*, 1981). human = *Homo sapiens*, musk shrew = *Suncus murinus*, shrew mole = *Urotrichus talpoides*, mole = *Mogera wogura*.

tion among major orders, with Chiroptera as many as three independently evolving lineages observed (Fig 3). The bootstrap probabilities of [(short-tailed shrew, musk shrew), greater spear-nosed bat] clad and {horse shoe bat, [Leshenult's rousette, (shrew mole, Japanese mole)]} clad are only 27% and 20%, respectively, which suggest the resolving power of the present data would not be enough to distinguish markedly these groups. This, in fact, may suggest that the relationship between Insectivora and Chiroptera may be very close. The close relationship of armadillo to Primates is also not in accord with the evidence from the complete mtDNA data of armadillo (Arnason *et al.*, 1997).

Despite the predominantly divergent nature of most molecular evolution, parallel and convergent evolution sometimes occur in the specific genes (Stewart *et al.*, 1987), and such a discrepancy could occur in 5% of the cases without any particular reason if the divergence among groups in question occurred within a short period.

Furthermore, the suborder Anthropeidea seems to be too distant from the other eutherians in Fig. 3. This may be probably due to the higher level of variation and the higher rate of amino acid replacement in this lineage.

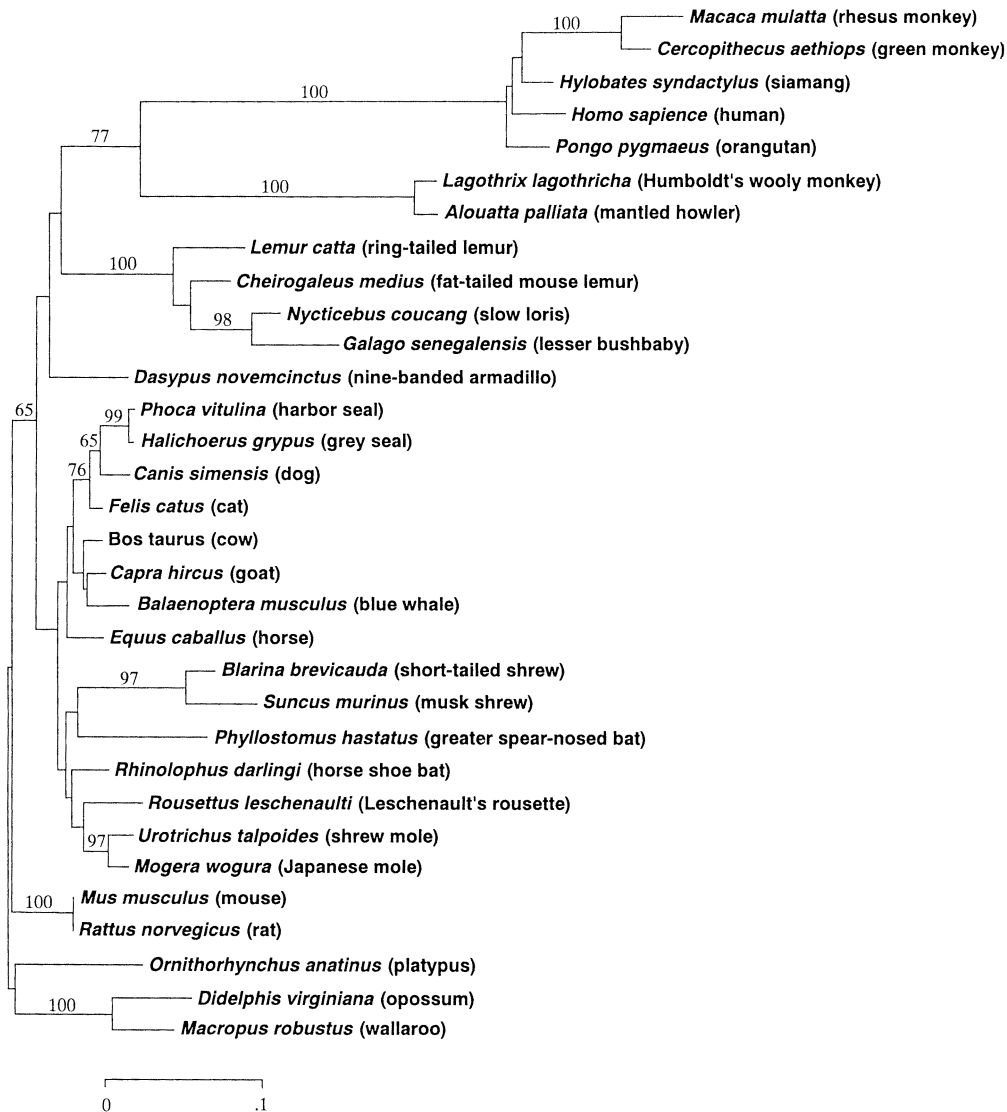
One of our purpose is to see the degree of interrelationship among the extant eutherian orders and to seek the most close relative of Insectivora. Therefore, we examined the data by maximum-likelihood (ML) method (Kishino *et al.*, 1990; Adachi and Hasegawa, 1992) for protein sequences with known consensus relationship of eutherians. The branching orders within each of the Primates, Ferungulata (Artiodactyla,

Cetacea, Perissodactyla), Insectivora, Chiroptera, Rodent, and outgroup are assumed as the consensus of the several morphological and molecular studies. Figure 4 shows the phylogenetic relationship among the seven major eutherian groups constructed from COII data alone by the PROTML. Although the bootstrap probability is not high, the ML tree with the highest boot strap probability (45.7%) locates Chiroptera closer to Insectivora than to the other eutherian orders.

From both NJ and ML phylogenetic analyses, the following interesting results were obtained. Firstly, the most closest relative of Insectivora seems to be the order Chiroptera. Secondly, there would be a sister-group relationship between Ferungulata and (Insectivora + Chiroptera).

Novacek (1986, 1989), from the analyses on skull characters, suggested the possibility that Insectivora may be part of a clade that includes Tubulidentates and Carnivorans. Miyamoto and Goodman (1986), from the analyses on amino acid sequence changes in several proteins, suggested that Insectivora may be most closely related to Carnivorans and Pholidotans. But these evidences supporting each association are admittedly weak. The insectivoran position has been not clear in eutherian cladogram yet. The COII gene trees obtained by the present study do not support either Novacek or Miyamoto and Goodman's hypothesis, and radically contradict the traditional view, which says that Chiroptera would be closely associated with Primates, flying lemur and tree shrew in the superorder Archonta (Novacek *et al.*, 1988 and Szalay, 1977).

We consider it appropriate to use molecular data because



**Fig. 3.** The neighbor-joining tree of amino acid sequence of COII gene. Bootstrap values 40% or greater (derived from 1000 replicates) are shown along branches. The horizontal length of each branch is proportional to the estimated number of amino acid substitutions.

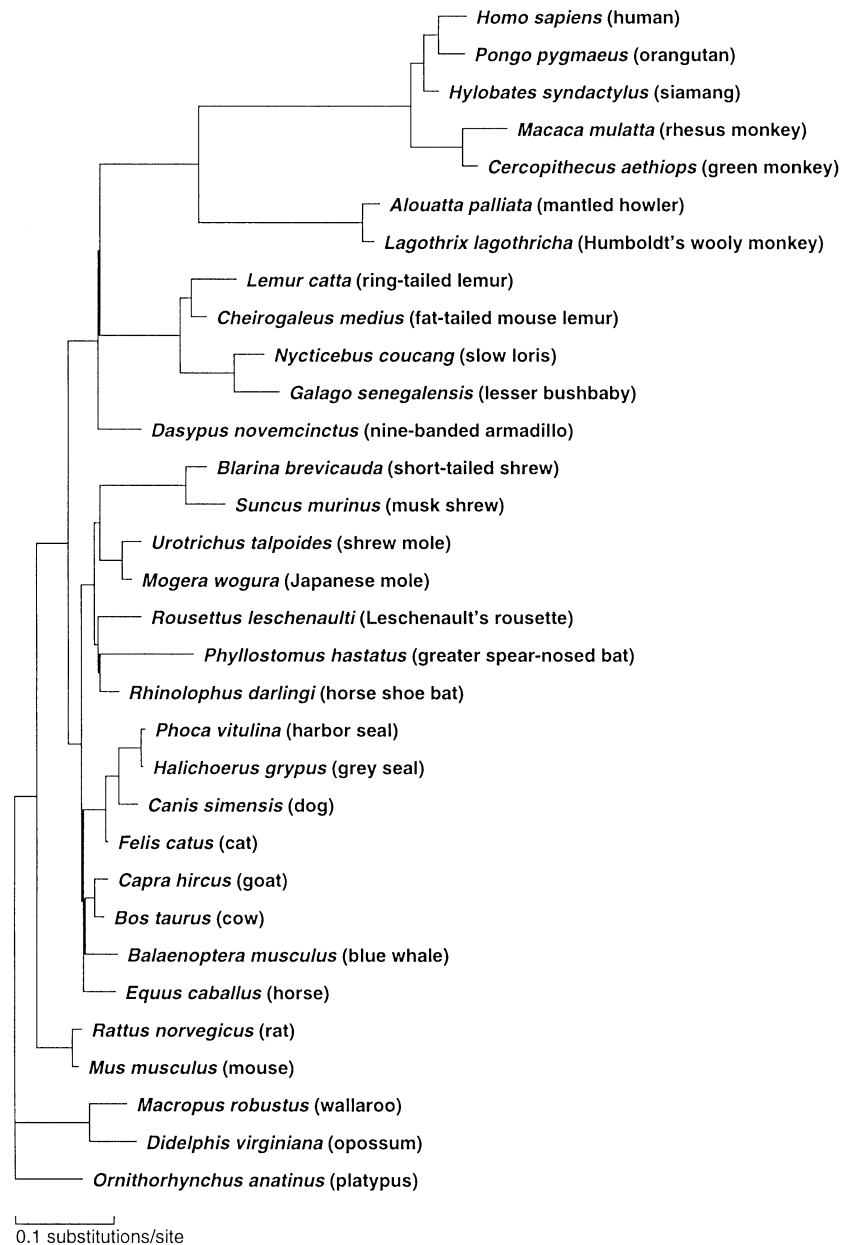
it seems that there are no recognizably non-primitive traits of Insectivora to distinguish them from other eutherians. Since analyses of individual genes could lead to an erroneous tree with an apparently significant confidence level, to avoid such possibility, we will carry out further analyses based on as many different genes as possible and synthesize the results.

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**Fig. 4.** The maximum likelihood tree of amino acid sequence of COII gene. The Prot ML program with the mtREV-F model was applied. The horizontal length of each branch is proportional to the estimated number of amino acid substitutions.

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