A Close Relationship of Chiroptera with Eulipotyphla (Core Insectivora) Suggested by Four Mitochondrial Genes

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ABSTRACT—We sequenced the mitochondrial cytochrome b, 12S rRNA and 16S rRNA genes from three insectivoran species (Japanese mole, shrew mole and musk shrew) and one chiropteran species(Japanese pipistrelles) and the cytochrome c oxidase subunit II (COII) gene from a chiropteran species. The phylogenetic relationship of core Insectivora or Eulipotyphla among major Eutherian orders was examined for these genes. A total evaluation of the maximum likelihood analyses of the four genes suggests that Chiroptera is the closest to Eulipotyphla among 4 eutherian groups (Primates, Eulipotyphla, Fereuungulata, Rodentia).

INTRODUCTION

Molecular phylogenetics has become a powerful tool in clarifying inter-ordinal relationships of placental mammals (for review, e.g., de Jong, 1998; Waddell et al., 1999a). One of the most surprising findings in this discipline is that, rather than to Primates as the traditional taxonomy suggested (Novacek 1992), Chiroptera (bats) is more closely related to Carnivora+Perissodactyla +Cetartiodactyla (Pumo et al., 1998; Teeling et al., 2000). The latter clade is often called Ferungulata, but, since Ferungulata as defined by Simpson (1945) includes Paenungulata, such as elephant, hyrax, dugong and aardvark, which turned out not to be directly related to this clade (Springer et al., 1997, 1999; Stanhope et al., 1998), Waddell et al. (1999b) proposed a superordinal taxon Fereuungulata (Carnivora + the true ungulates excluding Paenungulata) which consists of Cetartiodactyla, Perissodactyla, Carnivora, and Pholidota (pangolin).

Another surprising finding is that golden mole and tenrec which have been classified as Insectivora are more closely related to Paenungulata, Tubulidentata, and elephant shrew, together forming a clade called Afrotheria (Stanhope et al., 1998; Springer et al., 1997, 1999; de Jong, 1998). The sister-group relationship between Chiroptera and core insectivores or Eulipotyphla (hedgehog, shrew, solenodon and mole; Waddell et al., 1999a) has been suggested with mitochondrial COII sequences by Onuma et al. (1997, 1998).

To further strengthen the last finding, we determined the three other mitochondrial genes, cytochrome b(COB), 12S ribosomal RNA and 16S ribosomal RNA genes from three eulipotyphlan species (Japanese mole, Japanese shrew mole and musk shew), COB, 12S and 16S rRNA genes from a chiropteran species (Japanese pipistrelles), and further examined the phylogenetic relationship of the Eulipotyphla among eutherian orders.

MATERIALS AND METHODS

DNA sequencing

We determined the sequences of the COB, 12S rRNA and 16S rRNA mitochondrial genes from the three insectivoran species and COB, 12S and 16S rRNA genes from one chiropteran species (see Onuma et al., 1998). The entire COB, 12S rRNA, and16S rRNA genes were amplified with the following primers by the polymerase chain reaction. The sequences of the COB primer are L-1 (5'- CGA AGC TTG ATA TGA AAA ACC ATC GTT G), L-2 (5'-AAA AAG CTT CCA TCC AAC ATC TCA GCA TGA TGA A), H-1 (5'-ATG GAT CCT AGG ATG GCG TAT GCA AAT A), H-2 (5'-AAC TGC AGT CAT CTC CGG TTT ACA AGA C). The sequences of the12S rRNA primer are L-3 (5'-CTC AAG CTT GCA AGG CAC TGA AAA TGC TTA GA), L-4 (5'-AAA AAG CTT CAA ACT GGG ATT AGA TAC CCC ACT AT), H-3(5'-TGA CTG CAG AGG GTG ACG GGC GGT GTG T), H-4 (5'-CAT GGA TCC TTC TGG GTG TAG GCC AGA TGC TTT). The sequences of the16S rRNA primer are L-5 (5'-GGG AAG CAT ACT GGA AAG TG), L-5* (used only for Chiroptera) (5'-GCG TAA CAA GGT AAG TGT ACT GGA AAG TG), L-6 (5'-GAA CAT CTC AGC ACA GAG AAC TTA AG), L1990 (5'- CCA GAC CAG CTA TTC ATG AAC AGC), L-8 (5'- TTA AGC TTA GAA GTG ACA CTG CTG, L-9 (5'- CCC AGA GAT GGA ACT AAC TCC CAG A), L-10 (5'- ACA AAG CTT TAT ATC AGC CCG AAT ACC), L-11 (5'-TTT AAG CTT CTC TTA CTT CCA ATC AGT G), H-5 (5'- GGG GAT CCA AGT TCT CGT TAC TCA TAT), H-6 (5'- CAG GAT CCT TAG GGA GAG GAA TTG AAC). The

cytochrome c oxidase II gene of the Japanese pipistrelles (*Pipistrellus abramus*) was also determined.

Sequences have been deposited in DDBJ. Accession numbers are as follows: *Suncus murinus* 16S rRNA (AB032842), *Mogera wogura* 16S rRNA(AB032843), *Pipistrellus abramus* 16S rRNA-(AB032844), *Urotrichus talpoides* 16S rRNA(AB033608), *Suncus murinus* 12S rRNA(AB032845), *Mogera wogura* 12S rRNA(AB-032846), *Pipistrellus abramus* 12S rRNA(AB032847), *Urotrichus talpoides* 12S rRNA(AB033609), *Suncus murinus* cytochrome b(AB033610), *Mogera wogura* cytochrome b (AB033612), *Urotrichus talpoides* cytochrome b (AB033611), *Pipistrellus abramus* cytochrome c oxidase II (AB033723).

Sequence analyses:

Raw sequence data were analysed by software DNASIS-MAC and aligned by CLUSTAL-V. Both of the12S and16S rRNA sequence alignment were modified based on Gutell et al.'s (1993) models at hand. Positions with gaps and regions where the alignment was ambiguous were excluded from the analyses. NucML and ProtML programs in MOLPHY (Adachi and Hasegawa 1996b) and BaseML and AAML programs in PAML (Yang 1997) were used with the HKY model (Hasegawa et al., 1985) for nucleotide substitutions and mtREV-F model (Adachi and Hasegawa 1996a) for amino acid substitutions. In using BaseML and AAML programs, the discrete gamma-distribution (with 8 categories; Yang 1996) for site-heterogeneity was applied.

Since the branching order among Carnivora, Perissodactyla and Cetartiodactyla in Fereuungulata could not be resolved (Graur et al., 1997; Cao et al., 1999), trifurcation was assumed in our analyses.

Log-likelihoods of all the possible trees (105 trees) among Chiroptera, Eulipotyphla, Primates, Fereuungulata, and Rodentia with Marsupialia and Monotremata as an outgroup were estimated by us-

ing the NucML for 12S and 16S rRNAs and the ProtML for COB and COII by assuming the site-homogeneity, and the total log-likelihoods of the four genes were evaluated by the TotalML program in MOLPHY. Among these trees, those whose log-likelihoods are within 2SEs from the ML tree were analyzed by the BaseML and AAML programs with a gamma-distribution for the site-heterogeneity.

Data used in the analyses

We used the following species for both ML analysis and total ML analysis :

for the 12S rRNA gene: *Homo sapiens* (human, D38112), *Pan paniscus* (bonobo, D38116), *Hylobates lar* (common gibbon, X99256), *Halichoerus grypus* (grey seal, X72004), *Felis catus* (cat, U20753), *Equus caballus* (horse, X79547), *Rhinoceros unicornis* (Indian rhinoceros, X97336), *Bos taurus* (cow, J01394), *Balaenoptera musculus* (blue whale, X72204), *Blarina brevicauda* (short-tailed shrew, M95110), *Mogera wogura* (Japanese mole, AB032846), *Urotrichus talpoides* (shrew mole, AB033609), *Suncus murinus* (musk shrew, AB032845), *Pipistrellus abramus* (pipistrelles bat, AB032847), *Eptesicus fuscus* (brownbat, U61082), *Nyctimene albiventer* (tube-nosed bat, U61077), *Rattus norvegicus* (rat, X14848), *Mus musculus* (mouse, J01420), *Macropus robustus* (wallaroo, Y10524), *Didelphis virginiana* (opossum, Z29573), *Ornithorhynchus anatinus* (platypus, X83427).
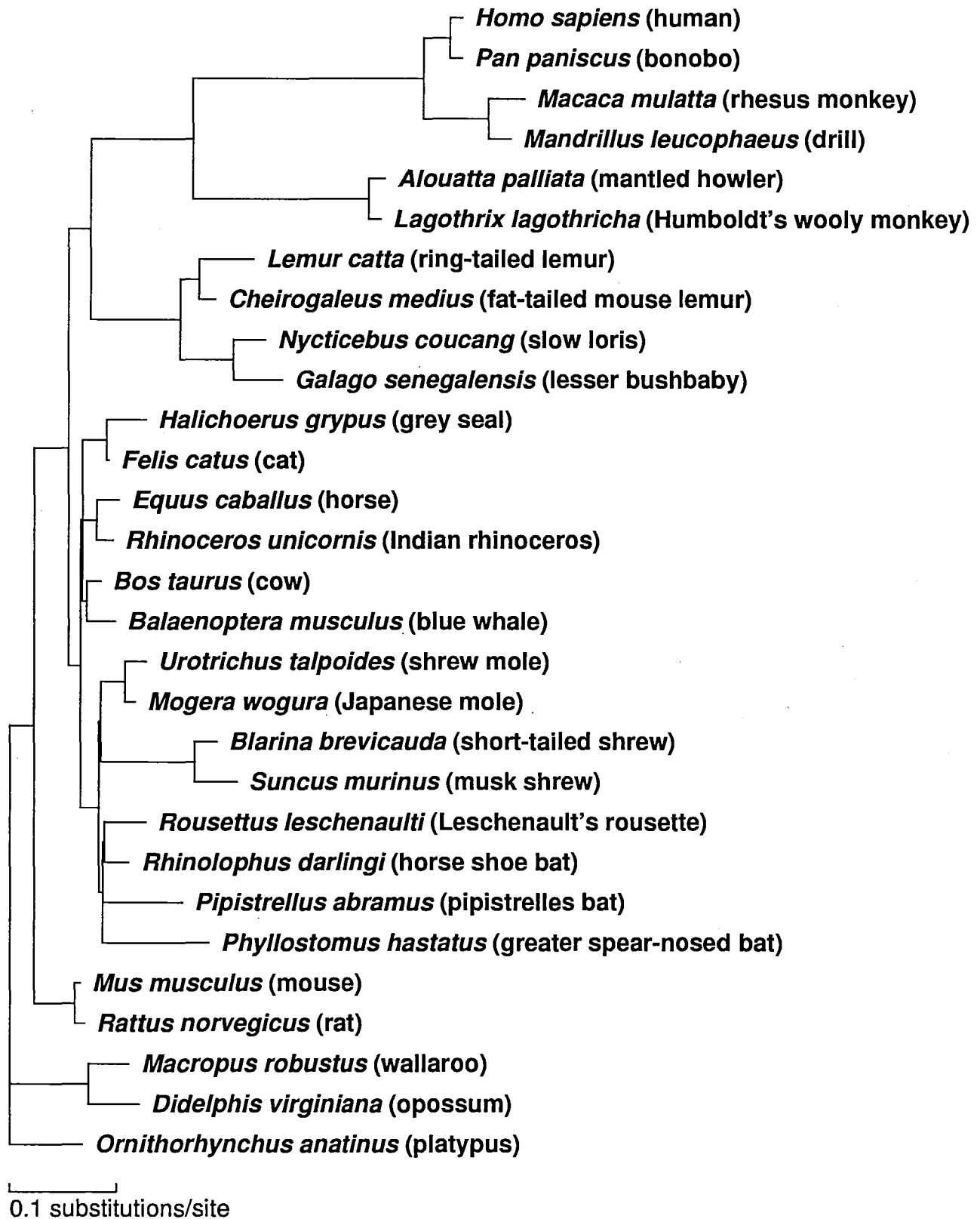
for the 16S rRNA gene: *Homo sapiens* (human, D38112), *Pan paniscus* (bonobo, D38116), *Pongo pygmaeus* (orangutan, D38115), *Hylobates lar* (common gibbon, X99256), *Halichoerus grypus* (grey seal, X72004), *Felis catus* (cat, U20753), *Equus caballus* (horse, X79547), *Rhinoceros unicornis* (Indian rhinoceros, X97336), *Bos taurus* (cow, J01394), *Balaenoptera musculus* (blue whale, X72204), *Urotrichus talpoides* (shrew mole, AB033608), *Mogera wogura* (Japanese mole, AB032843), *Suncus murinus* (musk shrew, AB032842),

Table 1. Log-likelihood value of the best tree in < >, and difference of log-likelihood of an alternative tree from the best with 1SE (following ±) estimated by the formula of Kishino and Hasegawa (1989). Bootstrap proportions among the 11 trees in () were estimated by the RELL method of Kishino et al. (1990) with 10,000 replications.

tree	12S	16S	COB	COII	COIItotal
without gamma					
1 ((P,(F,(E,C))),R)	−5.5±6.7	−0.3± 7.6	−8.9± 8.5	<−2,898.8>	<−23,768.6> (0.5443)
2 (((P,F),(E,C)),R)	−11.4±8.4	−7.4±13.9	<−5,610.4>	−2.3±4.0	−6.4±16.7 (0.2363)
3 (((P,(E,C)),F),R)	−11.5±8.3	−13.5±12.9	−12.5± 7.0	−2.3±3.3	−24.6±17.2 (0.0020)
4 ((((P,F),C),E),R)	−9.7±9.5	−8.0±13.9	−8.2± 8.1	−6.2±5.9	−17.4±19.6 (0.0661)
5 ((P,((F,C),E)),R)	−6.3±6.6	<−8,671.1>	−21.7±13.6	−7.7±6.1	−21.2±16.3 (0.0362)
6 ((P,((F,E),C)),R)	<−6,573.6>	−6.7± 5.3	−21.6±14.1	−7.7±6.1	−21.4±16.3 (0.0386)
7 ((((P,F),E),C),R)	−10.0±7.6	−5.2±14.4	−9.8± 7.5	−6.2±5.9	−16.6±18.8 (0.0626)
8 (((P,C),(F,E)),R)	−2.5±4.4	−16.5±10.2	−23.0±14.1	−12.4±8.1	−39.7±19.7 (0.0021)
9 (((P,(F,E)),C),R)	−2.4±4.4	−16.8±10.3	−22.3±13.6	−12.4±8.1	−39.2±19.4 (0.0009)
10 (((P,(F,C)),E),R)	−9.6±9.2	−14.8±10.0	−14.3±12.2	−12.4±8.1	−36.6±20.0 (0.0032)
11 ((((P,C),F),E),R)	−5.0±8.8	−15.6±12.0	−16.9±11.5	−12.4±8.1	−35.3±20.5 (0.0077)
with gamma					
1 ((P,(F,(E,C))),R)	−2.0±3.1	<−7,949.8>	−6.2±5.6	<−2,740.4>	<−2,1782.2> (0.4552)
2 (((P,F),(E,C)),R)	−2.0±3.1	−4.9±6.4	<−5,190.6>	−1.8±3.4	−0.5± 7.8 (0.3655)
3 (((P,(E,C)),F),R)	−2.0±3.1	−5.5±6.3	−7.1±5.0	−2.1±2.5	−8.5± 9.0 (0.0256)
4 ((((P,F),C),E),R)	−1.0±1.6	−5.4±7.2	−5.3±5.9	−4.3±5.1	−7.7±10.7 (0.0944)
5 ((P,((F,C),E)),R)	−2.0±2.2	−1.3±3.8	−14.5±9.1	−5.3±4.7	−14.8±11.2 (0.0177)
6 ((P,((F,E),C)),R)	−1.1±2.7	−2.8±2.6	−14.6±9.2	−5.3±4.7	−15.5±11.1 (0.0158)
7 ((((P,F),E),C),R)	−2.3±2.7	−5.6±7.0	−6.7±5.2	−4.3±5.1	−10.6±10.4 (0.0037)
8 (((P,C),(F,E)),R)	−0.9±2.4	−9.6±5.1	−14.8±9.3	−8.2±5.8	−25.3±12.4 (0.0001)
9 (((P,(F,E)),C),R)	−1.1±2.7	−9.5±5.2	−14.7±9.1	−8.2±5.8	−25.4±12.3 (0.0000)
10 (((P,(F,C)),E),R)	−1.0±1.6	−7.0±7.5	−8.6±8.2	−8.2±5.9	−16.6±12.7 (0.0157)
11 ((((P,C),F),E),R)	<−5,893.1>	−7.8±7.3	−9.7±7.9	−8.2±5.9	−17.4±12.3 (0.0063)

Abbreviation: C (Chiroptera), E (Eulipotyphla), P (Primates), F (Fereuungulata), R (Rodentia)
The HKY model was applied to 12S and 16S rRNAs, and the ML estimates of transition/transversion rate ratios are 7.8 and 4.1. The ML estimates of the shape parameter, alpha, are 0.24, 0.29, 0.38, and 0.38, respectively, for 12S, 16S, COB, and COII.

Homo sapiens (human)

Pan paniscus (bonobo)

Macaca mulatta (rhesus monkey)

Mandrillus leucophaeus (drill)

Alouatta palliata (mantled howler)

Lagothrix lagothricha (Humboldt's wooly monkey)

Lemur catta (ring-tailed lemur)

Cheirogaleus medius (fat-tailed mouse lemur)

Nycticebus coucang (slow loris)

Galago senegalensis (lesser bushbaby)

Halichoerus grypus (grey seal)

Felis catus (cat)

Equus caballus (horse)

Rhinoceros unicornis (Indian rhinoceros)

Bos taurus (cow)

Balaenoptera musculus (blue whale)

Urotrichus talpoides (shrew mole)

Mogera wogura (Japanese mole)

Blarina brevicauda (short-tailed shrew)

Suncus murinus (musk shrew)

Rousettus leschenaulti (Leschenault's rousette)

Rhinolophus darlingi (horse shoe bat)

Pipistrellus abramus (pipistrelles bat)

Phyllostomus hastatus (greater spear-nosed bat)

Mus musculus (mouse)

Rattus norvegicus (rat)

Macropus robustus (wallaroo)

Didelphis virginiana (opossum)

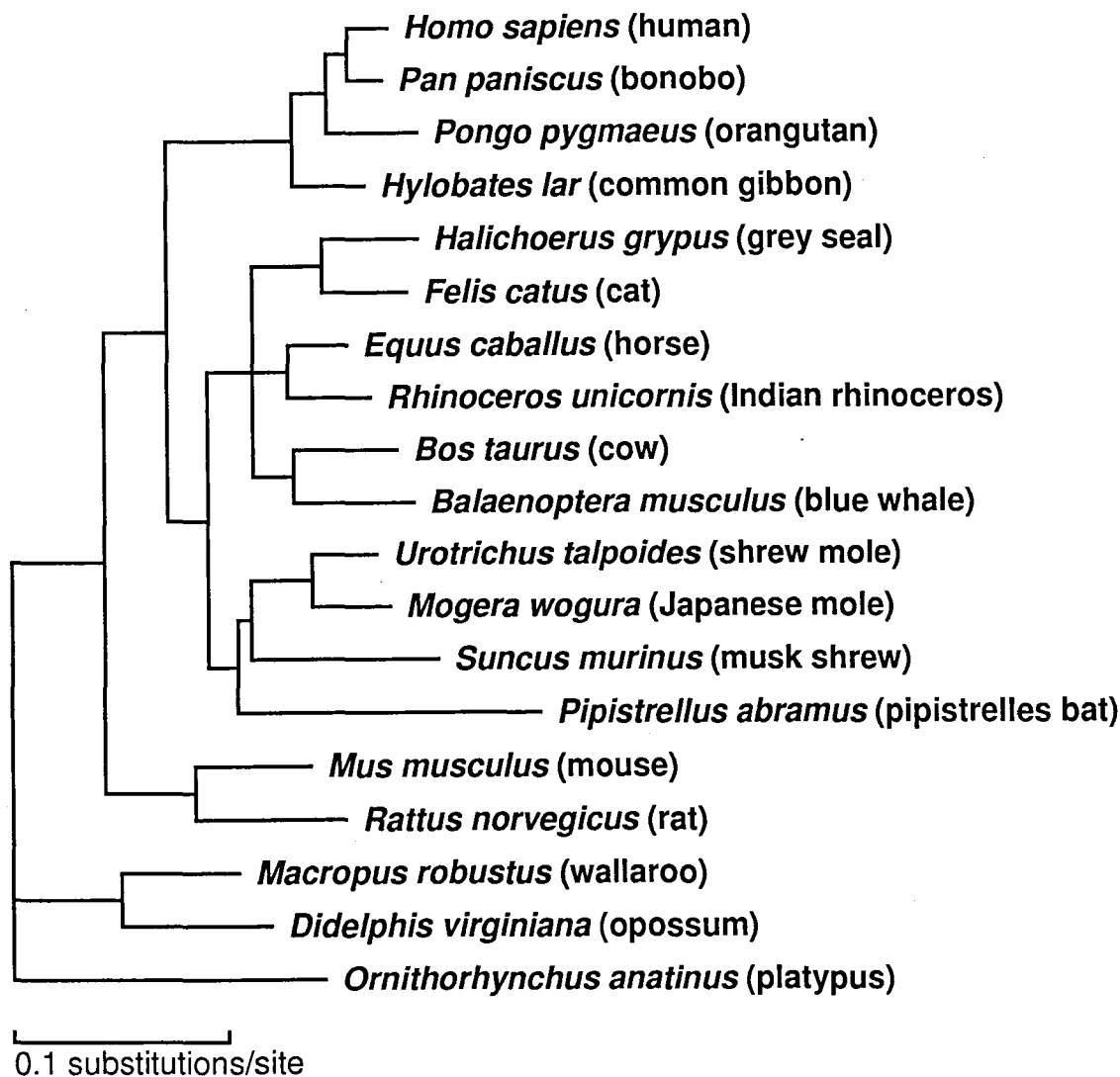Ornithorhynchus anatinus (platypus)

0.1 substitutions/site

Fig. 1.   The maximum likelihood tree of amino acid sequence of COII gene. The horizontal length of each branch is proportional to the estimated number of amino acid substitutions.

Pipistrellus abramus (pipistrelles bat, AB032844), *Mus musculus* (mouse, J01420), *Rattus norvegicus* (rat, X14848), *Macropus robustus* (wallaroo, Y10524), *Didelphis virginiana* (opossum, Z29573), *Ornithorhynchus anatinus* (platypus, X83427).

for the COB gene: *Chiroderma villosum* (shaggy haired bat, L28943), *Chiroderma trinitatum* (Goodwin's bat, L28942), *Platyrrhinus helleri* (Heller's bat, L28940), *Uroderma bilobatum* (tent-making bat, L28941), *Homo sapiens* (human, D38112), *Pan paniscus* (bonobo, D38116), *Pongo pygmaeus* (orangutan, D38115), *Hylobates lar* (common gibbon, X99256), *Lemur catta* (ring-tailed lemur, U53575), *Cheirogaleus major* (greater fat-tailed dwarf lemur, U53570), *Galago crassicaudatus* (greater bushbaby, U53579), *Nycticebus coucang* (slow loris, U53580), *Saimiri sciureus* (squirrel monkey, U53582), *Halichoerus grypus* (grey seal, X72004), *Felis catus* (cat, U20753), *Equus caballus* (horse, X79547), *Rhinoceros unicornis* (Indian rhinoceros, X97336), *Bos taurus* (cow, J01394), *Physeter macrocephalus* (sperm whale, X75589), *Balaenoptera musculus* (blue whale, X72204), *Suncus murinus* (musk shrew, AB033610), *Urotrichus talpoides* (Japanese shrew mole, AB033611), *Mogera wogura* (Japanese mole, AB033612) , *Rattus norvegicus* (rat, X14848), *Mus musculus* (mouse, J01420), *Macropus robustus* (wallaroo, Y10524), *Didelphis virginiana* (opossum, Z29573), *Ornithorhynchus anatinus*

(platypus, X83427).

for the COII gene: *Homo sapiens* (human, D38112), *Pan paniscus* (bonobo, D38116), *Macaca mulatta* (rhesus monkey, M74005), *Mandrillus leucophaeus* (drill, M74006), *Alouatta palliata* (mantled howler, L22774), *Lagothrix lagothricha* (Humboldt's wooly monkey, L22779), *Lemur catta* (ring-tailed lemur, L22780), *Cheirogaleus medius* (fat-tailed mouse lemur, L22775), *Nycticebus coucang* (slow loris, L22781), *Galago senegalensis* (lesser bushbaby, M80905), *Halichoerus grypus* (grey seal, X72004), *Felis catus* (cat, U20753), *Equus caballus* (horse, X79547), *Rhinoceros unicornis* (Indian rhinoceros, X97336), *Bos taurus* (cow, J01394), *Balaenoptera musculus* (blue whale, X72204), *Phyllostomus hastatus* (greater spear-nosed bat, M80906), *Rousettus leschenaulti* (Leschenault's rousette, M80908), *Rhinolophus darlingi* (horse shoe bat, U62580), *Blarina brevicauda* (short-tailed shrew, U62578), *Suncus murinus* (musk shrew, AB033720), *Urotrichus talpoides* (shrew mole, AB033722) , *Mogera wogura* (Japanese mole, AB033721) , *Pipistrellus abramus* (pipistrelles bat, AB033723) , *Rattus norvegicus* (rat, X14848), *Mus musculus* (mouse, J01420), *Macropus robustus* (wallaroo, Y10524), *Didelphis virginiana* (opossum, Z29573), *Ornithorhynchus anatinus* (platypus, X83427).

0.1 substitutions/site

Fig. 2. The maximum likelihood tree of nucleotide sequence of 16S rRNA gene. The horizontal length of each branch is proportional to the estimated number of nucleotid substitutions.

RESULTS AND DISCUSSION

After NucML and ProtML analyses were carried out separately for each four genes to examine the consistency of these phylogenetic trees, we tried to evaluate the total evidence of the ML analyses with the TotalML program in MOLPHY. Monophyly of each group of Eulipotyphla and Chiroptera is not always supported as the highest likelihood tree for each individual genes; e.g., closer relationship of shrew to Chiroptera rather than to mole was suggested by COB and 16S rRNA. However, the preference is only minor, and therefore the monphyly of each group was assumed in the subsequent analyses. The branching orders within each of the 5 major orders and within the outgroup were assumed as the consensus of the four genes.

In the possible 105 trees among Chiroptera, Eulipotyphla, Primates, Fereuungulata, and Rodentia, Tree-1, ((Primates, (Fereuungulata,(Eulipotyphla,Chiroptera))),Rodentia), is the highest likelihood tree from the total evidence of our analyses, and 10 trees have log-likelihood lower than Tree-1 only by less than 2SEs from the analysis without gamma. These 11 trees were subjected to more detailed analyses as shown in Table 1 by taking account of the site-heterogeneity with the gamma-distribution. Either without or with gamma, Tree-1 is consistently the highest likelihood tree from the total evidence, and Tree-2, (((Primates, Fereuungulata), (Eulipotyphla,-Chiroptera)),Rodentia), is the second highest likelihood tree with indistinguishable log-likelihood value (log-likelihood difference: -6.4 ± 16.7 and -0.5 ± 7.8 without and with gamma).

COII (Fig. 1) and 16S rRNA (Fig. 2) conform to the total evidence suggesting Tree-1 when the gamma distribution is applied, but COB prefers Tree-2, although Tree-1 has an indistinguishable log-likelihood value. Log-likelihoods of 12S rRNA are almost indistinguishable among the 11 trees, suggesting that this molecule has not sufficient information to address this problem. Subtotals of bootstrap probabilities of trees with the Chiroptera/Eulipotyphla clade (Trees-1, 2 and 3) are 0.7826 and 0.8463, respectively, without and with gamma.

Thus, although the bootstrap support is still not so high as to exclude other possibilities, the present study suggests that Chiroptera shares the closest phylogenetic relationship with Eulipotyphla, consistently with Onuma et al. (1998) who used only a more limited data set.

Before Pumo et al. (1998) demonstrated the close relationship of Chiroptera to Fereuungulta, Chiroptera had been considered to be closely related to Primates together with Dermoptera and Scandentia (the Archonta hypothesis; e.g., Novacek, 1992). Chiroptera is the most specialized group in Eutheria, together with Cetacea. The present study demonstrates an interesting possibility that bats might have derived from mole-like mammals. If it turns out to be true by further molecular phylogenetic investigations in the future, it must have a great implication in the study of eutherian evolution.

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