J Mol Evol (1995) 41:359–365

JOURNAL OF MOLECULAR EVOLUTION

© Springer-Verlag New York Inc. 1995

Molecular Phylogeny of Gibbons Inferred from Mitochondrial DNA Sequences: Preliminary Report

Seiji Hayashi,[1] Kenji Hayasaka,[2] Osamu Takenaka,[3] Satoshi Horai[2]

[1] Department of Planetary and Earth Sciences, School of Science, Nagoya University, Chikusa-ku, Nagoya 464-01, Japan
[2] Department of Human Genetics, National Institute of Genetics, 1,111 Yata, Mishima, Shizuoka 411, Japan
[3] Primate Research Institute, Kyoto University, Kanrin, Inuyama, Aichi 484, Japan

Received: 27 July 1994 / Accepted: 24 February 1995

Abstract. We analyzed the 896 base-pair (bp) mitochondrial DNA (mtDNA) sequences for seven gibbons, representative of three out of four subgenera. The result from our molecular analysis is consistent with previous studies as to the monophyly of subgenus *Hylobates* species, yet the relationship among subgenera remains slightly ambiguous. A striking result of the analysis is the phylogenetic location of Kloss's gibbon (*H. klossii*). Kloss's gibbon has been considered to be an initial offshoot of the subgenus *Hylobates* because of its morphological primitiveness. However, our molecular data strongly suggest that Kloss's gibbon speciated most recently within the subgenus *Hylobates*.

Key words: Gibbons — Mitochondrial DNA — Phylogeny

Introduction

A large amount of data has been accumulated to elucidate phylogenetic relationships within Hominoidea (humans and apes) (Brown et al. 1982; Koop et al. 1986; Sibley and Ahlquist 1987; Miyamoto et al. 1987; Horai et al. 1992, 1995, etc.). These studies revealed that the closest relatives to humans are African apes—ultimately, chimpanzees. The phylogenetic position of gibbons (family Hylobatidae) as a sister group to human and great apes was also supported by these studies.

Gibbons, genus *Hylobates,* inhabit a wide range of Southeast Asia, from eastern India to southern China on the mainland, as well as Borneo, Java, Sumatra, and the Mentawai Islands. Nine nominal species are generally accepted (Marshall and Sugardjito 1986; Groves 1989). They are the concolor gibbon (*H. concolor,* lives in Vietnam, Laos, Yunnan, and Hainan Island), the siamang (*H. syndactylus,* in Sumatra and Malaya), the hoolock gibbon (*H. hoolock,* in Assam, northern Burma, and Bangladesh), the Kloss's gibbon (*H. klossii,* in the Mentawai Islands), the white-handed gibbon (*H. lar,* in Thailand, southeastern Burma, Malaya, and northern Sumatra), the agile gibbon (*H. agilis,* in Malaya, central and southern Sumatra, and southwestern Borneo), the pileated gibbon (*H. pileatus,* in southwestern Thailand and Kampuchea), the silvery gibbon (*H. moloch,* in Java), and Muller's gibbon (*H. muelleri,* in Borneo). The former three species are placed in monotypic subgenera *Nomascus, Symphalangus,* and *Bunopithecus,* respectively. The latter six species are classified into the subgenus *Hylobates* (Prouty et al. 1983; Groves 1989). In the subgenus *Hylobates,* the latter five species, *lar, agilis, pileatus, moloch,* and *muelleri,* are called the *lar* group or *lar* species complex, because they share derived morphological characters, such as very dense fur and large ears (Groves 1984). *klossii* is excluded from the *lar* group due to lack of a synapomorphic characteristic of the *lar* group (Groves 1984). Fleagle (1984) has questioned the inter-

Correspondence to: S. Horai

specific relationship among gibbons. Recently, Garza and Woodruff (1992) presented phylogenetic relationships among 26 individuals of gibbons, representing seven species of three subgenera, deduced from the nucleotide sequences of a 252-base-pair (bp) partial fragment of the mitochondrial cytochrome *b* (cyt *b*) gene. However, the sequenced region was not long or variable enough to permit establishing relationships among the closely related gibbon species.

In this study, we sequenced and analyzed a 896-bp mtDNA fragment comprising partial sequences of ND4 and ND5 (NADH dehydrogenase complex) genes and an adjacent three complete tRNA genes ($tRNA^{His}$, $tRNA^{Leu(CUN)}$, and $tRNA^{Ser(AGY)}$, from seven gibbons representing three out of the four subgenera. The sequenced region has been corroborated to be variable enough to resolve phylogenetic relationships among primates with statistical reliability (Brown et al. 1982; Hayasaka et al. 1988). We constructed molecular phylogenetic trees and compared the results with the previous studies.

Materials and Methods

Blood samples from six species of captive gibbons, *H. concolor, H. klossii, H. pileatus, H. moloch, H. agilis,* and *H. lar,* were collected at Ragnan Zoo in Djakarta, Indonesia, and Dusit Zoo in Bangkok, Thailand. A B-cell line of *H. syndactylus* was provided through the courtesy of Dr. T. Ishida (University of Tokyo). Total genomic DNAs were extracted from blood and B-cell with standard procedures (Sambrook et al. 1989). The 896-bp mtDNA fragments were amplified by the polymerase chain reaction (PCR) (Saiki et al. 1988) from total DNAs using five pairs of oligonucleotide primers, A and F, B and F, B and E, C and E, and D and F. The sequences of primers are A: 5′ TC(T/C)TCA(A/G)T(T/C)AGCCACATAGC-3′ (L11644), B: 5′-ATCCAAACCCCCTGAAGCTT-3′(L11686), C: 5′-GCAAACTCAAACTACGAACG-3′(L11767), D: 5′-GGTGCAACTCCAAATAAAAG-3′(L12335), E: 5′-CTTTTATTTGGAGTTGCACC-3′(H12315R), and F: 5′-CTCTCAGCCGATGAAGAGTT-3′(H12752R). L and H refer to the sequence of light and heavy strands, respectively, and the numbers correspond to the 3′ end positions of the primers in the numbering system for human mtDNA (Anderson et al. 1981). In this numbering system the 896-bp sequence corresponds to the sequence from 11,680 to 12,575.

An amount of 20–30 ng of template DNA was subjected to 30 cycles of PCR amplification in 100 μl of reaction mixture containing 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatine, 200 mM each deoxynucleotide, 0.5 mM each primer, and 1 U *Taq* polymerase (Perkin Elmer Cetus). Each cycle consisted of 15 s at 94°C for denaturation, 15 s at 45–50°C for annealing, and 1 min at 72°C for extension.

The amplified fragments were separated on 1.5% agarose gel, excised from the gels, and purified by extraction through SUPREC-01 columns (Takara Shuzo, Co.). From 10 μl of the purified DNA, single-stranded DNAs were amplified by the asymmetric PCR method (Gyllensten and Erlich 1988). The second PCR products were desalted and concentrated using Centricon 30 microconcentrators (Amicon).

Using the second PCR products as template, nucleotide sequences were determined for both strands with a Sequenase sequencing kit (United States Biochemicals) and the primers just described. The determined sequences were aligned with the homologous sequences for the other hominoids and Japanese macaques (Brown et al. 1982; Hayasaka et al. 1988).

The number of nucleotide substitutions between each pair of species was estimated by the two-parameter method (Kimura 1980). The neighbor-joining (NJ) (Saitou and Nei 1987), maximum parsimony (MP) (Fitch 1971), and maximum likelihood (ML) (Felsenstein 1981) analyses were performed by the computer programs NJBOOT2 (Tamura 1992), PAUP3.0s (Swofford 1991), and DNAML in PHYLIP3.5c (Felsenstein 1993), respectively. For these analyses, we used a data set including information on the third codon position of protein coding genes, as these sites have little change in compositional bias among taxa.

Results and Discussion

Sequence Comparison

A single polymorphic site (site 301) was detected between two sequences of *H. lar,* the one reported previously (HLA1) (Brown et al. 1982) and the other determined in this study (HLA2) (Fig. 1). Sequence similarity among the species of gibbons ranges from 97.8% between *klossii* and *lar* to 87.3% between *klossii* and *concolor* (Table 1).

The number of species in the subgenus *Hylobates* has been controversial (Groves 1989). We tried to investigate this problem from the viewpoint of sequence similarities. According to the published 896-bp mtDNA sequences (Brown et al. 1982; Hayasaka et al. 1988), interspecific sequence similarity ranges from 87.6% (between Barbary and Japanese macaque) to 96.4% (between rhesus and Japanese macaques) and intergeneric sequence similarity ranges from 82.9% (between orangutan and chimpanzee) to 91.2% (between human and chimpanzee). Since the range of interspecific and intergeneric sequence similarity overlaps considerably, it is not possible to set a strict criterion for the relationship between taxonomic ranks and nucleotide similarity.

Sequence similarity is 88.5% between *Nomascus* (*concolor*) and *Symphalangus* (*syndactylus*), 89.4% between *Hylobates* and *Symphalangus* (an average of the six values between *syndactylus* and the species of *Hylobates*), and 87.9% between *Hylobates* and *Nomascus* (an average of the six values between *concolor* and the six species of *Hylobates*). These values are within the ranges of both the interspecific and intergeneric comparisons. Thus, the subgeneric classification of the gibbons seems appropriate.

Protein Coding Genes

In general, initiation codons for polypeptide coding genes are ATG and ATA in mammalian mtDNA. However, there are several exceptions. Especially the mammalian ND5 genes often use different codons as translational initiators. An ACA codon was observed at the first codon position in the orangutan sequence (Brown et al. 1982). In this study the ND5 translational initiator variation was found even within the gibbons; ACA (Thr) in

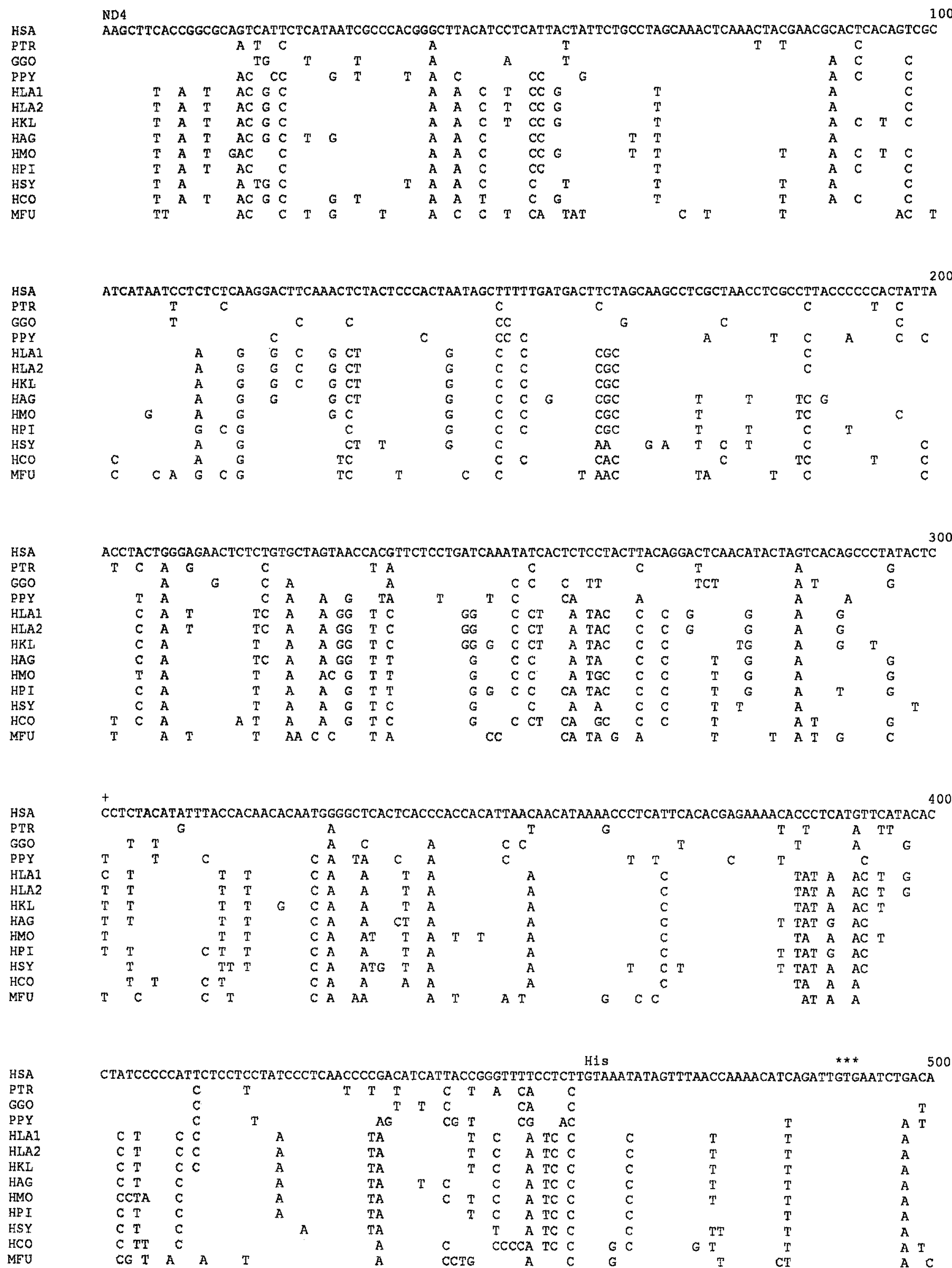

Fig. 1. Nucleotide sequences for 896-bp fragments of mtDNA from seven gibbons, orangutan, gorilla, chimpanzee, human, and Japanese macaque. Species names are abbreviated as follows. HSA = human, PTR = chimpanzee, GGO = gorilla, PPY = orangutan, HLA1 = *Hylobates lar* (Brown et al. 1982), HLA2 = *Hylobates lar* (this study), HKL = *H. klossii,* HAG = *H. agilis,* HPI = *H. pileatus,* HSY = *H. syndactylus,* HCO = *H. concolor,* and MFU = Japanese macaque (*Macaca fuscata.*). The whole nucleotides for the human sequence are shown on the top. For the other sequences, only nucleotides different from those in the human sequence are shown. + refers to a polymorphic site between two individuals of *H. lar* sequences. *Triple asterisks* (***) represent anticodons of the three tRNAs. *ND4, ND5, His, Ser,* and *Leu* above the sequences indicate the starting points for the 3′ region of ND4 gene, the 5′ region of ND5 gene, $tRNA^{His}$, $tRNA^{Leu(CUN)}$, and $tRNA^{Ser(AGY)}$ genes, respectively. A gap is denoted by a *dash* (–).

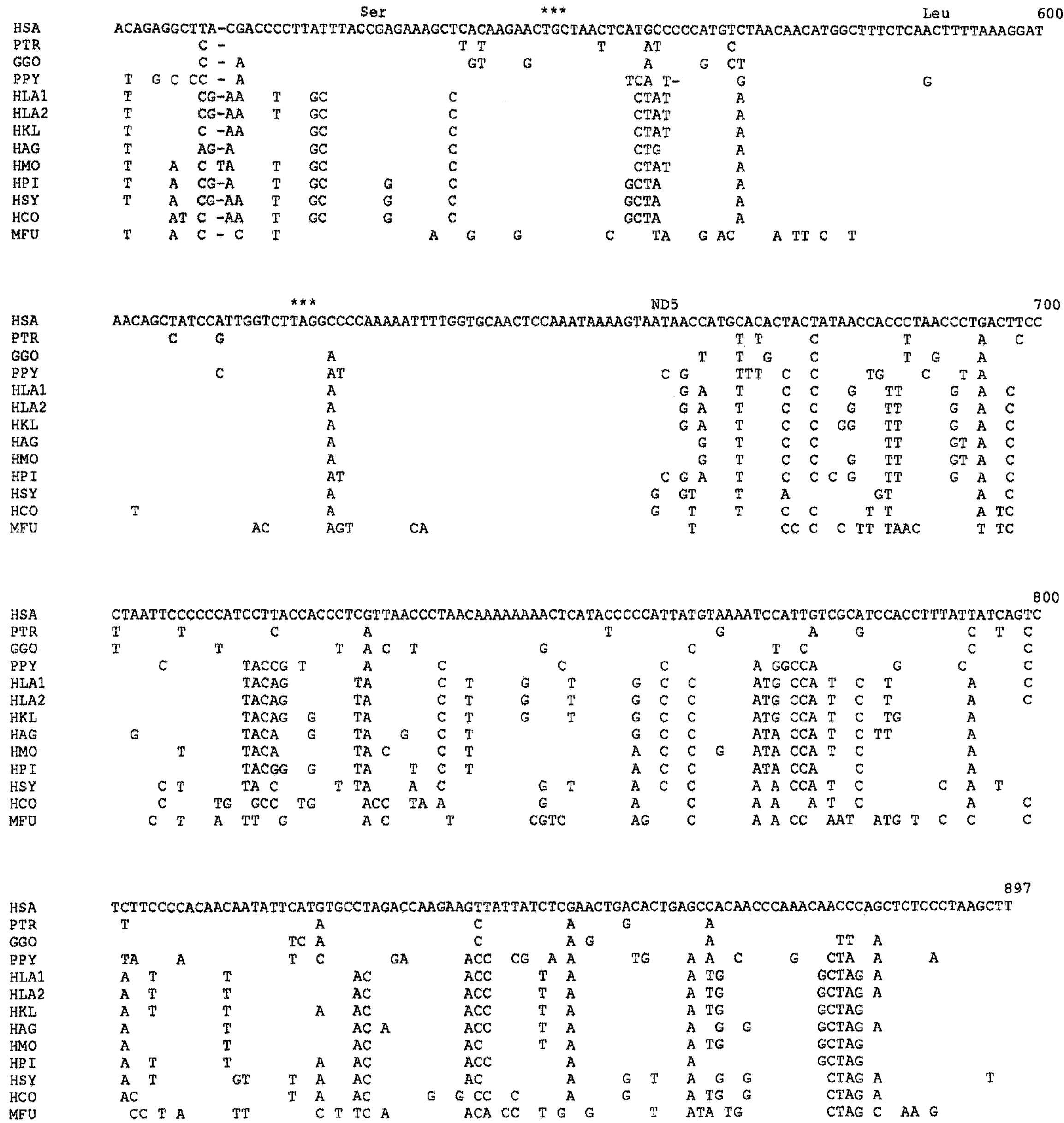

Fig. 1. Continued.

pileatus, GTA (Val) in *syndactylus* and *concolor,* and ATA (Met) in others. Brown et al. (1982) suggested, however, that an ATG codon located two codons downstream from the putative start codon (Anderson et al. 1981) might serve as an acceptable start codon for the ND5 gene. This codon proved to be highly conserved from human to New World monkey (Hayasaka et al. 1988). Our new data also support this view.

Phylogeny

Relationships Among the Subgenera

A consensus feature of the three molecular phylogenetic trees (Fig. 2) is the monophyly of the subgenus *Hylobates.* This monophyly was strongly supported with 100 and 98% bootstrap probabilities by the neighbor-joining and maximum parsimony analyses, respectively.

However, relationships among the three subgenera were problematic. Alternative hypotheses for the subgeneric relationship are shown in Fig. 3. Electrophoretic analyses of blood proteins (Lucotte et al. 1982; Cronin et al. 1984) resulted in yielding trichotomous trees for the three subgenera. In the maximum likelihood analysis of the cyt *b* sequence data (Garza and Woodruff 1992), tree-2 was most likely and tree-3 was significantly worse than tree-2 by Kishino and Hasegawa's two-standard-deviation criterion (Kishino and Hasegawa 1989). In contrast, tree-1 is most likely in this study, yet neither tree-2 nor tree-3 is excluded by Kishino and Hasegawa's test. The other analysis of our data failed to resolve this trichotomy. When using restricted data with exclusion of

Table 1. Percent silimarity of nucleotide sequences (above the diagonal) and estimated number of nucleotide substitutions per site between each pair of sequences (below the diagonal)[a]

	HSA	PTR	GGO	PPY	HLA	HKL	HAG	HMO	HPI	HSY	HCO	MFU
HSA		91.2	89.7	84.0	81.9	81.7	82.3	82.3	82.7	81.9	81.1	76.8
PTR	0.097		89.4	82.9	81.1	80.6	80.9	82.1	81.9	81.4	81.3	75.8
GGO	0.114	0.118		83.4	81.1	80.5	80.9	80.7	81.3	81.1	82.6	76.5
PPY	0.185	0.201	0.195		81.2	81.0	80.8	80.9	82.5	79.9	80.8	75.6
HLA	0.213	0.225	0.225	0.222		97.8	94.1	93.6	93.8	89.5	87.7	75.5
HKL	0.217	0.234	0.235	0.225	0.023		93.4	93.6	94.0	88.8	87.3	75.0
HAG	0.208	0.228	0.228	0.228	0.063	0.071		93.8	93.8	89.3	87.5	75.5
HMO	0.208	0.210	0.232	0.227	0.068	0.068	0.067		93.8	88.8	88.5	76.6
HPI	0.202	0.214	0.223	0.204	0.067	0.064	0.067	0.067		90.3	88.6	76.7
HSY	0.212	0.220	0.223	0.240	0.117	0.125	0.119	0.125	0.107		88.5	76.1
HCO	0.222	0.220	0.200	0.225	0.140	0.146	0.142	0.129	0.128	0.129		76.5
MFU	0.286	0.303	0.292	0.304	0.307	0.315	0.307	0.288	0.287	0.297	0.287	

[a]Abbreviation for the species names are given in Fig. 1. The HLA1 sequence (Fig. 1) was used in the calculation between *H. lar* and the other species

the third codon position of the protein coding genes (total of 662 bp), tree-3 was most likely. However, the difference in the log-likelihood values for the three alternative trees became smaller (Fig. 3). Thus, the available sequence data set cannot resolve relationships of the three subgenera with statistical significance.

Subgenus *Hylobates*

Relationships within the subgenus *Hylobates* are also complicated. Relationships among *agilis, moloch,* and the common ancestor of *lar* and *klossii* are polychotomous.

There is a clear discrepancy between our molecular trees and the morphological classification in the phylogenetic position of *klossii.* As described in the introduction, *klossii* is morphologically distinct from the *lar* group (Creel and Preuschoft 1984; Haimoff et al. 1984). By contrast, in our tree, *klossii* is within the *lar*-group clade and is placed as a sister lineage to *lar.* This is strongly supported with high bootstrap probabilities (both 100% in the NJ and MP trees). The 252-bp partial cyt *b* sequence analysis also placed *klossii* into the *lar*-group cluster. However, in the cyt *b* tree, unlike in our result, *klossii* was more closely related to *muelleri* and *pileatus* than to either *lar* or *agilis* (Garza and Woodruff 1992). A base compositional change among taxa causes conflict among data sets in molecular phylogenetic analyses. However, no compositional shift is observed in the cyt *b* data. Therefore, the contradiction between the two molecular analyses might result from statistical error associated with the small number of nucleotide sites used in the cyt *b* study. The number of phylogenetically informative sites in the 252-bp cyt *b* sequences is only 28, which is almost one-tenth of our data (247 sites). In the maximum likelihood analysis of our data, both the morphological trees, which locate *klossii* as a sister taxon of the *lar* group (Fig. 14 in Creel and Preuschoft 1984), and

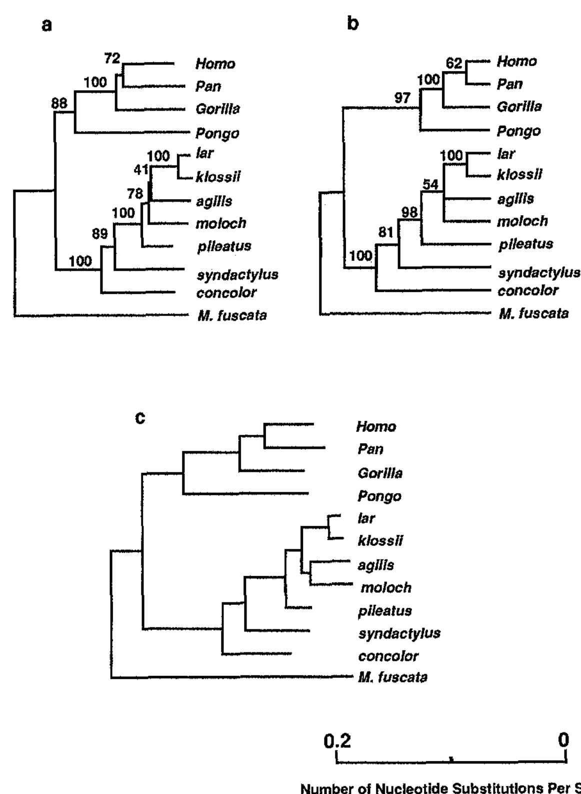

Fig. 2. Phylogenetic trees constructed by **(a)** the neighbor-joining (NJ), **(b)** maximum parsimony (MP), and **(c)** maximum likelihood (ML) trees for the seven gibbons and the other hominoids using Japanese macaque as an outgroup. The *numbers above nodes* in the NJ and MP trees are bootstrap probabilities (%) with 1,000 times of resamplings. A *scale bar* represents branch length in terms of substitutions per site for the NJ and ML trees. The consensus MP tree **(b)** was constructed on the condition of 50% majority rule in PAUP version 3.0s program (Swofford 1991). The most parsimonious tree (not shown) has the topology identical to the ML tree. The numbers of variable and informative sites are 394 and 247, respectively. The MP tree requires 745 substitutions with a consistency index (CI) of 0.642. In maximum likelihood tree **(c),** the t parameter (transition/transversion ratio) was set at 30. In the three methods, sites with insertion/deletion were excluded. In all the trees, *H. lar* is represented by the HLA1 sequence in Fig. 1.

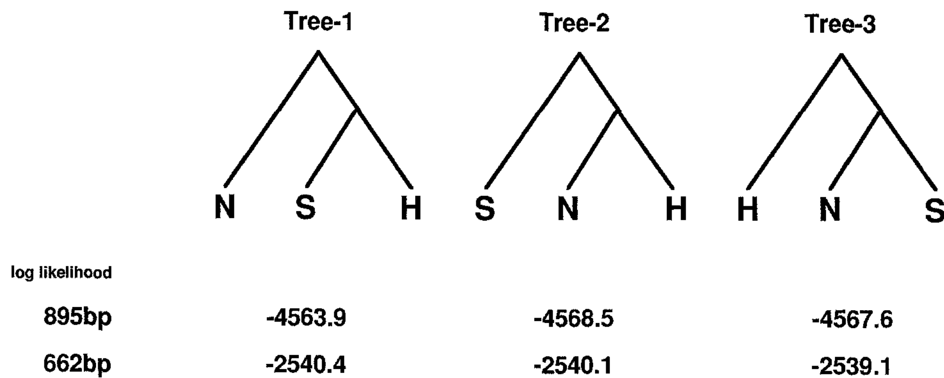

Fig. 3. Alternative phylogenetic trees for the three subgenera of gibbons with their log-likelihood values calculated from our sequence data. *N, S,* and *H* denote the subgenera *Nomascus, Symphalangus,* and *Hylobates,* respectively.

the 252-bp cyt *b* tree are statistically ruled out by Kishino and Hasegawa's test (Fig. 4). The close relationship between *lar* and *klossii* is not improbable considering that their geographic distributions are adjacent to each other, yet separated by the sea.

Sequence Diversity and Divergence Time

The level of sequence diversity (estimated by Kimura's two-parameter method, Table 1) among gibbons (0.146) is almost identical to that among hominines (human, chimpanzee, and gorilla) (0.118). The magnitude of sequence diversity among the members of the subgenus *Hylobates* (0.067) exceeds that between Japanese and rhesus macaques (0.037), but does not reach the level observed between human and chimpanzee (0.097). In their immunological analysis, Cronin et al. (1984) obtained a divergence time for the *lar* group of 1 million years ago. However, the divergence of the *lar* group may go back further in the past according to the mitochondrial clock. Assuming that human and chimpanzee diverged some 5 million years ago (Horai et al. 1992, 1995) and that there has been no extreme substitution-rate difference between the lineages of the great apes and gibbons, the dates for the divergences of the subgenera and the subgenus *Hylobates* are estimated at 6 and 3.5 million years ago, respectively.

Prospects

In the future, sequences from *hoolock* and *muelleri* are needed to further explore speciation events among gibbons. A larger set of sequence data is necessary to establish branching orders among the gibbons at a statistically significant level. Morphological variations are noted within species of gibbons. Thus, the investigation of their mtDNA polymorphism is of great interest from the viewpoint of population genetics, to assess the rates and mode of evolution in the mitochondrial genome.

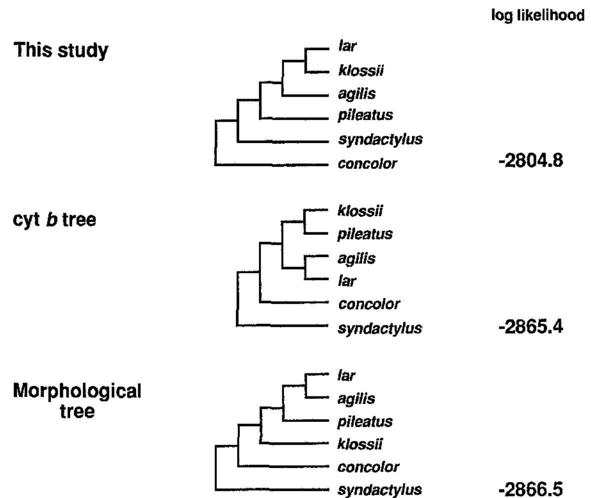

Fig. 4. Alternative phylogenetic trees for species of gibbons with their log-likelihood values calculated from our sequence data.

Acknowledgments. We deeply thank the Ragnan Zoo in Djakarta and the Dusit Zoo in Bangkok for blood samples of six gibbons, Dr. T. Ishida for the B-cell line of *H. syndactylus,* Drs. T. Ozawa and Y. Kumazawa for discussions and encouragement, Dr. R. Kondo for suggestions and computer analyses, and Drs. J. Felsenstein and K. Tamura for the computer programs. This work was supported by grants-in-aid from the Ministry of Education, Science and Culture, Japan, to S. Horai.

References

Anderson S, Bankier AT, Barrel BG, de Brujin MHL, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJH, Standen R, Young IC (1981) Sequence and organization of the human mitochondrial genome. Nature 290:457–465

Brown WM, Prager EM, Wang A, Wilson AC (1982) Mitochondrial DNA sequences of primates: tempo and mode of evolution. J Mol Evol 18:225–239

Creel N, Preushoft H (1984) Systematics of the lesser apes: a quantitative taxonomic analysis of craniometric and other variables. In: Preushoht H, Chivers DJ, Brockelman WY, Creel N (eds) The lesser apes—evolutionary and behavioral biology. Edinburgh University Press, Edinburgh, pp 562–613

Cronin JE, Sarich VM, Ryder O (1984) Molecular evolution and speciation. In: Preushoht H, Chivers DJ, Brockelman WY, Creel N

(eds) The lesser apes—evolutionary and behavioral biology. Edinburgh University Press, Edinburgh, pp 467–485

Felsenstein J (1981) Evolutionary trees from DNA sequences: a maximum likelihood approach. J Mol Evol 17:368–376

Felsenstein J (1993) PHYLIP (phylogeny inference package), version 3.5c. University of Washington, Seattle

Fitch WM (1971) Toward defining the course of evolution: minimal change for a specific tree topology. Syst Zool 20:406–416

Fleagle JG (1984) Are there any fossil gibbons? In: Preushoht H, Chivers DJ, Brockelman WY, Creel N (eds) The lesser apes—evolutionary and behavioral biology. Edinburgh University Press, Edinburgh, pp 431–447

Garza JC, Woodruff DS (1992) A phylogenetic study of the gibbons (*Hylobates*) using DNA obtained noninvasively from hair. Mol Phylogenet Evol 1:202–210

Groves CP (1984) A new look at the taxonomy and phylogeny of the gibbons. In: Preushoht H, Chivers DJ, Brockelman WY, Creel N (eds) The lesser apes—evolutionary and behavioral biology. Edinburgh University Press, Edinburgh, pp 542–561

Groves CP (1989) A theory of human and primate evolution. Clarendon Press, Oxford

Gyllensten UB, Erlich HA (1988) Generation of single-stranded DNA by the polymerase chain reaction and its application to direct sequencing of the HLA-DQA locus. Proc Natl Acad Sci USA 85: 7652–7656

Haimoff EH, Gittins SP, Whitten AJ, Chivers DJ (1984) A phylogeny and classification of gibbons based on morphology and ethology. In: Preushoht H, Chivers DJ, Brockelman WY, Creel N (eds) The lesser apes—evolutionary and behavioral biology. Edinburgh University Press, Edinburgh, pp 614–632

Hayasaka K, Gojobori T, Horai S (1988) Molecular phylogeny and evolution of primate mitochondrial DNA. Mol Biol Evol 5:626–644

Horai S, Satta Y, Hayasaka K, Kondo R, Inoue T, Ishida T, Hayashi S, Takahata N (1992) Man's place in Hominoidea revealed by mitochondrial DNA genealogy. J Mol Evol 34:32–43

Horai S, Hayasaka K, Kondo R, Tsugane K, Takahata N (1995) Recent African origin of modern humans revealed by complete sequences of hominoid mitochondrial DNAs. Proc Natl Acad Sci USA 92: 532–536

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120

Kishino H, Hasegawa M (1989) Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidea. J Mol Evol 29:170–179

Koop BF, Goodman M, Xu P, Chan K, Slightom JL (1986) Primate η-globin DNA sequences and man's place among the great apes. Nature 319:234–238

Lucotte G, Gautreau C, Smith DG (1982) Distance electrophoretiques entre trois sous-genres de gibbons. Ann Genet 25:11–13

Marshall J, Sugardjito J (1986) Gibbon systematics. In: Swindler D (ed) Comparative primate biology: systematics, evolution and anatomy. AR Liss, New York, pp 137–185

Miyamoto MM, Slightom JL, Goodman M (1987) Phylogenetic relations of humans and African apes from DNA sequences in the ψη-globin region. Science 238:369–373

Prouty LA, Buchanan PD, Pollitzer WS, Mootnick AR (1983) Bunopithecus: a genus-level taxon for the Hoolock gibbon (*Hylobates hoolock*). Am J Primatol 5:83–87

Saiki RK, Gelfand DH, Stoffel S, Schurf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988) Primer-detected enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239: 487–491

Saitou N, Nei M (1987) The neighbor-joining method: a new method of reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning. Cold Spring Harbor Laboratory, New York

Sibley CG, Ahlquist JE (1987) DNA hybridization evidence of hominoid phylogeny: results from expanded data set. J Mol Evol 26: 99–121

Swofford DL (1991) PAUP: phylogenetic analysis using parsimony, version 3.0s. Illinois Natural History Survey, Champaign, IL

Tamura K (1992) NJBOOT2, version 1.06. Pennsylvania State University, PA