A complete species-level phylogeny of the Hylobatidae based on mitochondrial *ND3–ND4* gene sequences

Zoltan Takacs [a], Juan Carlos Morales [a,b], Thomas Geissmann [c], Don J. Melnick [a,b,*]

[a] *Center for Environmental Research and Conservation, Columbia University, New York, NY 10027, USA*
[b] *Department of Ecology, Evolution, and Environmental Biology, Columbia University, New York, NY 10027, USA*
[c] *Anthropological Institute, University Zürich-Irchel, Winterthurerstrasse 190, CH–8057 Zürich, Switzerland*

Abstract

The Hylobatidae (gibbons) are among the most endangered primates and their evolutionary history and systematics remain largely unresolved. We have investigated the species-level phylogenetic relationships among hylobatids using 1257 bases representing all species and an expanded data set of up to 2243 bases for select species from the mitochondrial *ND3–ND4* region. Sequences were obtained from 34 individuals originating from all 12 recognized extant gibbon species. These data strongly support each of the four previously recognized clades or genera of gibbons, *Nomascus*, *Bunopithecus*, *Symphalangus*, and *Hylobates*, as monophyletic groups. Among these clades, there is some support for either *Bunopithecus* or *Nomascus* as the most basal, while in all analyses *Hylobates* appears to be the most recently derived. Within *Nomascus*, *Nomascus* sp. cf. *nasutus* is the most basal, followed by *N. concolor*, and then a clade of *N. leucogenys* and *N. gabriellae*. Within *Hylobates*, *H. pileatus* is the most basal, while *H. moloch* and *H. klossii* clearly, and *H. agilis* and *H. muelleri* likely form two more derived monophyletic clades. The segregation of *H. klossii* from other *Hylobates* species is not supported by this study. The present data are (1) consistent with the division of Hylobatidae into four distinct clades, (2) provide the first genetic evidence for all the species relationships within *Nomascus*, and (3) call for a revision of the current relationships among the species within *Hylobates*. We propose a phylogenetic tree as a working hypothesis against which intergeneric and interspecific relationships can be tested with additional genetic, morphological, and behavioral data.
© 2005 Elsevier Inc. All rights reserved.

Keywords: Hylobatidae; *Hylobates*; *Nomascus*; *Bunopithecus*; *Symphalangus*; Evolution; Mitochondrial DNA; Southeast Asia

1. Introduction

Gibbons or small apes (family Hylobatidae) are a relatively small and morphologically homogeneous group of primate species inhabiting closed canopy rain forests throughout Southeast Asia. The range of the Hylobatidae family is delineated by eastern India, southern China, Borneo, and Java. A distribution map of the genera is presented in Fig. 1 (for distribution maps of the species see Geissmann, 1995 and Geissmann et al., 2000).

Habitat loss and fragmentation, habitat degradation, hunting (food, medicine, and sport), and illegal trade (pets, medicine) are the top four threats which have seriously threatened gibbons throughout their range (Geissmann, 2003b).

While gibbons represent one of the three major adaptive radiations of anthropoid primates in Southeast Asia, and despite several revisions of gibbon systematics (e.g. Geissmann, 1995; Groves, 1972; Marshall and Sugardjito, 1986; Pocock, 1927) and various scenarios proposed to explain the radiation of this group (e.g., Chivers, 1977; Groves, 1993; Haimoff et al., 1982), their evolutionary history and systematics remain largely unresolved. Phylogenetic relationships, even among the
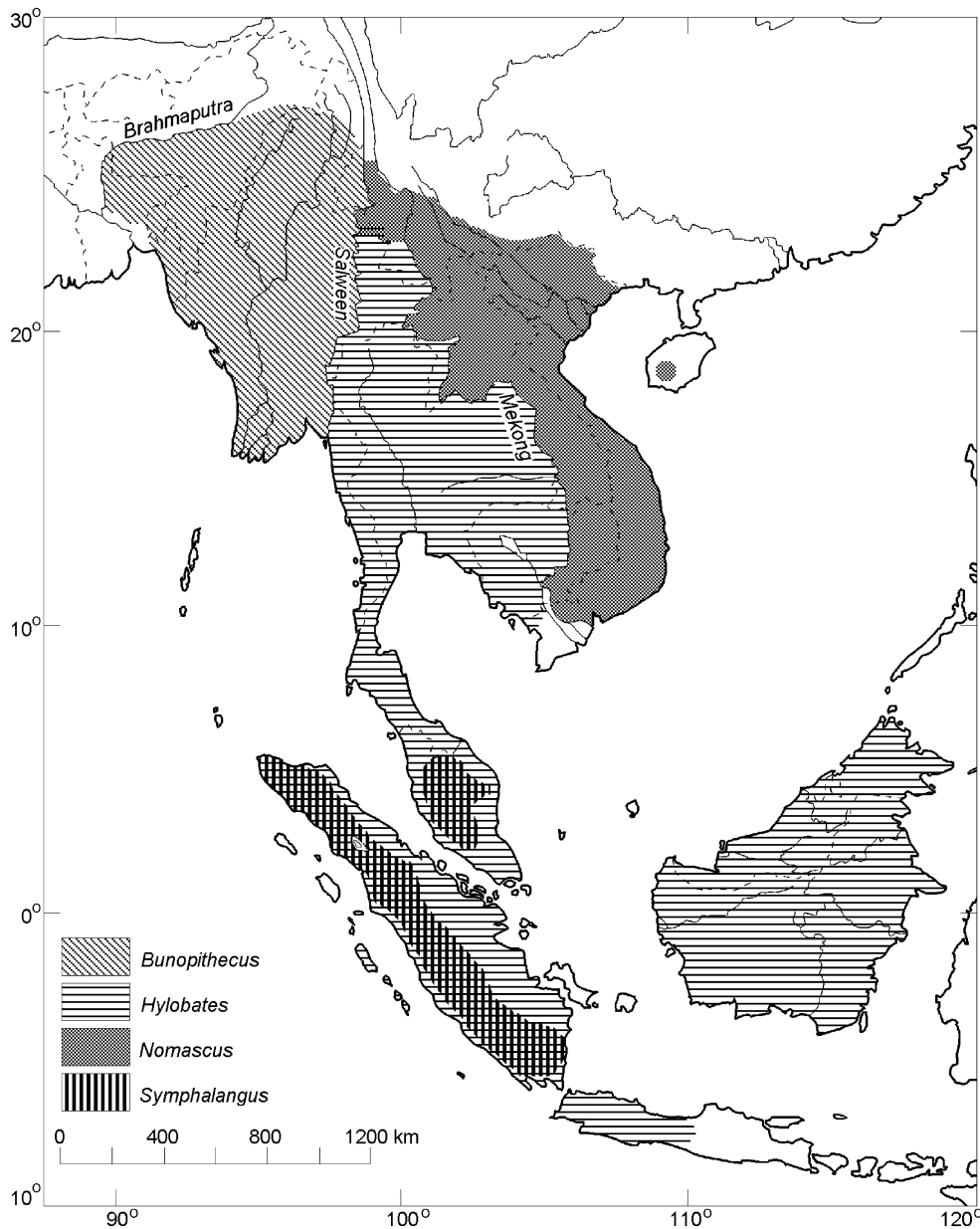
Fig. 1. Distribution of the gibbon genera: *Bunopithecus* (*B. hoolock*); *Hylobates* (6 species); *Nomascus* (4 species); and *Symphalangus* (*S. syndactylus*) (after Geissmann, 1995).

main divisions of the Hylobatidae family are unclear, and the total number of species is contested. Most published gibbon phylogenies are summarized in Fig. 2. The lack of resolution regarding hylobatid evolutionary history and systematics has been attributed to a lack of adequate sampling, inconsistencies among results obtained using different characters, and the effect of a presumed short time period during which gibbons have differentiated.

Fossil evidence applicable to gibbon evolution is very limited and its interpretation is considered problematic (Fleagle, 1984, 1999). Earlier studies applying morphological, behavioral or vocal characters to address the evolutionary relationships among gibbons have produced inconsistent results (Creel and Preuschoft, 1984; Geissmann, 1993, 2002a; Groves, 1972; Haimoff et al., 1982).

Genetic approaches to reconstructing the phylogeny of hylobatids have included cytogenetic studies and the sequencing of mitochondrial and nuclear genes. Cytogenetic studies based on unique karyotypes and diploid numbers divided the Hylobatidae into four groups often referred to as subgenera (Prouty et al., 1983), or more recently as genera (Brandon-Jones et al., 2004; Roos and Geissmann, 2001), namely *Hylobates*, *Bunopithecus*, *Symphalangus*, and *Nomascus*. The cytogenetic differentiation of these four groups is also supported by morphological (Marshall and Sugardjito, 1986; Prouty et al., 1983), and vocal data (Geissmann, 1995, 2002a). The
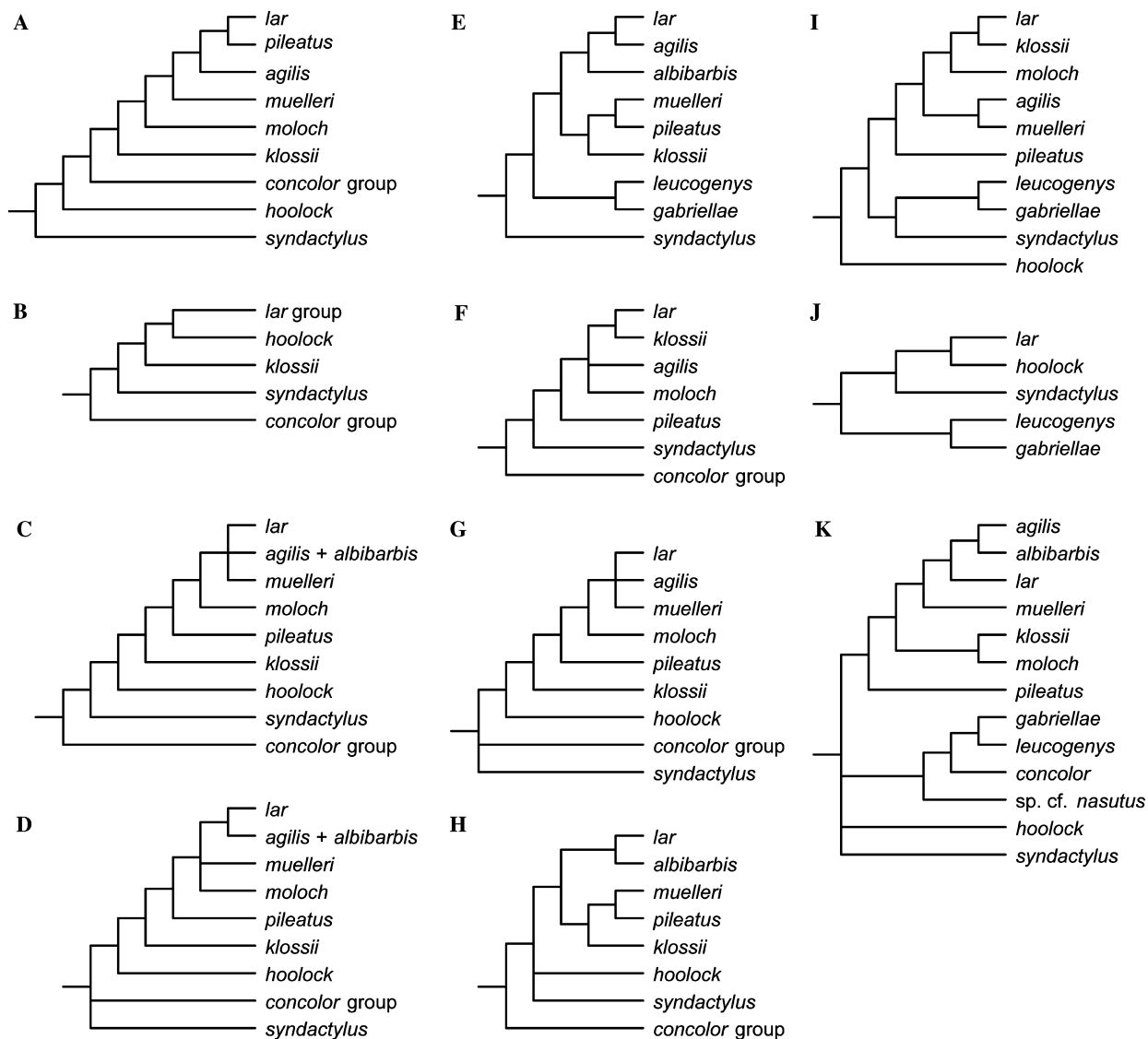
Fig. 2. Published representations of the phylogenetic relationships among gibbon taxa. (A) Groves (1972); (B) Chivers (1977); (C) Haimoff et al. (1982); (D) Creel and Preuschoft (1984); (E) Garza and Woodruff (1992); (F) Hayashi et al. (1995); (G) Purvis (1995); (H) Zhang (1997); (I) Zehr (1999); (J) Roos and Geissmann (2001); and (K) Geissmann (2002b, vocal data).

classification and the genus assignments used in the present study are based on the most recent consensus taxonomy for gibbons (Brandon-Jones et al., 2004; Geissmann, 2002b, 2003a; Geissmann et al., 2000) (Table 1).

DNA sequence analysis of various segments of the mitochondrial and nuclear genome was used to resolve the relationships among and within the four main divisions of Hylobatidae. The cytochrome *b* region of the mitochondrial genome that has been subject to separate studies (Garza and Woodruff, 1992; Hall et al., 1998) produced incomplete or inconsistent results regarding the phylogenetic relationship of the main gibbon groups and the species within those groups. Partial sequences of *ND4* and *ND5* regions using limited species representation also did not allow the complete reconstruction of species groups or subgenus

relationships (Hayashi et al., 1995). A consensus tree based on both previously published and new sequences of mitochondrial and nuclear DNA favored *Bunopithecus* as the first genus to diverge, with the next branch leading to *Symphalangus* and *Nomascus* as sister taxa (Zehr, 1999). Most recently, a study based on the fast evolving mitochondrial control region supported *Nomascus* as the most basal clade, followed by *Symphalangus*, with *Bunopithecus* and *Hylobates* as the last to diverge (Roos and Geissmann, 2001). In none of these studies, however, were all 12 recognized gibbon species across all four genera represented. In addition, these studies, were constrained by a lack of samples representing species from all four gibbon groups and a lack of enough variability in the relatively short segments of DNA analyzed.

Table 1
Main divisions of the genus *Hylobates* (from Geissmann, 2002b)

Genus	Diploid number of chromosomes	Other division names	Species	Common name
Hylobates	44	*lar* group	*H. agilis*[a]	Agile gibbon
			H. klossii	Kloss's gibbon
			H. lar	White-handed gibbon
			H. moloch	Silvery gibbon
			H. muelleri[b]	Müller's gibbon
			H. pileatus	Pileated gibbon
Bunopithecus	38		*B. hoolock*	Hoolock
Nomascus	52	*concolor* group, crested gibbons	*N. concolor*	Western black crested gibbon
			N. sp. cf. *nasutus*	Eastern black crested gibbon
			N. gabriellae	Yellow-cheeked crested gibbon
			N. leucogenys[c]	White-cheeked crested gibbon
Symphalangus	50		*S. syndactylus*	Siamang

[a] Including *H. agilis albibarbis.*
[b] Including *H. muelleri abbotti* and *H. muelleri funereus.*
[c] Including *N. leucogenys siki.*

In the present study, we investigated the phylogenetic relationships of the Hylobatidae at the tentative genus and species level. We sequenced the mitochondrial *ND3*, *ND4L*, and *ND4* region from 34 individuals representing all 12 recognized species of living Hylobatidae. We performed several analyses to reconstruct the phylogenetic relationships among and within the major clades of gibbons.

2. Materials and methods

2.1. Specimen information

A total of 34 specimens representing all 12 currently recognized species of Hylobatidae were genotyped and included in the present study (Table 2). Species identification was based on pelage, vocalization, morphology and geographical origin, and photographs were taken of most individuals. We did not include specimens where the identification of the species was questionable. Samples were collected from wild individuals or captive specimens maintained in zoos, rehabilitation centers, or as house pets within the distribution range of the species. Gibbons owned by private individuals as house pets were considered to be of unknown geographical origin unless the site of capture could be identified exactly.

2.2. Sample collection and DNA extraction

Blood samples were drawn from the femoral vein, mixed in 1:1 volume with easy blood buffer (100 mM Tris–HCl, pH 8.0, 100 mM EDTA, and 2% SDS), and transported at ambient temperature. Skin and muscle tissues from recently deceased zoo animals and fecal samples were saturated in 96% ethanol and transported at ambient temperature or as frozen material. Hair samples were stored either in ethanol or as dried samples at ambient temperature. Total genomic DNA was isolated from all tissues except hair using QIAamp DNA Blood Mini Kits (Qiagen) according to the manufacturer's protocols, except Proteinase K (Amresco) was substituted for protease in a few instances. Total genomic DNA from hair was isolated by the Proteinase K digestion, followed by standard phenol/chloroform extraction (Sambrook et al., 1989).

2.3. DNA sequencing

A 2243 base pair segment of the mtDNA *ND* complex (*ND3*, *ND4L*, and *ND4* region) including five tRNA genes (Gly and Arg on the opposite sides of *ND3*; Leu, Ser, and His flanking *ND4*) was sequenced, corresponding to nucleotide positions 9424 and 11,667 of the *Hylobates lar* mitochondrial sequence (Arnason et al., 1996, GenBank X99256). Polymerase chain reaction (PCR) was performed in a 50 μl total volume containing 3 μl (approximately 200 ng) total genomic DNA as template, 1 μM of each of primers L9424 and H11667 (Table 3), 0.2 mM dNTPs, 1.0–3.0 mM MgCl$_2$, and 0.4 U AmpliTaq DNA polymerase (Applied Biosystems). Thermal cycling was performed in GeneAmp PCR System 9700 (Applied Biosystems) thermal cycler with the following protocol: hot-start at 94 °C for 3 min, followed by 25–35 cycles of denaturation at 94 °C for 30 s, annealing at 54–60 °C for 45 s, and extension at 72 °C for 30–105 s, ended by a final step at 72 °C for 7 min. For suboptimal samples (museum skins, feces) smaller, overlapping fragments were amplified using internal primer pairs (Table 3). A negative control containing no DNA was included in every set of reactions.

After electrophoresis in a 0.8–1.5% agarose gel stained with ethidium bromide, the PCR product was

Table 2
List of hylobatid specimens included in the present study

ID	Genus	Species	DNA #	Tissue source[a]	Tissue	Bases sequenced	Sex	GenBank Accession No.
1	*Hylobates*	*lar*	HLMITCSEQ	N.A.	X99256	2243		X99256
2	*Hylobates*	*lar*	tg045	W—Knie's Kinderzoo Rapperswil, Switzerland	Skin	1703	F	AY961003
3	*Hylobates*	*agilis*	tg414	W—Zoo Erfurt, Germany	Hair	2223	M	AY961013
4	*Hylobates*	*agilis*	5181	U—Taman Safari, Indonesia	Blood	2243		AY961014
5	*Hylobates*	*moloch*	5173	U—Taman Safari, Indonesia	Blood	2173		AY961004
6	*Hylobates*	*moloch*	9754	U—Central Java, Indonesia	Blood	2225		AY961005
7	*Hylobates*	*moloch*	9755	U—West Java, Indonesia	Blood	2216		AY961006
8	*Hylobates*	*moloch*	tg427	C—Münster Zoo, Germany	Placenta	1533	F	AY961007
9	*Hylobates*	*klossii*	4618	U—Taman Safari, Indonesia	Blood	2243		AY961008
10	*Hylobates*	*klossii*	4617	U—Taman Safari, Indonesia	Blood	2222		AY961009
11	*Hylobates*	*klossii*	4619	U—Taman Safari, Indonesia	Blood	2225		AY961010
12	*Hylobates*	*klossii*	tg026	W—Basle Zoo, Switzerland	Muscle	2238	M	AY961011
13	*Hylobates*	*klossii*	tg324	W (Siberut)—Basle Zoo, Switzerland	Skin	1657	F	AY961012
14	*Hylobates*	*muelleri*	4624	U—Columbia Univ, USA	Blood	2236		AY961015
15	*Hylobates*	*muelleri*	4625	U—Columbia Univ, USA	Blood	2243		AY961016
16	*Hylobates*	*muelleri*	tg426	W—Münster Zoo, Germany	Hair	1834	M	AY961017
17	*Hylobates*	*pileatus*	tg424	C—Zürich Zoo, Switzerland	Skin	2219	M	AY961018
18	*Hylobates*	*pileatus*	tg046	C—Zürich Zoo, Switzerland	Skin	780	F	AY961019
19	*Bunopithecus*	*hoolock*	tg434	W—Perth Zoo, Australia	Hair	1294	M	AY961034
20	*Bunopithecus*	*hoolock*	tg435	Perth Zoo, Australia	Hair	1407	F	AY961035
21	*Symphalangus*	*syndactylus*	tg047	C—Zürich Zoo, Switzerland	Skin	2018	M	AY961020
22	*Symphalangus*	*syndactylus*	4601	U—Columbia Univ, USA	Blood	2243		AY961021
23	*Symphalangus*	*syndactylus*	4602	U—Columbia Univ, USA	Blood	2243		AY961022
24	*Symphalangus*	*syndactylus*	4603	U—Columbia Univ, USA	Blood	2243		AY961023
25	*Symphalangus*	*syndactylus*	4600	U—Columbia Univ, USA	Blood	2243		AY961024
26	*Nomascus*	*gabriellae*	tg615	W—Mulhouse Zoo, France	Muscle	2172	M	AY961025
27	*Nomascus*	*gabriellae*	tg418	W—Hong Kong Zoo, Hong Kong	Hair	1967	F	AY961026
28	*Nomascus*	*gabriellae*	tg340	W (Laos)—Budapest Zoo, Hungary	Hair	2219	F	AY961027
29	*Nomascus*	*leucogenys*	tg614	W—Mulhouse Zoo, France	Muscle	2225	F	AY961028
30	*Nomascus*	*leucogenys*	tg022	W—Zoo Hellabrunnd, Munich, Germany	Hair	2139	F	AY961029
31	*Nomascus*	*leucogenys*	tg502	W—Mulhouse Zoo, France	Hair	2186	M	AY961030
32	*Nomascus*	*leucogenys*	tg280	W—Mulhouse Zoo, France	Blood	2243	M	AY961031
33	*Nomascus*	*concolor*	tg530	W—Twycross Zoo, U.K.	Feces	1257	M	AY961032
34	*Nomascus*	sp. cf. *nasutus*	tg433	W (Vietnam)—Zool. Museum Humboldt Univ. ZMB 7003	Museum skin	1485	F	AY961033

[a] *Abbreviations:* C, captive-born; W, wild-born; U, unknown.

visualized under UV light. The PCR product was purified using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions, except the final product was dissolved in 18–36 μl of water depending on the intensity of the PCR band in the agarose gel. Cycle sequencing reactions were performed in 9 μl total volume containing 4.5 μl of the purified PCR product and 1 μM of sequencing primer (Table 3) in a BigDye reaction mixture (Applied Biosystems). Thermal protocols were 96 °C for 3 min followed by 30 cycles of 96 °C for 10 s, 54–62 °C for 45 s, 72 °C for 2 min 30 s, and a final 7 min at 72 °C. The product was purified using a Sephadex G-50 Centrisep column, and dried under vacuum for 15 min at 60 °C. The dried sample was dissolved in 4 μl of 1:4 mixture of blue dextran/EDTA loading buffer and deionized formamide solution and stored at

−80 °C for not more than 48 h before loading to the sequencing gel.

Sequencing was performed on an ABI 377 Automated DNA Sequencer using a 36 cm TBE polyacrylamide gel for a collection time of 8 h. Typically, one run yielded 400–700 base pairs data for each reaction. The DNA was sequenced in both directions. Output ABI files, overlapping in sequence, were assembled using AutoAssembler program (Parker, 1997), then consensus sequences were imported into a PAUP version 4.0b6 (Swofford, 1999) matrix and aligned using the *Hylobates lar* complete mtDNA sequence (GenBank X99256). Due to either limited template DNA or suboptimal samples, we were unable to obtain the full *ND3–ND4* sequence from all specimens (Table 2). However, except in the case of *N.* sp. cf. *nasutus* and *N. concolor* where only single

Table 3
Primers used to sequence Hylobatidae mitochondrial *ND3–ND4* region

H/L	Position	Sequence
L	9424	5′-GAGGATCCTACTCTTTTAGTATAA-3′
L	9465	5′-CAATTAACCAGCTTCGATAACGCT-3′
H	9871	5′-ATTTGATAAGTATGGTTGCCA-3′
L	9891	5′-TGGCAACCATACTTATCAAAT-3′
H	10261	5′-ATAATTAGRCTGTGGGTGGT-3′
L	10280	5′-ACCACCCACAGYCTAATTAT-3′
H	10602	5′-ATGAGCCTGCGTTTAGGCGT-3′
L	10811	5′-AAAATRCCCCTTTACGG-3′
H	10876	5′-AGTTTTARGAGTACTGC-3′
H	11089	5′-GTRAAGCTTCAGGGGGTTTG-3′
L	11180	5′-CTYGCAAACTCAAACTA-3′
H	11404	5′-TGTGTTATRATRAATATGTA-3′
H	11553	5′-ATTAAACTATGTTTACAGGGA-3′
H	11654	5′-CCATGTTGTTATACATGGGATAGT-3′
H	11667	5′-AAAGTTGAGAAAGCCATGTTGTTA-3′

Note. Primer position is given by the 3′ end that indicates the position in the *Hylobates lar* mitochondrial genome, X99256 (Arnason et al., 1996). H and L designate heavy-strand and light-strand primers, respectively.

samples were available, partial sequences for some specimens were always accompanied by at least one complete sequence from the same species. The samples with partial sequences consistently clustered with the complete sequences of the same species in all phylogenetic analyses.

2.4. Phylogenetic analyses

Thirty-four gibbon sequences were aligned by eye and assembled into a data matrix. These were then aligned with published sequences for *Homo sapiens* (D38112), *Pan troglodytes* (D38113), *Gorilla gorilla* (X93347), and *Pongo pygmaeus* (D38115), which were used for outgroup comparisons. For each of the first, second, and third codon positions of the protein coding sequences the number of transitions and transversions were plotted against pairwise uncorrected *p*-distances among mtDNA haplotypes to assess the levels of saturation (not shown). No significant differences were observed among saturation levels in the different codon positions, and therefore, all characters were weighted equally in all subsequent analyses.

To evaluate evolutionary relationships among aligned mtDNA *ND3–ND4* sequences, unique haplotypes were analyzed using parsimony criteria. Heuristic searches for the most parsimonious trees were conducted using 10 replications of random addition of taxa with PAUP version 4.0b6 algorithm (Swofford, 1999). A strict consensus tree was constructed from the set of equally parsimonious trees recovered from the search. Branch support was estimated with 1000 replications of non-parametric bootstrap analysis, each with a single replication of random addition of taxa.

Maximum likelihood (ML) analyses were also performed using the general time reversible model that allows for transition bias and does not assume equal base frequencies (Hasegawa et al., 1985). In this analysis, a proportion of the nucleotide sites were assumed invariable, and rates at variable sites were assumed to have a gamma distribution (Sullivan and Swofford, 1997). For the ML analysis, the starting topology was the consensus tree from the 45 equally parsimonious trees, and then we allowed PAUP to randomly dichotomize the tree to begin the parameter estimation under the likelihood optimality criterion. Using the heuristic method (Swofford et al., 1996), the gamma-shape parameter, the proportion of invariable sites, and the substitution rate matrix based on the most parsimonious tree topologies from the cladistic analysis with PAUP were estimated. The parameters of the most likely tree were used to perform likelihood analysis with nucleotide frequencies estimated from the data yielding a new, more likely tree. Thereafter, the parameters were estimated again from this tree and subjected to a second likelihood analysis using these new parameters. This procedure was repeated until additional iterations of parameter estimation and likelihood analysis did not further optimize the overall tree likelihood.

Finally, because the original matrix was very large, and since all species were monophyletic, we also used one representative per taxon, and the more thorough branch and bound search under the parsimony criterion, to see if we could improve the optimization of phylogenetic relationships.

3. Results

3.1. Sequence variation

Thirty-four specimens representing all extant species of hylobatids were sequenced for the mitochondrial *ND3*, *ND4L*, and *ND4* region corresponding to base pairs from 9424 to 11,667 in *Hylobates lar* (GenBank X99256). Comparison of the aligned sequences with tRNAs removed from the ingroup taxa revealed 683 variable sites including 515 phylogenetically informative sites (903 variable with 682 phylogenetically informative sites including the outgroups) of the total 2016 bases sequenced (variable sites can be phylogenetically informative, all genetically informative sites are variable). Within-genus uncorrected sequence divergence means and ranges (in parenthesis) were 0.03867 (0.00240–0.08307) in *Nomascus*, 0.01096 (0.00044–0.01641) in *Symphalangus*, 0.00665 in *Bunopithecus*, and 0.059970 (0.00000–0.09156) in *Hylobates*. When compared to the outgroup sequences, the only insertion or deletion that was present among the Hylobatidae was a 3-base deletion in the species of *Nomascus* at position

10320–10322 within the *ND4* gene. The unambiguous transition/transversion ratio was 5.289 in the full sequence, and 5.261 in the coding region. Within the coding region, the transition/transversion ratio was 3.255, 2.800, and 7.170 for the 1st, 2nd, and 3rd codon positions, respectively.

3.2. Phylogenetic relationships

Maximum parsimony analysis of all positions with all characters weighted equally generated 45 equally parsimonious trees (length = 2108 steps, C.I. = 0.546, R.I. = 0.815; Fig. 3). Bootstrap support values are indi-

cated over the branches, and show strong support for all major clades. ML analyses (Fig. 4; −ln$L$ = 12861.75419, I = 0.446216; Γ = 1.723376) resulted in the same general pattern of relationships as the maximum parsimony analyses *Bunopithecus* was placed as the most basal taxon, followed sequentially by the branching of *Nomascus*, *Symphalangus*, and then *Hylobates*. Within *Nomascus*, the ML tree shows *N*. sp. cf. *nasutus* as the most basal branch, followed by *N. concolor*, and then by a *N. leucogenys* and *N. gabriellae* as the most recently diverged clade. Similarly, the ML analysis presented the same relationship within the genus *Hylobates* as the maximum parsimony analysis. *Hylobates pileatus* was the most basal
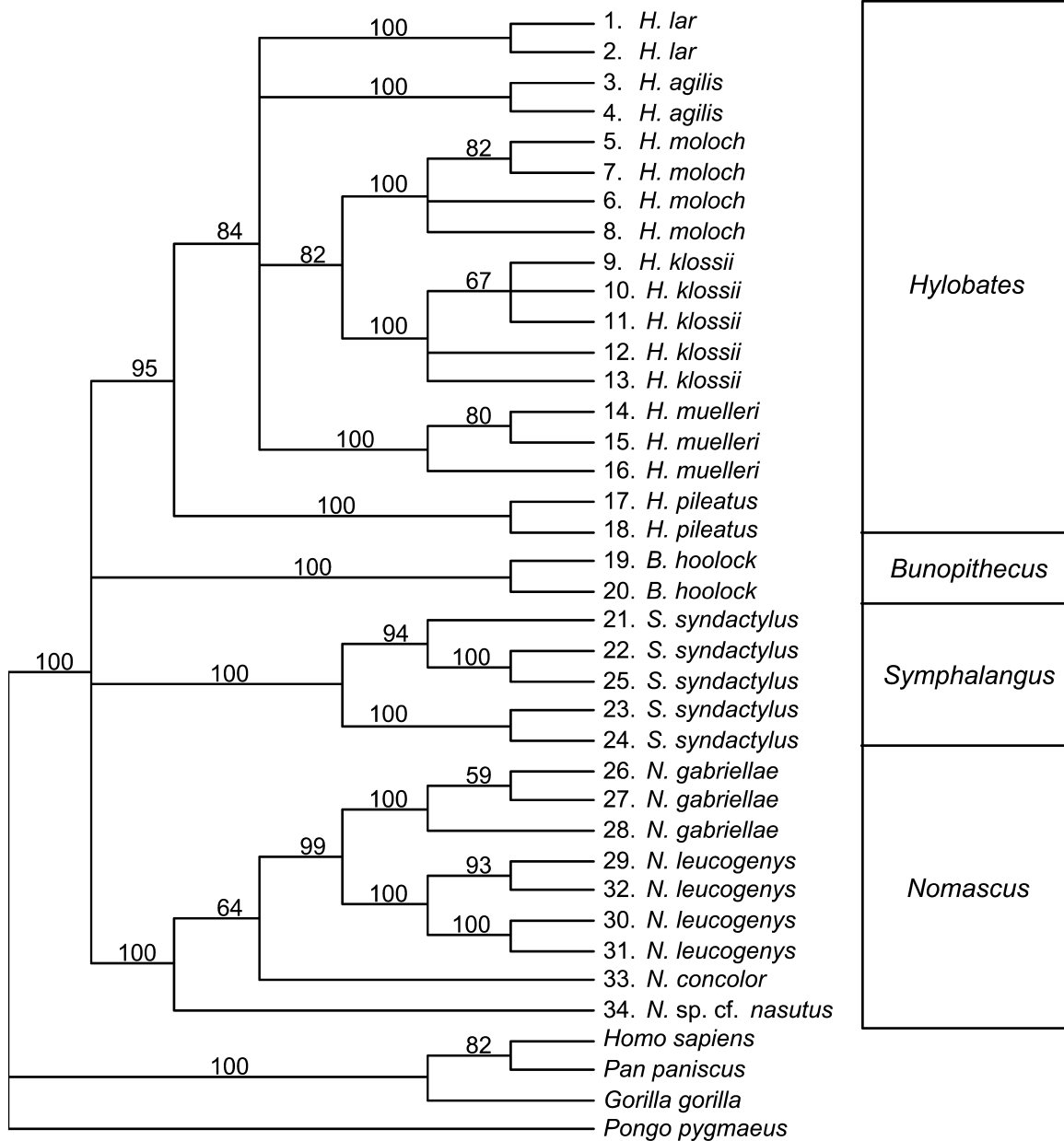

Fig. 3. Bootstrap analysis of *ND3–ND4* sequences from all species of the Hylobatidae. Numbers above the branches represent percentage bootstrap support for 1000 replicates.
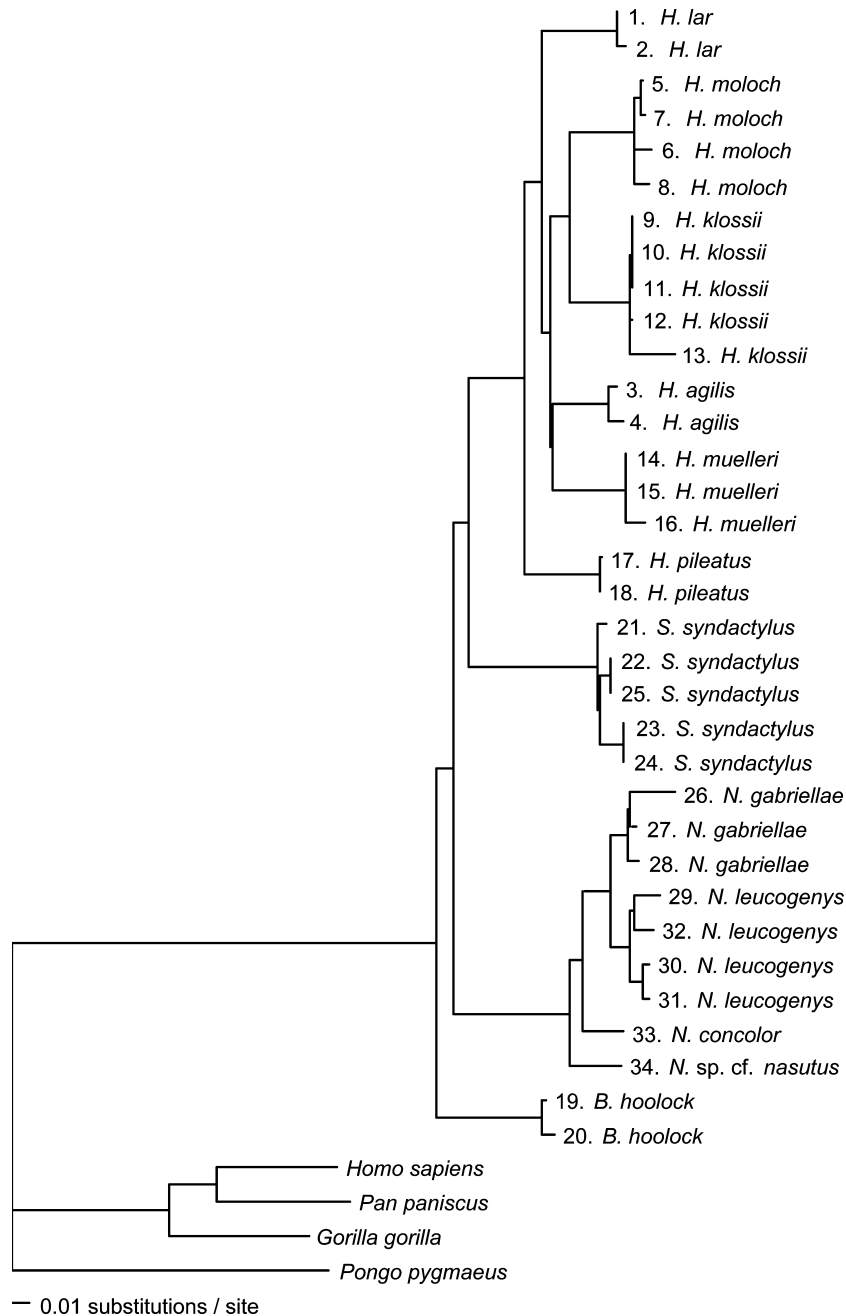
Fig. 4. Maximum likelihood tree among mtDNA *ND3–ND4* sequences form all species of Hylobatidae. A strict consensus tree from the 45 equally parsimonious trees was used in PAUP as a starting point for ML parameter estimation. PAUP was allowed to randomly dichotomize this tree before parameter estimation could proceed.

taxon, then *H. lar* diverged from the clade, followed by a split that leads on one end to a *H. agilis* and *H. muelleri* clade, and on the other end to a better resolved *H. klossii* and *H. moloch* clade. A branch and bound search using a pruned dataset, including one representative species for each genus, was also performed. The resulting tree places *Nomascus* and *Hylobates* as sister taxa, while still leaving *Bunopithecus* as basal to the family (Fig. 5A). However, a bootstrap analysis of the same pruned matrix resulted in an unresolved tetrachotomy among the four genera (Fig. 5B), exactly the same pattern as shown in Fig. 3.

4. Discussion

4.1. Relationship among the genera

In the present study, we analyzed the phylogenetic relationship of all 12 currently recognized extant gibbon species using the mitochondrial *ND3–ND4* region. Based on morphological, vocal, biochemical, and karyotypic evidence, it has been long recognized that the family Hylobatidae is composed of two (Schultz, 1933; Simonetta, 1957; Napier and Napier, 1967), and more recently, four
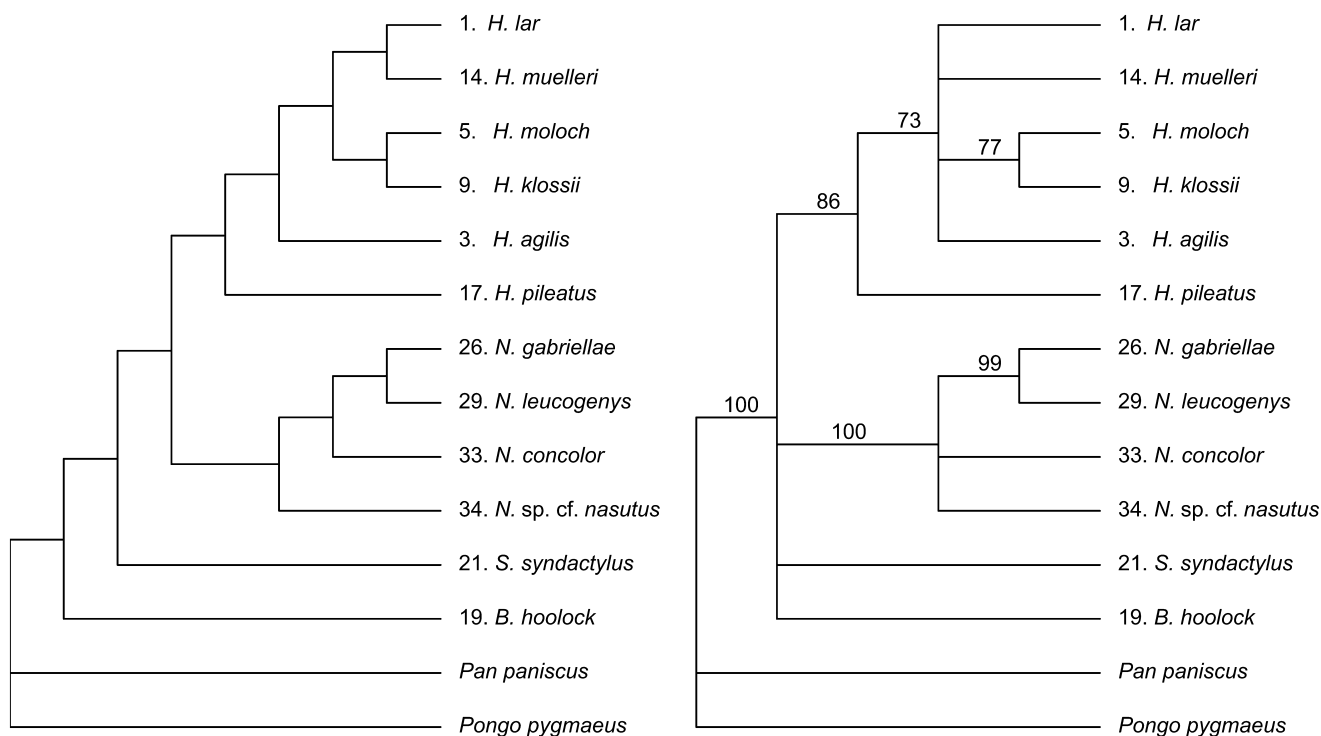
Fig. 5. Branch and bound (A), and bootstrap (B) analysis of *ND3–ND4* sequences based on a pruned dataset using one specimen per species.

distinct clades, often referred to as subgenera (Geissmann, 1995; Groves, 2001; Hayashi et al., 1995; Marshall and Sugardjito, 1986; Nowak, 1999; Rowe, 1996; Prouty et al., 1983). Most recently, it was recommended that these clades be given genus status on the basis of molecular differences among them that are at the same or higher level than those between *Homo* and *Pan* (Brandon-Jones et al., 2004; Roos and Geissmann, 2001). In agreement with this suggestion, the present study shows four distinct, monophyletic clades within the Hylobatidae, namely *Nomascus*, *Symphalangus*, *Bunopithecus*, and *Hylobates*, corresponding exactly with those defined on the basis of phenotypic characters. However, we recognize, that assigning generic-level status to these four clades remains open to debate. In addition, due to lineage specific substitution rate heterogeneity, our sequence divergence estimates alone are inadequate to establish taxonomic level, and should be interpreted along with other characters.

The monophyly of each of the four gibbon genera is highly supported. Of course, two of the genera (*Bunopithecus* and *Symphalangus*) are monospecific and thus are monophyletic by definition. However, the monophyly of *Hylobates* is supported by high (86–95%) bootstrap probabilities. Similar results were also obtained for partial sequences of *ND4* and *ND5* regions (Hayashi et al., 1995). *Nomascus*, the other polyspecific genus, is similarly highly supported as a monophyletic clade by 100% bootstrap probabilities.

The relationship among clades is only partially resolved. Maximum parsimony analysis (Fig. 3) fails to resolve the relationships among the four gibbon genera. Maximum likelihood analysis results in either *Bunopithecus* or *Nomascus* as the basal clade of hylobatids (Fig. 4 and data not shown). In contrast, *Symphalangus* and *Hylobates* are consistently the last genera to diverge.

Whereas most previous reconstructions of hylobatid phylogeny agree in the distal position of the genus *Hylobates*, they differ in placing *Symphalangus* (Groves, 1972), *Nomascus* (Chivers, 1977; Haimoff et al., 1982; Roos and Geissmann, 2001; Zhang, 1997) or *Bunopithecus* (Zehr, 1999) in the most basal position (see review of phylogenies in Geissmann, 2002a).

It is important to note that, similar to the present study, the exact relationships among hylobatid genera could not be resolved conclusively using partial mtDNA cytochrome *b* sequences (Roos and Geissmann, 2001; Hall et al., 1998), partial mtDNA *ND4* and *ND5* sequences (Hayashi et al., 1995), and complete mtDNA control region sequences (Roos and Geissmann, 2001). Likewise, the various segments of mitochondrial and nuclear DNA analyzed by Zehr (1999) produced inconsistent results, although the consensus tree calculated by that author favored the genus *Bunopithecus* in the basal position. A possible explanation for this lack of resolution may be the fact that despite the early differentiation of gibbons from other apes at approximately 16–23 million years ago (Sibley and Ahlquist, 1987), the subsequent cladogenic events that led to the four distinct genera may have occurred much later and over a very short period of time. Thus, there were few changes in

mtDNA sequences between each subsequent cladogenic event and the internodes cannot be resolved accurately or consistently. This hypothesis is supported by the relatively short branch lengths separating the four genera in the maximum likelihood analyses (Fig. 4).

4.2. Relationship within the genera

The *Nomascus* clade (also referred to as the *concolor* group, crested gibbons, or genus *Nomascus*) was considered to be monotypic with only *N. concolor* (Marshall and Sugardjito, 1986; Napier and Napier, 1985). More recently it has been defined as containing three (Groves, 1997), four (Geissmann, 1997; Geissmann, 2002a,b; Geissmann et al., 2000) or five species (Groves, 2001). The present study follows the four-species framework presented in Table 1. Beside this present study, the only other molecular phylogenetic work that included the four species of *Nomascus*, albeit not labeled as such, was that of Zhang (1997). These four species were also the subject of a phylogenetic analysis using vocal, fur coloration, and anatomical data, of which the vocal data produced the most reliable and best resolved trees (Geissmann, 2002b). Consistently throughout our various analyses, all four species were reciprocally monophyletic. Similarly, *N.* sp. cf. *nasutus* was always the most basal branch in the clade, followed by *N. concolor*, and then the more derived *N. leucogenys* and *N. gabriellae* clade. The same topology is supported, though weakly, by a recent analysis of vocal data of the same four species (Geissmann, 2002b). The presence of four distinct clades within *Nomascus* was also supported by sequence analysis of the cytochrome *b* region of the mitochondrial genome, although no reference to species was made (Zhang, 1997).

In contrast to *Nomascus*, the phylogenetic relationships within *Hylobates* are not well resolved (Creel and Preuschoft, 1984). The currently recognized six species of *Hylobates* have minimal morphological differences (Groves, 1984), and share virtually identical karyotypes (Jauch et al., 1992; Stanyon et al., 1987). In the present study, *H. pileatus* was consistently placed as the most basal branch in the *Hylobates* clade, a topology that is also supported by other work using DNA sequences (Hayashi et al., 1995; Zehr, 1999) and by vocal data (Geissmann, 2002a,b). Similarly, in all of our analyses *H. moloch* and *H. klossii* were always resolved as sister taxa, again in agreement with the vocal data (Geissmann, 2002a,b). Finally, in all maximum likelihood analyses, whether the approximations to estimate the most parsimonious tree were based on 0, 10, or 50% third position weighting, *H. agilis* and *H. muelleri* were also resolved as sister taxa. The maximum parsimony analysis neither supports nor contradicts this relationship. In general, because the internodes are small within *Hylobates*, maximum parsimony analysis resolves only a few intrageneric relationships. However, maximum likelihood resolves several

other such relationships, which are not contradicted by the maximum parsimony analysis. Thus, we suggest that the maximum likelihood topology for *Hylobates* is a reasonable hypothesis to be tested with other data.

It has been postulated that *H. klossii* was the first species to have differentiated from the main stock of *Hylobates* because of its morphological primitiveness and lack of synapomorphic characters present in other *Hylobates* species, including large ears and dense fur (Chivers, 1977; Creel and Preuschoft, 1976; Creel and Preuschoft, 1984; Groves, 1989; Groves, 1972; Haimoff et al., 1982; Haimoff, 1983; Haimoff et al., 1984; Purvis, 1995). Consequently, all other species within the genus *Hylobates*, except *H. klossii*, namely *H. lar*, *H. agilis*, *H. pileatus*, *H. moloch*, and *H. muelleri*, were long believed to be a monophyletic group and were often referred to as the *lar* group or *lar* species complex (Brockelman and Gittins, 1984; Groves, 1972, 1984; Haimoff et al., 1984; Marshall and Sugardjito, 1986; Marshall et al., 1984). Furthermore, the *lar* group has been considered a single species, *H. lar* (Creel and Preuschoft, 1984), while other studies recognized four (Groves, 1984) or five closely related species (Chivers, 1977; Chivers and Gittins, 1978; Haimoff, 1983; Geissmann, 1995; Haimoff et al., 1982, 1984; Marshall et al., 1984; Marshall and Sugardjito, 1986). The exclusion of *H. klossii* from the *lar* group and its basal placement within the *Hylobates* clade is clearly not supported by our results. Similarly, other recent studies of gibbon vocalizations (Geissmann, 1995, 2002a,b) and mtDNA sequences (Garza and Woodruff, 1992; Hayashi et al., 1995; Zehr, 1999), indicate that not only is *H. klossii* well within the *lar* group, but that it is unlikely to be the most basal species of *Hylobates*. However, the single *H. klossii* specimen used by Hayashi et al. (1995) probably is a misidentified *H. agilis* (Geissmann, unpublished data). Because Zehr (1999) used the same *H. klossii* sequence for part of her study, her consensus phylogeny must also be regarded with caution. Misidentified gibbons are (or were) common in both the literature on gibbon genetics, anatomy and karyology, and in zoos (e.g. Chen et al., 2004; Marshall and Marshall, 1975; Schilling, 1984). One of us (TG) has seen numerous misidentified gibbons himself in European, American and Asian zoos. Most of the so-called "Kloss's gibbons" in zoos turned out to be Agile gibbons upon close inspection.

Both vocal data (Geissmann, 2002a,b) and our DNA sequence data identify *H. klossii* as the sister taxon of *H. moloch*. This affinity is contradicted by the results of Hayashi et al. (1995) and Zehr (1999), but again, their results suffer from inclusion of a probably misidentified "*H. klossii*."

4.3. Summary and further directions

We have estimated the phylogenetic relationship among all 12 recognized species of living hylobatids. Our results show that the Hylobatidae are subdivided into

four well-supported monophyletic clades or tentative genera: *Nomascus, Bunopithecus, Symphalangus,* and *Hylobates.* Within *Nomascus, N.* sp. cf. *nasutus* is the most basal branch, followed by *N. concolor,* and then by a clade of *N. leucogenys* and *N. gabriellae.* Within *Hylobates, H. pileatus* is the most basal branch, while *H. moloch* and *H. klossii* form a more derived monophyletic clade. The possible monophyly of *H. agilis* and *H. muelleri* remains to be tested by other datasets. We propose the maximum-likelihood tree in Fig. 4 as a working hypothesis of gibbon relationships. To confirm or refine these relationships, we believe the collection of additional sequence data from different regions of the mitochondrial genome, as well as autosomal and Y-chromosome genes will be necessary.

References

Arnason, U., Gullberg, A., Xu, X., 1996. A complete mitochondrial DNA molecule of the white-handed gibbon, *Hylobates lar,* and comparison among individual mitochondrial genes of all hominoid genera. Hereditas 124, 185–189.

Brandon-Jones, D., Eudey, A.A., Geissmann, T., Groves, C.P., Melnick, D.J., Morales, J.C., Shekelle, M., Stewart, C.-B., 2004. An Asian primate classification. Int. J. Primatol. 25, 97–164.

Brockelman, W.Y., Gittins, S.P., 1984. Natural hybridization in the *Hylobates lar* species group: implications for speciation in gibbons. In: Preuschoft, H., Chivers, D.J., Brockelman, W.Y., Creel, N. (Eds.), The Lesser Apes. Evolutionary and Behavioural Biology. Edinburgh University Press, Edinburgh, pp. 498–532.

Chen, H.-C., Geissmann, T., Chen, P.-C., 2004. A survey of the taxonomic status of captive gibbons in Taiwan. Raffles Bull. Zool. 52, 265–269.

Chivers, D.J., Gittins, S.P., 1978. Diagnostic features of gibbon species. Int. Zoo Yearb. 18, 157–164.

Chivers, D.J., 1977. The lesser apes. Primate conservation. In: Bourne, G.H. (Ed.), Prince Rainier III of Monaco. Academic Press, New York, pp. 539–598.

Creel, N., Preuschoft, H., 1976. Cranial morphology of the lesser apes. A multivariate study. In: Rumbaugh, D.M. (Ed.), Gibbon and Siamang, vol. 4. Karger, Basel and New York.

Creel, N., Preuschoft, H., 1984. Systematics of the lesser apes: A quantitative taxonomic analysis of craniometric and other variables. In: Preuschoft, H., Chivers, D.J., Brockelman, W.Y., Creel, N. (Eds.), The Lesser Apes: Evolutionary and Behavioural Biology. Edinburgh University Press, Edinburgh, pp. 562–613.

Fleagle, J.G., 1984. Are there any fossil gibbons. In: Preuschoft, H., Chivers, D.J., Brockelman, W.Y., Creel, N. (Eds.), The Lesser Apes: Evolutionary and Behavioural Biology. Edinburgh University Press, Edinburgh, pp. 431–447.

Fleagle, J.G., 1999. Primate Adaptation and Evolution, second ed. Academic Press, London.

Garza, J.C., Woodruff, D.S., 1992. A phylogenetic study of the gibbons (*Hylobates*) using DNA obtained non-invasively from hair. Mol. Phylogen. Evol. 1, 202–210.

Geissmann, T., 1993. Evolution of communication in gibbons (Hylobatidae), Ph.D. thesis, Anthropological Institute, Philosoph. Faculty II, Zürich University.

Geissmann, T., 1995. Gibbon systematics and species identification. Int. Zoo News 42, 467–501.

Geissmann, T., 1997. New sounds from the crested gibbons (*Hylobates concolor* group): First results of a systematic revision. In: Zissler, D. (Ed.), Verhandlungen der Deutschen Zoologischen Gesellschaft: Kurzpublikationen–Short Communications, 90. Jahresversammlung 1997 in Mainz. Gustav Fischer, Stuttgart, p. 170.

Geissmann, T., 2002a. Duet-splitting and the evolution of gibbon songs. Biological Reviews 77, 57–76.

Geissmann, T., 2002b. Taxonomy and evolution of gibbons. In: Soligo, C., Anzenberger, G., Martin, R.D. (Eds.), Anthropology and primatology into the third millennium: The Centenary Congress of the Zürich Anthropological Institute, Evolutionary Anthropology 11 (Suppl. 1). Wiley-Liss, New York, pp. 28–31.

Geissmann, T., 2003a. Vergleichende Primatologie. Springer, Heidelberg and New York.

Geissmann, T., (compiler) 2003b. Symposium on Gibbon Diversity and Conservation: Concluding resolution. Asian Primates 8(3–4), 28–29.

Geissmann, T., Nguyen Xuan Dang, Lormée, N., Momberg, F., 2000. Vietnam primate conservation status review 2000—Part 1: Gibbons, Fauna and Flora International, Indochina Programme, Hanoi.

Groves, C.P., 1972. Systematics and phylogeny of gibbons. In: Rumbaugh, D.M. (Ed.), Gibbon and Siamang, vol. 1. Karger, Basel, pp. 1–89.

Groves, C.P., 1984. A new look at the taxonomy and phylogeny of the gibbons. In: Preuschoft, H., Chivers, D.J., Brockelman, W.Y., Creel, N. (Eds.), The Lesser Apes. Evolutionary and Behavioural Biology. Edinburgh University Press, Edinburgh, pp. 542–561.

Groves, C.P., 1989. A Theory of Human and Primate Evolution. Clarendon Press, Oxford, pp. 219–303.

Groves, C.P., 1993. Speciation in living hominoid primates. In: Kimbel, W.H., Martin, L.B. (Eds.), Species, Species Concepts, and Primate Evolution. Plenum Press, New York and London, pp. 109–121.

Groves, C.P., 1997. Taxonomy and phylogeny of primates. In: Blancher, A., Klein, J., Socha, W.W. (Eds.), Molecular Biology and Evolution of Blood Group and MHC Antigens in Primates. Springer-Verlag, Berlin, pp. 3–23.

Groves, C.P., 2001. Primate Taxonomy. Smithsonian Institution Press, Washington, DC and London.

Haimoff, E.H., Chivers, D.J., Gittins, S.P., Whitten, T., 1982. A phylogeny of gibbons (*Hylobates* spp.) based on morphological and behavioural characters. Folia Primatol. 39, 213–237.

Haimoff, E.H., 1983. Gibbon songs: an acoustical, organizational, and behavioural analysis. Ph.D. thesis, University of Cambridge..

Haimoff, E.H., Gittins, S.P., Whitten, A.J., Chivers, D.J., 1984. A phylogeny and classification of gibbons based on morphology and ethology. In: Preuschoft, H., Chivers, D.J., Brockelman, W.Y.,

Creel, N. (Eds.), The Lesser Apes. Evolutionary and Behavioural Biology. Edinburgh University Press, Edinburgh, pp. 614–632.

Hall, L.M., Jones, D.S., Wood, B.A., 1998. Evolution of the gibbon subgenera inferred from cytochrome *b* DNA sequence data. Mol. Phylogenet. Evol. 10, 281–286.

Hasegawa, M., Kishino, H., Yano, T., 1985. Dating of the human–ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. 22, 160–174.

Hayashi, S., Hayasaka, K., Takenaka, O., Horai, S., 1995. Molecular phylogeny of gibbons inferred from mitochondrial DNA sequences: preliminary report. J. Mol. Evol. 41, 359–365.

Jauch, A., Wienberg, J., Stanyon, R., Arnold, N., Tofanelli, S., Ishida, T., Cremer, T., 1992. Reconstruction of genomic rearrangements in great apes and gibbons by chromosome painting. Proc. Natl. Acad. Sci. USA 89, 8611–8615.

Marshall, J.T., Marshall, E.R., 1975. Letter to the editor. Mammal Chromosomes Newslett. 16, 30–33.

Marshall, J.T., Sugardjito, J., Markaya, M., 1984. Gibbons of the lar group: relationships based on voice. In: Preuschoft, H., Chivers, D.J., Brockelman, W.Y., Creel, N. (Eds.), The Lesser Apes. Evolutionary and Behavioural Biology. Edinburgh University Press, Edinburgh, pp. 533–541.

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.

Napier, J.R., Napier, P.H., 1967. A Handbook of Living Primates. Academic Press, London and New York.

Napier, J.R., Napier, P.H., 1985. The Natural History of the Primates. MIT Press, Cambridge, MA.

Nowak, M., 1999. Walker's Primates of the World. John Hopkins University Press, Baltimore and London.

Parker, S.R., 1997. AutoAssembler sequence assembly software. Methods Mol. Biol. 70, 107–117.

Pocock, R.I., 1927. The gibbons of the genus *Hylobates*. Proc. Zool. Soc. London 2, 719–741.

Prouty, L.A., Buchanan, P.D., Pollitzer, W.S., Mootnick, A.R., 1983. *Bunopithecus*: a genus-level taxon for the hoolock gibbon (*Hylobates hoolock*). Am. J. Primatol. 5, 83–87.

Purvis, A., 1995. A composite estimate of primate phylogeny. Philosop. Trans. R. Soc. London B 348, 405–421.

Roos, C., Geissmann, T., 2001. Molecular phylogeny of the major hylobatid divisions. Mol. Phylogen. Evol. 19, 486–494.

Sambrook, J., Fritsch, E., Maniatis, T., 1989. Molecular Cloning—A Laboratory Manual. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York.

Rowe, N., 1996. The Pictorial Guide to the Living Primates. Pogonias Press, East Hampton, New York.

Schilling, D., 1984. Letter to the editor. Primate Report 11, 36.

Schultz, A.H., 1933. Observations on the growth, classification and evolutionary specialization of gibbons and siamangs. Hum. Biol. 5, 212–255, 385–428.

Sibley, C.G., Ahlquist, J.E., 1987. DNA hybridization evidence of hominid phylogeny: results from an expanded data set. J. Mol. Evol. 26, 99–121.

Simonetta, A., 1957. Catalogo e sinonimia annotata degli ominoidi fossili ed attuali (1758–1955). Atti Soc. Toscana Sci. Nat., Pisa, Ser. B 64, 53–113.

Sullivan, J., Swofford, D.L., 1997. Are guinea pigs rodents. The importance of adequate models in molecular phylogenetics. J. Mammal. Evol. 4, 77–86.

Stanyon, R., Sineo, L., Chiarelli, B., Camperio-Ciani, A., Haimoff, E.H., Mootnick, A.R., Suturman, D.R., 1987. Banded karyotypes of the 44-chromosome gibbons. Folia Primatol. 48, 56–64.

Swofford, D.L., Olsen, G.J., Waddell, P.J., Hillis, D.M., 1996. Phylogenetic inference. In: Hillis, D.M., Moritz, C., Mable, B.K. (Eds.), Molecular Systematics, second ed. Sinauer Associates, Sunderland, MA, pp. 407–543.

Swofford, D.L. 1999. PAUP*, Phylogenetic analysis using parsimony (∗ and other methods), version 4, Sinauer Associates, Sunderland.

Zehr, S.M., 1999. A nuclear and mitochondrial phylogeny of the lesser apes (Primates, genus *Hylobates*), Ph.D. thesis, Harvard University, Cambridge, Massachusetts.

Zhang, Y., 1997. Mitochondrial DNA sequence evolution and phylogenetic relationships of gibbons. Acta Genet. Sin. 24, 231–237 (Chinese text, English summary).