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Resolution of the *Hylobates* phylogeny: Congruence of mitochondrial D-loop sequences with molecular, behavioral, and morphological data sets

Danielle J. Whittaker [a,*], Juan Carlos Morales [b,c,d], Don J. Melnick [b,c,d]

[a] *Department of Biology, Indiana University, Bloomington, IN 47405, USA*
[b] *Department of Ecology, Evolution, and Environmental Biology, Columbia University, New York, NY 10027, USA*
[c] *New York Consortium in Evolutionary Primatology (NYCEP), USA*
[d] *Center for Environmental Research and Conservation (CERC), Columbia University, New York, NY 10027, USA*

Abstract

Gibbons of the genus *Hylobates* likely speciated very rapidly following isolation by rising sea levels during the Pleistocene. We sequenced the hypervariable region I (HV-I) of the mitochondrial D-loop to reconstruct the phylogeny of this group. Although the results clearly supported monophyly of each of the six species, the relationships among them were not clearly resolved by these data alone. A homogeneity test against published data sets of a coding mitochondrial locus (ND3–ND4 region), behavioral characters (vocalizations), and morphological traits (including skeletal and soft tissue anatomy) revealed no significant incongruence, and combining them resulted in a phylogenetic tree with much stronger support. The Kloss's gibbon (*H. klossii*), long considered a primitive taxon based on morphology, shares many molecular and vocal characteristics with the Javan gibbon (*H. moloch*), and appear as the most recently derived species. The northernmost species (*H. lar* and *H. pileatus*) are the most basal taxa. These data suggest that ancestral gibbons radiated from north to south. Unlike other markers, the HV-I region can accurately identify members of different gibbon species much like a DNA barcode, with potential applications to conservation.
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1. Introduction

The gibbons (Family Hylobatidae) are the small apes, endemic to the forests of South and Southeast Asia, known for their musical morning calls and their brachiating locomotion. There are currently 12 recognized species in four genera: the siamang, *Symphalangus syndactylus*, the hoolock gibbon, *Hoolock hoolock* (formerly *Bunopithecus*), the crested gibbons, genus *Nomascus* (four species), and the genus *Hylobates*, formerly known as the lar group (six species) (Brandon-Jones et al., 2004; Mootnick and Groves, 2005). The relationships among these four genera are not yet fully agreed upon; however, most analyses show *Hylobates* to be the most derived taxon within this radiation.

The genus *Hylobates* includes six species: *lar*, *agilis*, *moloch*, *muelleri*, *pileatus*, and *klossii*. The first five have long been considered closely related, and are virtually indistinguishable on the basis of cranial characters (hence, "lar group"). Past analyses have disagreed on the Kloss's gibbon's placement in this genus; however, we agree on its inclusion in this group based on a variety of shared characters, including cranial shape, intermembral index, genital features, and especially chromosome number ($2n = 44$) (Groves, 2001).

* Corresponding author. Fax: +1 812 855 6785.
E-mail address: djwhitta@indiana.edu (D.J. Whittaker).

The Kloss's gibbon (*Hylobates klossii*) was first described as a "dwarf siamang" due to its invariant black pelage and small size (relative to the siamang), and was placed in the genus *Symphalangus* (Miller, 1903) and later in the subgenus *Symphalangus* within the genus *Hylobates* (Groves, 1968). Later observations and morphological studies led to the conclusion that the Kloss's gibbon is neither a dwarf nor a siamang (Groves, 1972; Tenaza and Hamilton, 1971), but related to the so-called lar group (Chivers, 1977; Creel and Preuschoft, 1984; Haimoff et al., 1982; Marshall and Sugardjito, 1986). For instance, in a multiple discriminant analysis of 90 cranial and dental variables by Creel and Preuschoft (1984), *H. klossii* clusters with the lar group, far from the siamang, the hoolock, or the crested (*Nomascus*) gibbons. These later studies still note the features of the Kloss's gibbon identified as primitive (such as reduced hair density, lack of facial markings, higher average number of vertebrae) in earlier studies and still place this species as basal to the rest of the group. However, Geissmann's (1993, 2002a) cladistic analysis of morphological and karyological "non-communicatory" characters (which excludes pelage characteristics) disagrees with this conclusion, and places the Kloss's gibbon as a sister taxon to *H. agilis* at an internal node.

Vocalizations have been considered reliable taxonomic indicators of closely related species (Gautier, 1988; Oates et al., 2000; Snowdon et al., 1986; Zimmermann, 1990). Geissmann (1993, 2002a) has analyzed gibbon phylogenetic relationships using vocal traits. Based on these characters, *H. klossii* is considered the sister taxon of the Javan silvery gibbon (*H. moloch*) because these two species, unlike all other gibbon taxa, do not sing duets; the males and females have separate songs (Geissmann, 1993, 2002a). Geissmann suggests that duet-splitting (partners singing at different times of the day) is a derived characteristic, evolving after song-splitting (partners singing different parts of a duet) (Geissmann, 2002b). A more detailed study on Javan gibbon calling behavior has demonstrated that like Kloss's gibbons, all Javan gibbon males in an area chorus before dawn, while the females chorus after dawn (Geissmann and Nijman, 2006; Tenaza, 1976).

A number of molecular studies have included the Kloss's gibbon, but all produce different results (reviewed in Takacs et al., 2005). Most recently, Takacs et al. (2005) constructed a molecular phylogeny of all 12 recognized species using the ND3–ND4 region of the mitochondrial genome. In this study, the relationships among members of the genus *Hylobates* remain largely unresolved, though *H. klossii* and *H. moloch* appear as sister taxa. The lack of resolution in this and other molecular studies is likely due to the fact that while more slowly evolving genes such as the mitochondrial cytochrome *b* gene are often appropriate for phylogenetic analysis of temporally deep branches, gibbons radiated over a relatively short time span, and thus a more quickly evolving locus is necessary to identify intrageneric species relationships. The hypervariable region I (HV-I) of the displacement loop, or D-loop, has a faster mutation rate than any other part of the primate mitochondrial genome. This region is useful for examining intraspecific relationships and evolutionary relationships between closely related species (Avise, 2000) and may be more appropriate than slower-evolving genes for the phylogenetic analysis of gibbons (Chatterjee, 2001; Roos and Geissmann, 2001). This locus was used to attempt to resolve the previously unclear relationships between the four subgenera or genera of gibbons (*Hylobates*, *Hoolock*, *Nomascus*, and *Symphalangus*) (Roos and Geissmann, 2001), as well as relationships among populations of *H. lar* (Woodruff, 1993; Woodruff et al., 2005), *H. moloch* (Andayani et al., 2001), and *H. klossii* (Whittaker, 2005).

Alternatively, the difficulty of resolving the gibbon phylogeny may suggest that gibbons may not have speciated in a strict bifurcating or branching pattern, as assumed by phylogenetic methods. Instead, gibbon phylogenetic relationships may in fact represent a "hard" polytomy, an actual simultaneous or nearly simultaneous multiple speciation event (as opposed to a "soft" polytomy, which is a result of insufficient data to resolve speciation patterns within an analysis). One scenario is that populations of a single ancestral species were simultaneously isolated on islands by rising sea levels (vicariance) and subsequently differentiated into multiple species. Marshall and Sugardjito (1986) have observed that with the exception of the siamang, all gibbons are ecologically and behaviorally similar, and because of their non-overlapping distributions have not needed to adapt to different niches. Thus, the primary differences between the gibbon species are characters that aid in species identification, including coloration and vocalizations.

The present study aims to resolve the phylogeny of the genus *Hylobates*. We included samples from wild Kloss's gibbons throughout their range in the Mentawai Islands of Indonesia (Whittaker, 2005), as well as samples collected from pet *H. moloch* and *H. agilis* of known origin (Andayani et al., 2001) to eliminate errors due to species misidentification. We chose to sequence the HV-I region of the mitochondrial D-loop to increase the likelihood of picking up a phylogenetic signal from a relatively fast evolutionary event. We then tested the incongruence of our D-loop data set with three other data sets (molecular, vocal, and morphological) using the partition homogeneity test. Data sets that were not significantly incongruent were combined to produce a combined evidence phylogeny, which provided much stronger support for the resulting phylogenetic reconstruction.

2. Methods

2.1. D-loop sequencing

2.1.1. H. klossii samples

From January to May 2001 and August to December 2003, one of us (DJW) collected fecal samples

non-invasively from unhabituated wild Kloss's gibbons at each of seven sites across the four Mentawai islands. These samples were also used to test whether geographically isolated populations of *H. klossii* have genetically diverged (Whittaker, 2005). Samples were collected from 2–8 groups for each site, for a total of 32 gibbon groups sampled, and stored in either lysis buffer or RNA*later*® RNA Stabilization Solution (Ambion, Inc.). DNA was extracted using standard phenol–chloroform procedures (Sambrook et al., 1989) or Qiagen Stool Kits®.

The HV-I region of the D-loop was amplified and sequenced using the gibbon-specific primers GIBDLF3 (5′-CTT CAC CCT CAG CAC CCA AAG C-3′) and GIB DLR4 (5′-GGG TGA TAG GCC TGT GAT C-3′) (Andayani et al., 2001) which correspond to the human primers L15996 (Vigilant et al., 1989) and H16498 (Kocher et al., 1989). The loci were amplified using optimized Polymerase Chain Reaction (PCR) protocols (Palumbi, 1996) in 50 μL reactions and processed on Perkin-Elmer® thermocyclers with an annealing temperature of 55 °C. Because of low concentration of DNA in each extraction, large quantities of template DNA (up to 8 μL) were used in each reaction. (Not all samples could be successfully amplified due to extremely low DNA concentration, resulting in a final set of 21 *H. klossii* sequences.) Bovine Serum Albumin was added to each reaction to overcome any remaining PCR inhibitors. PCR products were purified with Qiagen PCR purification kits and cycle-sequenced using Perkin-Elmer's ABI Prism™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kits. The ABI Prism™ 377 Automated Sequencer and ABI 3730 XL 96-well Capillary Sequencer were used for sequencing. Consensus sequences for each individual were generated using the ABI software package AutoAssembler© as well as the Sequencher 3.1 program. All sequences were deposited in GenBank (Accession Nos. EF363486 through EF363506).

2.1.2. Other Hylobatid species

HV-I sequences from other gibbon species were obtained from GenBank (*H. agilis*: 2; *H. lar*: 2; *H. moloch moloch*: 5; *H. moloch pongoalsoni*: 8) (Table 1). The *H. moloch* and *H. agilis* samples were obtained from captive animals of known origin (Andayani et al., 2001), while the *H. lar* and outgroup specimens were from carefully identified zoo animals (Roos and Geissmann, 2001). Sequences for *H. muelleri* and *H. pileatus*, as well as *H. agilis albibarbis*, were not available on GenBank. We sequenced DNA from blood samples from zoo specimens (*H. muelleri*: JP92, JP93 [GenBank accession nos. EF363507 and EF363508]; *H. pileatus*: JP99 [EF363509]; and a putative *H. agilis albibarbis*: JP90 [EF363485]) following the same protocols as above. One sequence from each of the other three gibbon genera (*Hoolock*, *Nomascus gabriellae*, and *Symphalangus*) were also obtained from GenBank and used as outgroups for the phylogenetic analyses (Table 2).

Table 1
List of sequences retrieved from GenBank

Taxon	Sample ID	GenBank acquisition number	Reference
H. agilis	NAN04, NAN39	AF338876, AF338905	Andayani et al. (2001)
H. lar	lar2, lar3	AF311724, AF311723	Roos and Geissmann (2001)
H. moloch moloch	NAN08, NAN12, NAN14, NAN26, NAN41	AF338880, AF338884, AF338886, AF338897, AF338906	Andayani et al. (2001)
H. moloch pongoalsoni	NAN06, NAN07, NAN10, NAN13, NAN28, NAN30, NAN33, NAN35	AF338878, AF338879, AF338882, AF338885, AF338899, AF338900, AF338902, AF338904	Andayani et al. (2001)
B. hoolock	Bunopithecus	AF311725	Roos and Geissmann (2001)
S. syndactylus	Symphalangus	AF311722	Roos and Geissmann (2001)
N. gabriellae	Nomascus	AF193804	Roos and Geissmann (2001)

Table 2
Significance values from pairwise partition homogeneity tests

	D-loop	ND3–ND4	Vocalizations	Morphology
D-loop	—	All four together: $p = 0.16$		
ND3–ND4	$p = 0.29$	—		
Vocalizations	$p = 0.06$	$p = 0.80$	—	
Morphology	$p = 0.14$	$p = 0.18$	$p = 0.10$	—

2.2. Phylogenetic analysis

Sequences were aligned using the CLUSTAL X Multiple Sequence Alignment Program, version 1.81 (Jeanmougin et al., 1998). We used PAUP*4.0b10 (Swofford, 2002) to perform maximum parsimony and maximum likelihood phylogenetic inference analyses.

We used unweighted maximum parsimony analysis to find the most parsimonious tree. A heuristic search was performed with 1000 bootstrap replications. To determine which model of evolutionary change best fit the data for the maximum likelihood analysis, the program MODEL-TEST 3.6 was employed (Posada and Crandall, 1998), using the hierarchical likelihood ratio test (hLRT) to choose which of 56 models best fit the data. A heuristic

maximum likelihood search was performed with 100 bootstrap replications.

Finally, because the data matrix is large, and because the sampling was uneven across taxa, we chose a single representative for each species and performed an exhaustive search using the maximum parsimony criterion. For *H. klossii*, the most common haplotype was chosen (five individuals [PL04, SB04, SB06, SB19, and SP08] had the same haplotype). For taxa with smaller sample sizes (*H. agilis*, *H. lar*, *H. muelleri*, *H. moloch*), a single representative was chosen at random. Only one sequence was available for *H. pileatus*.

2.3. Congruence with other data sets

A "total evidence" approach, in which different kinds of data (e.g., morphological, molecular, and behavioral; or mitochondrial and nuclear DNA sequences) are combined into a single analysis, can be employed to increase explanatory power (DeSalle and Brower, 1997; Eernisse and Kluge, 1993). To overcome disparities among data sets due to different mutation rates or selection pressures, the data can be partitioned and statistically compared to look for conflicting phylogenetic signals. The Incongruence Length Difference test (ILD, also known as the partition homogeneity test) examines whether the difference between data partitions is greater than that expected by chance, and can be particularly useful for determining whether data can be combined into a single analysis (Cunningham, 1997a,b; Farris et al., 1995).

We tested the D-loop sequences from this study for congruence with three other data sets: (1) the more slowly evolving, protein-coding mitochondrial ND3–ND4 region (Takacs et al., 2005), with a total of 2274 basepairs; (2) 29 vocalization characters (Geissmann (1993, 2002a)); and (3) Geissmann's (1993, 2002a) "non-communicatory" data set which includes 26 skeletal, postcranial, soft parts anatomy, and karyological characters (Geissmann, 1993; Groves, 1972; Marshall and Sugardjito, 1986; Prouty et al., 1983; Stanyon et al., 1987; van Tuinen and Ledbetter, 1983). All four data sets were combined into a single data set and partitioned. We used the Partition Homogeneity Test in PAUP* 4.0b10 to examine whether the data sets displayed significantly incongruent phylogenetic signals (Farris et al., 1995; Swofford, 2002).

A single representative of each species was used in the analysis. Takacs et al. (2005) did not include *H. agilis albibarbis* as a separate taxon, so it was excluded from the congruence analysis. The morphological and vocalization characters were ordered (Wagner parsimony) (Geissmann, 2002a), while the genetic characters were unordered (Fitch parsimony). Uninformative characters were excluded (Lee, 2001), leaving 125 characters in the D-loop data set, 258 in the ND3–ND4 set, 21 vocal characters, and 19 morphological characters. The partition homogeneity test was run using heuristic parsimony (100 replicates) with random stepwise addition of sequences, and the Goloboff fit crite-

rion ($K = 2$) was employed to reduce the effect of homoplasy on the tree (Goloboff, 1993). We conducted the partition homogeneity test both as a simultaneous four-way test and as each possible pairwise comparison.

Several authors agree that if no significant conflict is observed among different data sets, they can be combined into a single data set and analyzed as one (Cunningham, 1997a; DeSalle and Brower, 1997; Flynn and Nedbal, 1998; Omland, 1994; Remsen and DeSalle, 1998; Vogler and Pearson, 1996). When no significant incongruence was found among the four data sets, we produced a combined evidence phylogenetic tree using maximum parsimony analysis.

3. Results

3.1. D-loop phylogeny

The length of the amplified sequence was 487–491 bp in *Hylobates* species, longer in outgroup species (*Hoolock*: 502, *Nomascus*: 507, *Symphalangus*: 520); the alignment including gaps was 528 bp long, including 404 uninformative sites. Average within-species pairwise sequence divergence estimates were as follows (range in parentheses): *H. agilis*, 7.6% (5.3–8.9%); *H. klossii*, 2.5% (0.2–4.5%); *H. lar*, 2.9%; and *H. moloch*, 3.3% (1.2–5.7%). Average between-species sequence divergence within the genus *Hylobates* was 10.9% (range 7.1–17.2%), and average intergeneric divergence was 21.5% (range 17.0–26.3%). The highest interspecific divergences within the genus *Hylobates*, overlapping with intrageneric divergence, were observed between *H. pileatus* and the other species (13.1–17.2%), with the greatest distance found between *H. pileatus* and *H. agilis*.

Using D-loop sequences, the genus *Hylobates* is monophyletic with 98–100% bootstrap support in all analyses, and species with multiple samples also show monophyly with high support. *Hylobates klossii* clusters with *H. moloch* and *H. agilis*, inside the lar group, and not basal to the lar group. *H. pileatus* and *H. lar* appear as the basal taxa in most analyses.

3.1.1. Maximum parsimony

The heuristic search found 167 equally parsimonious trees. *H. klossii* clusters with *H. moloch*, with *H. agilis* as the next most closely related species. The bootstrap maximum parsimony analysis (1000 replications) resulted in an unresolved polytomy of the entire genus *Hylobates* (data not shown).

3.1.2. Maximum likelihood

Using the hLRT, the best fitting nucleotide substitution model was HKY + G (Hasegawa et al., 1985). This model assumes that transitions are more likely than transversions, that purine and pyrimidine transitions are equally likely, and that the substitution rate follows a gamma distribution. Using a heuristic search in PAUP*, two equally likely

trees were produced. In a strict consensus tree, *H. klossii* and *H. agilis* are shown as sister taxa, with *H. moloch* as the next most closely related (not shown), however, in a bootstrap tree, *H. klossii* is part of an unresolved four-way polytomy with *H. moloch*, *H. agilis*, and *H. muelleri* (Fig. 1).

3.1.3. Exhaustive maximum parsimony search

The exhaustive maximum parsimony search using one representative of each taxon found a tree with two clades: on one branch, *H. klossii* and *H. moloch* are sister taxa, with *H. agilis* and *H. agilis albibarbis* as a sister clade, and on the other branch, *H. muelleri, H. pileatus* and *H. lar* form a clade with *H. lar* as the basal taxon (tree not shown).

3.2. Partition homogeneity test and combined evidence tree

No significant incongruence was found between the D-loop data and the other data sets ($p > 0.05$ in all comparisons, Table 2), so we combined them in a maximum parsimony analysis. The maximum parsimony tree (1000 bootstrap replications) is shown in Fig. 2. The pattern in this analysis closely follows that of the D-loop analyses, but with much stronger support. *H. klossii* and *H. moloch*
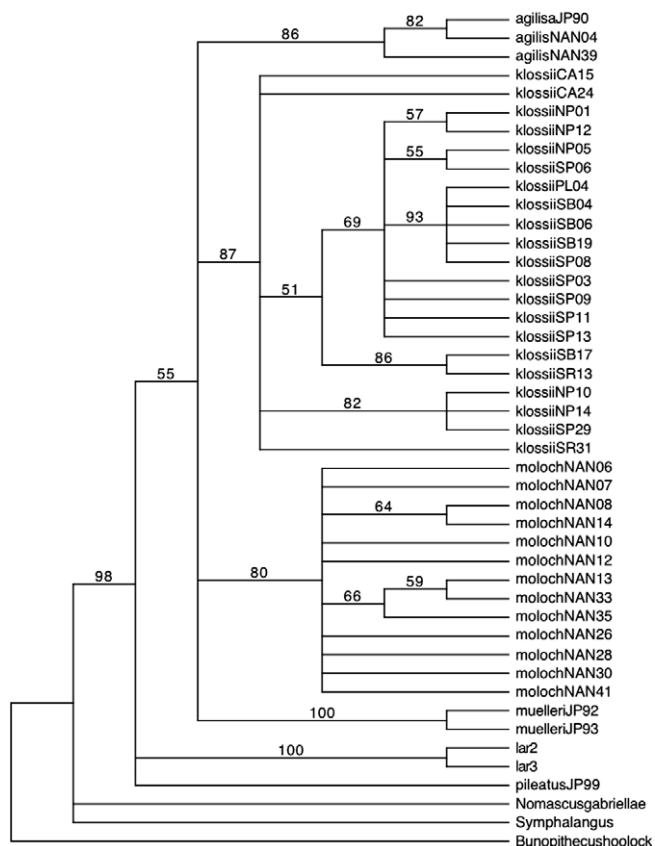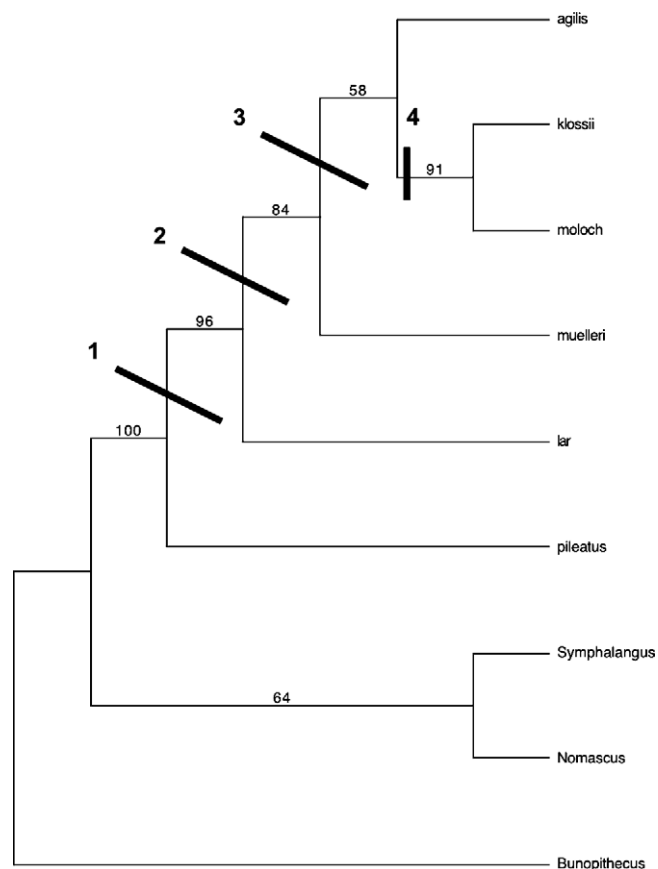

Fig. 2. Combined evidence tree produced by maximum parsimony, 1000 bootstrap replications. Heavy lines numbered 1–4 correspond to biogeographic breaks.

are sister taxa (91% bootstrap support), with *H. agilis* as the next most closely related species. *H. pileatus* is the most basal taxon in the *Hylobates* radiation, followed by *H. lar*.

4. Discussion

The mitochondrial D-loop appears to be an appropriate locus for assisting in the reconstruction of the phylogeny of the genus *Hylobates*. These gibbon species are closely related and probably diverged in a short period of time, and by using this fast-evolving locus we were able to discern a phylogenetic signal. However, on its own the D-loop does not provide strong enough resolution, as evident in the extremely low bootstrap support values in the maximum parsimony and maximum likelihood analyses. Combining the D-loop sequences with other types of phylogenetically informative data resulted in a much more strongly supported tree. Importantly, the signal from the D-loop did not significantly conflict with information from other sources. The data presented here suggest that the Kloss's gibbon clusters with Javan silvery gibbon and the agile gibbon within the *Hylobates* genus, with the pileated gibbon in the most basal position of this radiation. The lar gibbon and the Bornean gibbon occupy intermediate positions.

Fig. 1. Tree produced by maximum likelihood analysis of HV-I sequences (100 bootstrap replications).

4.1. Combining the data sets

The use of the ILD test to determine combinability of data has been a source of great controversy in the literature (Barker and Lutzoni, 2002; Bull et al., 1993; Hipp et al., 2004). We chose to proceed in this manner for the following reasons: (1) the test revealed no significant incongruence among the data sets, and (2) a visual inspection of the trees produced by the original analyses reveals many similarities to the D-loop phylogeny such as the clustering of *H. klossii* and *H. moloch* (ND3–ND4, vocalizations) and the basal position of *H. pileatus* (vocalizations, morphology). The independent D-loop and ND3–ND4 analyses did not provide sufficient resolution, as is true of most molecular gibbon phylogenies. By combining different types of information into a single analysis, we were able to increase the accuracy of our phylogenetic reconstruction.

Several authors have examined the gibbon phylogeny using molecular markers: cytochrome *b* (Garza and Woodruff, 1992; Hall et al., 1998; Zhang, 1997), ND4–ND5 region (Hayashi et al., 1995), COII (Zehr, 1999), and the nuclear G6PD locus (Zehr, 1999). While all genetic analyses, regardless of locus, have found *H. klossii* to cluster inside the *Hylobates* genus (and not basal as previously suggested by morphological studies), there has been no agreement on the most closely related taxon. Across past analyses, every species of the lar group has been suggested as a sister taxon to *H. klossii*: *H. moloch* (Takacs et al., 2005), *H. agilis* (G6PD: Zehr, 1999), *H. muelleri* and *H. pileatus* (cytochrome *b*: Garza and Woodruff, 1992; Zhang, 1997), and *H. lar* (ND4–ND5: Hayashi et al., 1995). However, the specimens used in most analyses are from zoo animals, some of which may have been misidentified. Thus, the results of some of these studies may be unreliable, as the specimen identified as *H. klossii* in some analyses (Hayashi et al., 1995; same specimen used in Zehr, 1999) may in fact be a misidentified *H. agilis* (as discussed in Takacs et al., 2005).

With such varying conclusions, any single locus will not sufficiently support a phylogenetic hypothesis in the gibbons. For reasons discussed above, the D-loop locus may be more likely to recover an accurate phylogenetic signal. To test the resulting reconstruction, we compared the fast-evolving, non-coding HV-I region of the D-loop to a more slowly evolving coding gene (ND3–ND4), a set of behavioral characters (vocalization characters), and a set of morphological characters. We found no significant incongruence among these data sets. We then combined them and produced a tree very similar to the D-loop tree with much stronger branch support. Combining data in this way can increase the support of a tree because different characters evolve at different rates and will support different parts of the tree.

The combined analysis presented here shows the Kloss's gibbon to be most closely related to the Javan silvery gibbon (*H. moloch*) and the agile gibbon (*H. agilis*). When combined, the signal from the D-loop data is not significantly different than that from the ND3–ND4, vocal, and morphological data, providing additional support for this phylogenetic hypothesis.

4.2. Monophyly of taxa and DNA barcoding

All recognized species with multiple samples were monophyletic in the D-loop analyses, with very high bootstrap support (values ranging from 78% to 100%). The genus *Hylobates* is generally supposed to have speciated over a short period of time, as evidenced by overall phenotypic similarity, comparatively low levels of genetic sequence divergence, and difficulty in resolving phylogenetic relationships. However, in this short time span, not only have species-specific markers emerged, but sequence divergence among species (7.1–17.2%) has increased to the point that, with the single exception of variation within *H. agilis*, it no longer overlaps with divergences found within species (0.2–5.7%, excluding two *H. agilis* distances). Agile gibbon populations display unusual diversity, and some authors have suggested raising *H. agilis albibarbis* to a full species (Groves, 2001; Hirai et al., 2004; Tanaka et al., 2004). Two pairwise comparisons in the present study revealed large intraspecific distances of 8.6% and 8.9% within this species. However, the putative *H. agilis albibarbis* specimen in the present study clusters within the other *H. agilis* specimens, supporting the maintenance of *albibarbis* as a subspecies of *agilis*. The six *Hylobates* species also hybridize easily: three hybrid zones exist where the ranges of different species meet (*H. lar* and *H. pileatus* in Thailand, *H. lar* and *H. agilis* in Malaysia, and *H. agilis* and *H. muelleri* in Borneo) (Marshall and Sugardjito, 1986). The data presented here suggest that, although hybridization is observed in wild populations, gene flow among species has historically been minimal.

The genus *Hylobates* is also monophyletic with 98–100% bootstrap support in all analyses, suggesting a long separation of the gibbon genera with no gene flow among them. This suggestion is supported by the different chromosome numbers of the four genera, which may act as a postzygotic reproductive isolating mechanism (King, 1993), as well as the lack of intergeneric hybrids observed. The genetic distances among the genera (17–26%), which just barely overlap with intrageneric distances (7.1–17.2%), further support their classification as full genera.

The ability of the HV-I region to separate species suggests that it may be useful as a "DNA barcode." The mitochondrial COI locus has been presented as such a barcode, as conspecifics show a consistent level of sequence variation that is much lower than that found among species (Hebert et al., 2003; Hebert, 2004). Critics have commented that closely related sister taxa have not been adequately sampled to show whether this locus can be used to differentiate species that may have diverged recently or very quickly (Moritz and Cicero, 2004). While the COI gene has not yet been sequenced for all gibbon species, the

HV-I region presented here clearly separates species into monophyletic groups. This locus could thus be useful for identifying zoo animals or even bushmeat specimens, and could prove to be a powerful tool for gibbon conservation.

4.3. A biogeographic scenario

In the analyses presented here, the basal taxon of the radiation is *H. pileatus*, followed by *H. lar*; the two species with the northernmost geographic distributions. The southernmost species (*H. agilis*, *H. klossii*, and *H. moloch*) are the most derived, suggesting that the ancestral stock followed a single north-to-south radiation (Fig. 3). During Pleistocene glacial maxima, populations were likely isolated in retracted forest patches and subsequently differentiated vicariantly; when the forest expanded, the populations expanded until they met barriers, such as the Sunda river systems (Marshall and Sugardjito, 1986). Later, rising sea levels contributed to the present species distribution.

Past scenarios have had to invoke multiple migration and speciation events to explain the phylogenetic patterns observed in past analyses (e.g., Chatterjee, 2001). The scenario inferred from the present study is simple and straightforward. At least one other group of forest-dependent southeast Asian taxa follow a similar pattern: in an analysis of murine rodents on the Sunda shelf, the mainland populations are the most basal taxa, while the southern island populations diverged following vicariance events caused by rising sea levels (Gorog et al., 2004). Speciation events in more ecologically flexible taxa, such as macaques (genus *Macaca*), appear to have a much more complex history that likely took place over a longer period of time (Abegg and Thierry, 2002; Tosi et al., 2003).
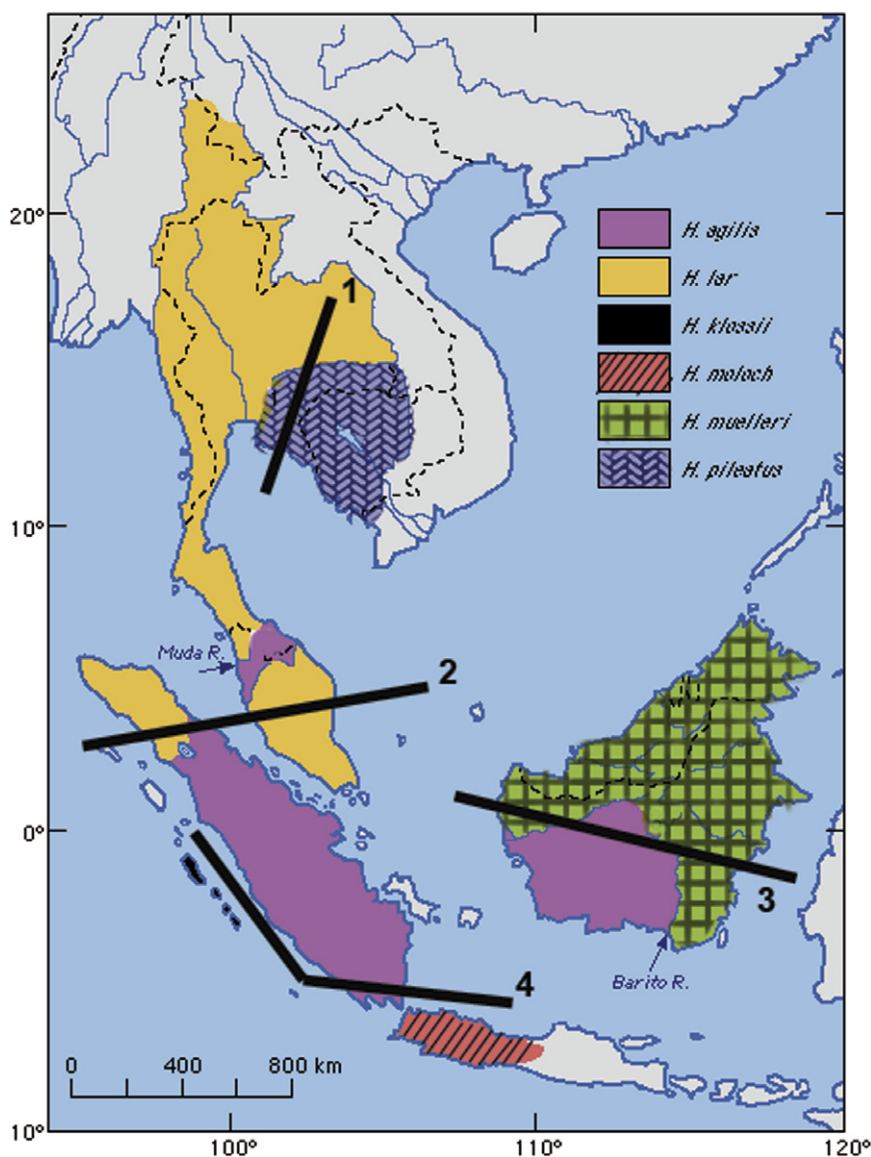

Fig. 3. Map showing inferred biogeographic scenario based on speciation patterns. Adaptation of a map by Thomas Geissmann (http://www.gibbons.de), with permission. Biogeographic breaks correspond with numbered internodes in Fig. 2.

5. Conclusions

Past phylogenetic analyses of *Hylobates* species have led to inconsistent and often poorly resolved phylogenetic trees. A major cause of this problem may be choice of locus for analysis: in a fast-evolving group of species, a fast-evolving gene is needed to detect a phylogenetic signal. The mitochondrial D-loop appears to be useful for sorting out relationships among the *Hylobates* species. The resulting phylogeny was strengthened by the inclusion of other data sets (molecular, behavioral, and morphological), none of which were incongruent with the D-loop data set based on the incongruence length difference test. The results suggest a speciation pattern that agrees intuitively with the region's geographic history: the northernmost species are basal to the radiation while the southernmost are the most derived.

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