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Molecular systematics of Indochinese primates

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Summary

The systematics of primates distributed in the Indochinese bioregion is still controversial. Based on molecular data, we established a solid platform for the classification of all the Indochinese primate taxa.

Accordingly, among strepsirrhines, two slow loris species (*N. pygmaeus* and *N. bengalensis*) occur in Indochina. The only cercopithecines distributed in Asia are members of the genus *Macaca*. Among them, five species occur in Indochina (*M. arctoides*, *M. assamensis*, *M. fascicularis*, *M. mulatta* and *M. leonina*). The distinct species status for all of them is confirmed and two subspecies are provisionally recognised for *M. fascicularis*.

The colobines of Indochina are divided into the three genera *Pygathrix*, *Rhinopithecus* and *Trachypithecus*. The douc langurs (*Pygathrix*) are represented by the three monotypic species *P. cinerea*, *P. nemaeus* and *P. nigripes*. Within the snub-nosed monkeys (genus *Rhinopithecus*), the distinct species status for the four recognised species (*R. avunculus*, *R. bieti*, *R. brelichi*, *R. roxellana*) is confirmed, but further investigations are necessary to elucidate the taxonomic position of the three described *R. roxellana* subspecies. In Indochina, the genus *Trachypithecus* is represented by three species groups [*T. obscurus*], [*T. cristatus*] and [*T. francoisi*]. Based on the molecular data, the only Indochinese representative of the [*T. obscurus*] group, *T. crepusculus*, is mitochondrially more closely related to the [*T. francoisi*] group. However, in the Y-chromosomal data set, the taxon forms a clade together with the other [*T. obscurus*] group members, indicating that the species is the result of ancestral hybridisation between the two species groups. The [*T. cristatus*] group in Indochina is represented by the two species *T. margarita* and *T. germaini*, most likely separated from each other by the Mekong River. Within the [*T. francoisi*] group, the four taxa *T. delacouri*, *T. francoisi*, *T. poliocephalus* and *T. laotum* should be recognised as distinct species, with *leucocephalus* and *hatinhensis* being subspecies of *T. poliocephalus* and *T. laotum*, respectively.

For the crested gibbons (genus *Nomascus*), we propose that *N. hainanus*, *N. nasutus*, *N. concolor*, *N. leucogenys* and *N. gabriellae* be recognized as distinct species. The subspecies of *N. concolor* are downgraded as synonyms of the nominate form, and *N. siki* is provisionally classified as subspecies of *N. leucogenys*, although further research is needed on this question.

Hệ thống phân loại học phân tử các loài linh trưởng Đông dương

Tóm tắt

Hệ thống phân loại học các loài linh trưởng phân bố ở Đông dương vẫn còn là vấn đề đang được tranh luận. Trên cơ sở nghiên cứu cấu trúc phân tử, chúng tôi đã thiết lập một nền tảng vững chắc để phân loại toàn bộ các loài linh trưởng ở Đông dương. Đầu tiên là phân bộ tiền linh trưởng, ở Đông dương có hai loài culi gồm (*N. pygmaeus* và *N. bengalensis*). Tiếp đó là giống Macaca thuộc họ

Cercopithecines phân bố ở Châu Á. Giống này có năm loài được tìm thấy ở Đông dương (*M. arctoides*, *M. assamensis*, *M. fascicularis*, *M. mulatta* và *M. leonina*). Cả năm loài này được xác định là các loài riêng biệt. Riêng loài *M. fascicularis* có hai phân loài được tạm thời xác nhận.

Khỉ ăn lá ở Đông dương có ba giống *Pygathrix*, *Rhinopithecus* và *Trachypithecus*. Giống voọc chà vá (*Pygathrix*) đại diện bởi ba loài riêng biệt *P. cinerea*, *P. nemaeus* và *P. nigripes*. Giống voọc mũi hếch (*Rhinopithecus*), có bốn loài riêng biệt gồm (*R. avunculus*, *R. bieti*, *R. brelichi*, và *R. roxellana*). Riêng với loài *R. roxellana* cần có thêm những nghiên cứu để giải thích vị trí phân loại của ba phân loài thuộc loài này. Giống *Trachypithecus* được biết đến với ba nhóm loài gồm [*T. obscurus*], [*T. cristatus*] và [*T. francoisi*]. Nghiên cứu cho thấy, nhóm loài [*T. obscurus*] chỉ có một đại diện là loài *T. crepusculus* có yếu tố di truyền ty thể quan hệ gần với nhóm loài [*T. francoisi*]. Tuy nhiên, số liệu về yếu tố di truyền trong nhân của nhiễm sắc thể Y lại chỉ ra rằng loài này là con lai của hai nhóm loài trên. Đối với nhóm loài [*T. cristatus*] có hai loài *T. margarita* và *T. germaini* phân bố biệt lập bên hai bờ sông Mekong. Nhóm loài [*T. francoisi*], có bốn loài phân biệt gồm *T. delacouri*, *T. francoisi*, *T. poliocephalus*, và *T. laotum*. Loài *poliocephalus* có phân loài là *leucocephalus* và loài *T. laotum* có phân loài là *T. hatinhensis*.

Đối với vượn (giống *Nomascus*), chúng tôi đề xuất việc thừa nhận các loài *N. hainanus*, *N. nasutus*, *N. concolor*, *N. leucogenys* và *N. gabriellae*. Phân loài của *N. concolor* không nên công nhận, và *N. siki* tạm thời xác định là phân loài của *N. leucogenys*. Tuy nhiên những nghiên cứu tiếp theo trên hai đối tượng này là cần thiết.

Introduction

The Indochinese bioregion harbours a large number of endemic animal and plant species and is well known for its different primate species including slow lorises, macaques, doucs, snub-nosed monkeys, leaf monkeys and gibbons. However, the Indochina ecosystem and its inhabitants are endangered because of the negative impacts of poaching, logging, and habitat destruction. Especially for primates, hunting for traditional medicine and food as well as habitat loss significantly lowered the populations over the last decades, so that today five species of the region are included in the 25 most endangered primate species of the world (Mittermeier *et al*., 2006). One of the major issues facing those working on the conservation of Indochinese primates is the taxonomy of these creatures which continues to be debated. Several classification schemes based on morphology, fur colouration or genetics are at hand (e.g. Brandon-Jones *et al*., 2004; Groves, 2001; Roos, 2004), but currently no consensus exists. A clear definition of how many taxa exist and knowledge about their exact distribution however is urgently required to establish efficient conservation action plans.

To elucidate the phylogenetic relationships among Indochinese primates and their systematic classification, samples of a large number of all Indochinese primates and their relatives in other countries were collected. From these samples, we sequenced a fragment or the complete coding region of the mitochondrial cytochrome b gene for a number of lorises (*Nycticebus*), colobines (*Trachypithecus*, *Rhinopithecus*, *Pygathrix*) and gibbons (*Nomascus*). For macaques (*Macaca*), a region spanning the mitochondrial 12S-16S rRNA was selected. From some representatives, also nuclear DNA, e.g. Y-chromosomal loci, were analysed. Based on pair-wise genetic differences between and among taxa, we established a solid platform for the systematic classification of the primates distributed in the Indochinese bioregion. Moreover, we reconstructed phylogenetic trees, allowing detailed insights into the evolutionary and phylogeographic history of these species.

Materials and Methods

Hair, blood, fresh, dried or smoked tissue, museum skins and fecal samples were collected in primate keeping facilities, museums or during field surveys. Samples were stored until further processing in plastic or paper bags (hair, dried or smoked skin), or in 80% ethanol (all other sample types). DNA from the samples was extracted using methods as outlined in Sambrook *et al*. (1989), Walsh *et al*. (1991), or by applying the Qiagen DNA Mini Kit or Qiagen DNA Stool Mini Kit. Depending on genera, a fragment or the complete mitochondrial cytochrome b gene or a region spanning the 12S-16S rRNA was amplified via PCR using methods and oligonucleotide primers as described earlier (Geissmann *et al*., 2004; Nadler *et al*., 2005; Roos 2003, 2004; Roos & Nadler,

2001; Roos *et al.*, 2001, 2003, 2004; Tosi *et al.*, 2002; Ziegler *et al.*, 2007). From some representatives, also nuclear DNA, e.g. Y-chromosomal or autosomal loci, were analysed. The resulting PCR products were separated on 1% agarose gels, subsequently excised and the DNA extracted using the Qiagen Gel Extraction Kit. Direct sequencing reactions were performed with the BigDye Terminator Cycle Sequencing Kit following the manufacturer's recommendations. Sequence determination was performed on an automated ABI377 gel or an ABI3100-Avant capillary sequencer. Further details about laboratory procedures are available upon request.

To expand the different data sets (mainly those for macaques), further sequences deposited at GenBank were included. Sequences were checked for their potential to be correctly transcribed to exclude pseudogene contaminations of the data sets. Sequence alignments were carried out with ClustalW (Thompson *et al.*, 1994) and subsequently checked by eye. In the 12S-16S rDNA alignment, several indels were detected, which were removed with the Gblocks software (Castresana, 2000). Pairwise genetic differences were calculated with PAUP 4.0b10 (Swofford, 2002). Phylogenetic tree reconstructions were carried out using the maximum-parsimony (MP), and neighbor-joining algorithms (NJ) as implemented in PAUP. Maximum-likelihood (ML) analyses were performed with TREEPUZZLE 5.0 (Strimmer & von Haeseler, 1996). For MP analyses, all characters were treated as unordered and equally weighted throughout. A heuristic search was performed with the maximum number of trees set to 100. For NJ and ML reconstructions, different models of sequence evolution as well as the best-fitting model, estimated by MODELTEST 3.06 (Posada & Crandall, 1998), were applied. Support of internal branches was either determined by bootstrap analyses (MP and NJ) on the basis of 1,000 replications or indicated by the ML quartet puzzling support values (10,000 puzzling steps).

Results and Discussion

Lorises (*Nycticebus*)

The complete (1,140 bp) mitochondrial cytochrome b gene was sequenced for 40 slow loris individuals as well as from one slender loris (*Loris tardigradus*), which was used as outgroup for phylogenetic tree reconstructions. Among the 40 slow lorises studied, 27 different haplotypes were detected. Based on pairwise difference analyses, the genus can be divided into four major groups (*N. pygmaeus*, *N. bengalensis*, *N. coucang* and *N. menagensis*), which differ in 4.3-12.8% from each other (Table 1). Largest differences were detected between *N. pygmaeus* and the remaining groups (11.1-12.8%), whereas smallest differences between major groups can be found between *N. menagensis* and *N. bengalensis* (4.3-4.9%). Variations within the four major groups are comparatively low and do not exceed 2.0%. The pairwise differences between the type specimen of *N. intermedius* and *N. pygmaeus* individuals fall within this range (data not shown).

Table 1. Pairwise differences (in %) within and between *Nycticebus* taxa

	1	2	3	4
(1) *N. pygmaeus*	0.2-1.7			
(2) *N. bengalensis*	11.4-12.2	0.1-2.0		
(3) *N. coucan*g	12.2-12.8	7.2-8.0	0.3-0.4	
(4) *N. menagensis*	11.1-11.5	4.3-4.9	7.9-8.5	0.4-0.9

The phylogenetic relationships obtained from the data set are completely resolved and the branching patterns are significantly supported (Fig. 1), so that the depicted relationships most likely represent the real evolutionary relationships of the genus *Nycticebus*, although samples of *N. javanicus* are necessary to definitively explain the complete evolutionary history of the genus. According to the data, *N. pygmaeus* was the first to split off, followed by *N. coucang*, whereas *N. menagensis* and *N. bengalensis* were the last to diverge. The type specimen of *N. intermedius* forms a clade together with the *N. pygmaeus* representatives.

Based on the data, it is justified to recognise at least four distinct species (*N. pygmaeus*, *N. coucang*,

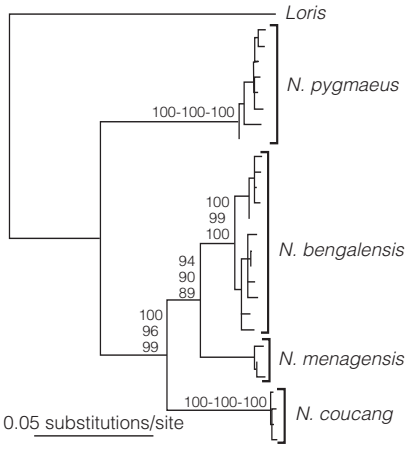

Fig. 1. Phylogenetic relationships among slow loris (*Nycticebus*) haplotypes as obtained from complete mitochondrial cytochrome b sequence data. Branch lengths are based on the NJ tree with numbers on branches indicating internal support (first: ML, second: NJ, third: MP).

N. menagensis and *N. bengalensis*) within the genus with two of them, *N. pygmaeus* and *N. bengalensis*, distributed in the Indochinese bioregion. *N. intermedius* should be regarded as synonym of *N. pygmaeus*. This opinion is supported by recent observations of seasonal weight and colour changes in pygmy lorises (Streicher, 2004).

Macaques (*Macaca*)

A 1,506 bp long fragment of the mitochondrial genome spanning the 3' end of the 12S rDNA, tRNA-Val and the 5' end of the 16S rDNA was analysed for one representative of all currently known macaque species with the exception of the recently described *M. munzala*. *Papio hamadryas* was used as the outgroup. Indels in the data set were removed, so that the final alignment comprises 1,467 bp. Variation between macaque species ranges from 0.6-7.6% (Table 2). Largest differences with at least 3.7% were detected between the four species groups [*M. sylvanus*], [*M. silenus*], [*M. sinica*] and [*M. fascicularis*] (including *M. arctoides*), which are comparable with those observed within the [*M. silenus*] group between the Sulawesi macaques and the remaining members of the species group (3.7-5.9%). Within the [*M. silenus*] group (excluding the Sulawesi macaques), five major lineages were detected, which differ in 1.6-3.1%, with the lower distance observed between *M. leonina* and *M. silenus*. Among the four analysed members of the [*M. sinica*] group, variation of 0.6-4.3% was detected. Within the [*M. fascicularis*] group, the five species vary in 1.5-3.5%, with lowest differences observed between *M. mulatta*, *M. cyclopis* and *M. fuscata*.

Table 2. Pairwise differences (in %) within and between *Macaca* taxa

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)
(1) M. sylvanus	-															
(2) Sulawesi macaques	5.0-6.1	0.9-3.3														
(3) M. silenus	6.0	4.6-5.6	-													
(4) M. leonina	5.9	4.2-5.2	1.6	-												
(5) M. nemestrina	6.4	4.8-5.8	2.5	2.2	-											
(6) M. siberu	6.1	4.9-5.9	2.5	2.5	2.5	-										
(7) M. pagensis	4.8	3.7-4.6	2.8	2.3	3.1	3.0	-									
(8) M. radiata	5.8	5.4-6.3	6.1	5.8	6.1	5.9	5.5	-								
(9) M. sinica	6.7	6.2-7.6	7.0	6.5	6.7	6.9	6.1	4.3	-							
(10) M. thibetana	7.0	6.0-7.2	6.5	6.4	6.5	6.7	5.9	3.8	4.1	-						
(11) M. assamensis	6.9	6.1-7.3	6.5	6.3	6.5	6.6	6.1	3.9	4.2	0.6	-					
(12) M. mulatta	5.5	5.3-6.5	6.0	5.7	6.1	5.8	5.3	4.2	4.6	5.0	5.1	-				
(13) M. fascicularis	5.2	4.8-6.1	5.6	5.0	5.6	5.4	4.5	4.6	5.0	5.2	5.3	3.5	-			
(14) M. fuscata	6.0	5.5-6.7	6.1	5.7	6.3	6.0	5.5	4.3	4.8	5.5	5.5	2.0	3.1	-		
(15) M. cyclopis	5.7	5.1-6.5	5.7	5.1	5.7	5.2	4.9	3.7	4.7	5.0	5.1	1.8	2.9	1.5	-	
(16) M. arctoides	5.5	5.7-6.9	5.9	5.7	5.9	5.9	5.2	3.7	4.7	5.2	5.4	3.3	3.4	3.3	2.7	-

The evolutionary relationships obtained from the different phylogenetic tree reconstruction methods are completely resolved and for most branching patterns significantly supported (supports not shown, Fig. 2). Based on the tree topology, the distinction of the macaque genus into the four species groups can be confirmed, with the sole African species *M. sylvanus* representing a sister clade to the Asian representatives. Among Asian macaques, the [*M. silenus*] group split off first, while the [*M. sinica*] and [*M. fascicularis*] groups diverged later from each other. Within the [*M. silenus*] group, the Sulawesi macaques form a monophyletic sister clade to the remaining species. Among the latter, *M. pagensis* split of first, before the other four species diverged during a radiation-like splitting event. Although not significantly supported, the closest relative of *M. leonina* is *M. silenus* and not the other pig-tailed

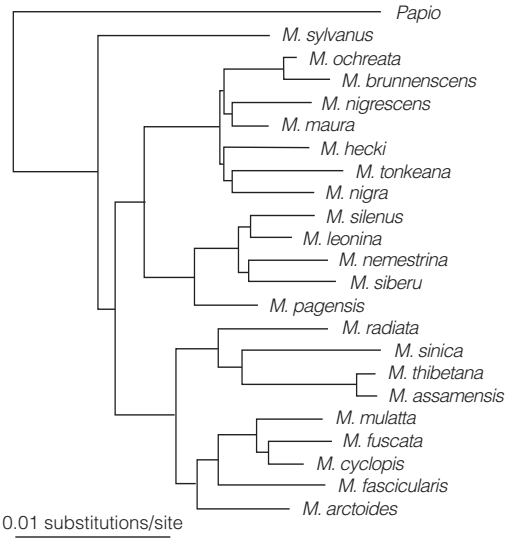

Fig. 2. Phylogenetic relationships among macaque (*Macaca*) species as obtained from the 12S-16S rRNA sequence data. Branch lengths are based on the NJ tree.

macaques. Within the [*M. sinica*] group, *M. radiata* represents the first splitting event, followed by *M. sinica*. In contrast, *M. thibetana* and *M. assamensis* diverged relative recently. The [*M. fascicularis*] group comprises five species. Among them, *M. arctoides* was the first species to be separated, before *M. fascicularis* diverged. This result however represents only the mitochondrial gene tree. In contrast, in Y chromosomal based trees, *M. arctoides* forms a sister clade to *M. assamensis*/*M. thibetana*, suggesting a hybrid origin of *M. arctoides* from progenitors of *M. fascicularis* and *M. assamensis*/ *M. thibetana* (Tosi *et al.*, 2000). The split between *M. mulatta*, *M. cyclopis* and *M. fuscata* occurred relative recently, with ongoing hybridisation between *M. fascicularis* and *M. mulatta* in overlapping regions (Tosi *et al.*, 2002). The relationships based on mitochondrial data confirm earlier results and are in agreement with other classification schemes (e.g. Deinard & Smith, 2001; Delson, 1980; Fooden, 1975; Groves, 2001; Hayasaka *et al.*, 1996; Morales & Melnick, 1998; Roos *et al.*, 2003; Tosi *et al.*, 2003; Ziegler *et al.*, 2007).

Based on our data, we recognise five macaque species for Indochina (*M. arctoides*, *M. assamensis*, *M. fascicularis*, *M. mulatta* and *M. leonina*). However, several subspecies are described and variation among populations is great, so that further studies are required to verify the taxonomy within species. E.g. for rhesus macaques, it is well know that individuals from India and China differ significantly in their response to SIV infections, although all these populations are recognised as *M. m. mulatta*. For *M. fascicularis*, two subspecies are described for the Indochinese bioregion, with one occurring on the mainland (*M. f. fascicularis*) and one on Con Dao Island (*M. f. condorensis*). Our preliminary data, indicate distinct subspecies status, however further studies are necessary.

Leaf monkeys (*Trachypithecus*)

A 573 bp long fragment of the cytochrome b gene was sequenced from 123 individuals representing all recognised species of the genus *Trachypithecus* and one *Presbytis fluviatilis*, which was used as the outgroup. *T. geei*, *T. pileatus*, *T. johnii* and *T. vetulus* were excluded from the data set, since the four species are mitochondrially closer related to *Semnopithecus* than they are to *Trachypithecus* (Geissmann *et al.*, 2004), with *T. vetulus* and *T. johnii* being members of

Table 3. Pairwise differences (in %) within and between *Trachypithecus* taxa

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)
(1) T. auratus	0.3-1.5													
(2) T. cristatus	2.4-4.3	0.5-1.7												
(3) T. germaini	3.3-4.7	3.1-4.2	0.2-1.0											
(4) T. margarita	2.8-4.0	2.4-3.5	2.6-3.1	0.2										
(5) T. p. poliocephalus	8.5-9.4	8.7-9.4	8.9-9.5	8.0-8.5	0.2-0.3									
(6) T. p. leucocephalus	9.2-10.1	9.4-10.4	9.7-10.4	9.2-9.7	1.8-2.1	0.2-0.5								
(7) T. francoisi	8.7-9.9	8.7-9.9	9.0-9.7	8.9-9.4	2.4-2.6	2.3-2.6	0.2-0.7							
(8) T. delacouri	8.3-10.1	8.0-9.4	9.0-9.9	8.9-9.5	4.3-5.2	4.2-4.9	3.3-4.3	0.2-1.0						
(9) T. l. laotum	8.2-9.0	8.2-8.9	9.2-9.5	9.0-9.2	5.2-5.6	5.7-5.9	5.0-5.4	3.1-3.8	-					
(10) T. l. hatinhensis	8.5-10.4	8.7-10.1	9.2-10.1	9.5-10.4	5.4-6.4	5.9-6.6	5.2-6.2	3.3-4.7	1.2-1.9	0.2-1.2				
(11) T. crepusculus	8.2-9.9	8.0-9.9	8.7-10.1	8.2-9.4	7.6-8.7	7.3-8.5	7.1-8.5	6.4-8.2	6.2-7.1	7.1-8.7	0.3-2.1			
(12) T. obscurus	6.6-8.3	6.6-8.0	7.5-8.5	6.2-7.1	8.9-9.7	9.7-11.1	9.0-10.4	8.3-9.4	8.2-8.9	8.3-9.4	7.6-9.9	0.3-1.7		
(13) T. phayrei	7.5-8.7	6.9-8.2	7.6-8.0	6.8-7.1	8.7-9.0	9.7-10.2	8.9-9.5	8.3-9.4	8.9-9.0	9.0-9.9	7.3-9.2	3.8-4.5	0.5	
(14) T. barbei	6.9-8.0	6.6-7.1	7.1-7.5	6.6-6.8	9.9-10.4	9.9-10.9	9.9-10.6	8.9-9.0	8.3	8.7-9.4	8.9-10.2	4.3-5.2	4.3-4.5	-

Semnopithecus, and *T. geei* and *T. pileatus* being the result of ancestral hybridization between the two genera (data not shown). Among the 123 analysed individuals, 66 haplotypes were detected. Variation within taxa range from 0.2-2.1%, whereas between taxa, the maximum variation detected was 11.1% (Table 3). Accordingly, the analysed taxa can be divided into four major groups [*T. cristatus*] group, [*T. obscurus*] group, [*T. francoisi*] group and *T. crepusculus*, which differ by 6.6–11.1%. Within the *T. cristatus* group, *T. auratus*, *T. cristatus*, *T. germaini* and *T. margarita* are different by 2.4–4.7%. The members of the [*T. obscurus*] group, *T. obscurus*, *T. phayrei* and *T. barbei* differ by 3.8–5.2%. Within the [*T. francoisi*] group, the highest differences of 5.0-6.6% were detected between the northern members (*T. francoisi*, *T. p. policephalus* and *T. p. leucocephalus*) and the southern taxa (*T. l. laotum* and *T. l. hatinhensis*). *T. delacouri* differs from all other members of the group by 3.1–5.2%. Within the northern clade, *T. francoisi* and *T. p. poliocephalus*/*T. p. leucocephalus* are different by 2.3-2.6%. The latter two taxa show pairwise differences of 1.8-2.1%, which are only slightly higher than those observed between *T. l. laotum* and *T. l. hantinhensis* (1.2-1.9%). The two analysed black langurs, representing the recently described *T. auratus ebenus*, are identical with one *T. l. hatinhensis* haplotype or differ only in 0.2% from other haplotypes. *T. crepusculus* differs from all other species groups in 6.2-10.2%.

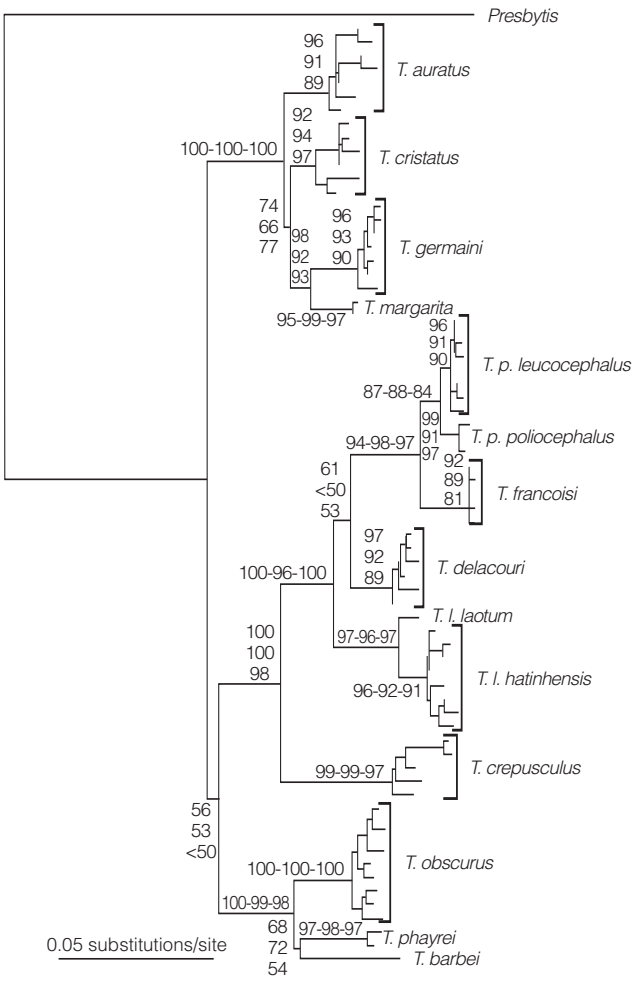

Fig. 3. Phylogenetic relationships among leaf monkey (*Trachypithecus*) haplotypes as obtaind from 573bp long sequences of the mitochondrial cytochrome b gene. Branch lengths are drawn according to the NJ tree with numbers on branches indicating internal support (first: ML, second: NJ, third: MP).

The evolutionary relationships as obtained from the phylogenetic tree reconstructions are completely resolved and significantly supported for most branching patterns (Fig. 3). Accordingly, the genus can be divided into the [*T. cristatus*] group, the [*T. obscurus*] group and the *T. francoisi* group, which significantly clusters together with *T. crepusculus*. The relationships among these three major groups however are not well resolved. Within the [*T. cristatus*] group, four reciprocal monophyletic clades were detected, which represent *T. auratus* (Java), *T. cristatus* (Sumatra, Borneo, Malaysian peninsular) and the mainland populations, which can be divided into two subgroups. These are most likely separated by the Mekong River and were recently classified as distinct species (Nadler *et al*., 2005). Although a monophyletic origin of the two mainland species *T. germaini* and *T. margarita* is confirmed by the data, the relationship among those and *T. auratus* and *T. cristatus* is not settled yet. Although the relationships are not well resolved, the [*T. obscurus*] group can be divided into three major groups, representing the species *T. obscurus*, *T. phayrei* and *T. barbei*. The last major group includes *T. crepusculus* and the members of the “limestone langurs”. This significantly supported relationship is based on a maternal inherited marker, however, molecular data based on paternal

inherited marker systems (data not shown) show that *T. crepusculus* is more closely related to the *T. obscurus* group. These findings indicate that *T. crepusculus* is the result of introgression or ancestral hybridization between the two species groups. Within the *T. francoisi* group, a northern, a central and a southern group can be distinguished, although the relationships among them are not well resolved and contradict with earlier results (Roos, 2004). The northern group is divided into three monophyletic clades, which represent *T. francoisi*, *T. p. poliocephalus* and *T. p. leucocephalus*, whereas the latter two form a sister clade to *T. francoisi*. The central group consists only of *T. delacouri*, and the southern clade comprises *T. l. laotum*, *T. l. hatinhensis* and *T. a. ebenus*, whereas the two analysed *T. a. ebenus* specimens cluster within the *T. l. hatinhensis* clade.

Based on the pairwise differences and phylogenetic relationships, we propose to recognise *T. poliocephalus, T. francoisi, T. delacouri, T. laotum, T. crepusculus, T. barbei, T. phayrei, T. obscurus, T. germaini, T. margarita, T. cristatus* and *T. auratus* as distinct species. Because of the low differences between *T. leucocephalus* and *T. poliocephalus* and between *T. laotum* and *T. hatinhensis*, *T. leucocephalus* and *T. hatinhensis* should be recognised only as subspecies of *T. poliocephalus* and *T. laotum*, respectively. Since the studied black langurs (*T. a. ebenus*) are not separated from *T. l. hatinhensis*, the taxon is here classified as synonym of *T. l. hatinhensis*. Moreover, there is information that both forms occur sympatrically (Ruggieri & Timmins, 1995), which led us to the conclusion that the black langur may be a melanistic morphe of *T. l. hatinhensis*. However, recent field studies indicate that both forms may occur allopatrically (Le Khac Quyet, 2004), doubting the synonymy of the black langur with *T. l. hatinhensis*. Although provisionally downgraded as synonym of the Hatinh langur, further research is needed to definitively solve the taxonomic position of the black langur.

Douc langurs (*Pygathrix*)

To analyse pairwise differences and phylogenetic relationships among douc langurs, a 573 bp long fragment of the mitochondrial cytochrome b gene was sequenced for 83 individuals, as well as one *Rhinopithecus avunculus* used as the outgroup. Among the 83 douc langurs sequences, 42 different haplotypes were detected. Based on pairwise differences (Table 4), douc langurs can be classified into three major groups, which represent the three recognised taxa *P. nemaeus*, *P. nigripes* and *P. cinerea*. The lowest differences among them were detected between *P. nemaeus* and *P. cinerea* (2.8–3.4%) and the largest between the latter two and *P. nigripes* (7.6–8.9%). Within groups, variation is comparatively low, ranging from 0.2-1.6%. One individual sampled at the Lang Bian Peak (Lam Dong Province, Vietnam), the type locality of *P. moi* shows 0.4–1.6% difference to *P. nigripes*, which is typical for intra-species variety.

Phylogenetic trees derived from the dataset reveal three statistically supported clades, which represent the three different taxa (Fig. 4). The relationships among them are also significantly supported. Accordingly, *P. nigripes* represents the first splitting event whereas *P. cinerea* and *P. nemaeus* diverged later from each other. *P. moi* clusters within the *P. nigripes* clade.

Based on these results, we conclude that *P. nemaeus*, *P.cinerea* and *P. nigripes* should be

Table 4. Pairwise differences (in %) within and between *Pygathrix* taxa

	1	2	3
(1) P. nemaeus	0.2-1.4		
(2) P. cinerea	2.8-3.4	0.2-0.9	
(3) P. nigripes	7.6-8.9	7.7-8.6	0.2-1.6

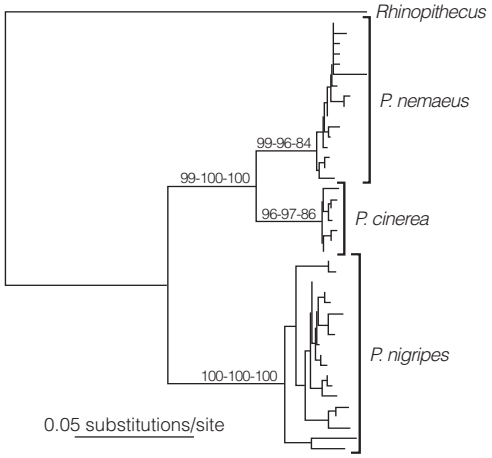

Fig. 4. Phylogenetic relationships among douc langur (*Pygathrix*) haplotypes as obtained from 573bp long sequences of the mitochondrial cytochrome b gene. Branch lengths are based on the NJ tree with numbers on branches indicating internal support (first: ML, second: NJ, third: MP).

recognised as distinct species. The taxon *P. moi* is not phylogenetically separated from *P. nigripes* and hence should be regarded only as synonym of *P. nigripes*.

Snub-nosed monkeys (*Rhinopithecus*)

573 bp long sequences of the mitochondrial cytochrome b gene were generated from 21 snub-nosed monkey individuals representing all four recognised species and one red-shanked douc langur (*Pygathrix nemaeus*) used as the outgroup. Among them, only eleven haplotypes were detected. Pairwise differences show that the analysed individuals can be divided into the four traditionally recognised species, which differ in 5.7–7.5% from each other (Table 5). Within species only low differences of a maximum of 0.9% were observed, with the exception of *R. bieti*, were two major subgroups were detected, which differ in 3.4-3.6%.

Phylogenetic tree reconstructions (Fig. 5) confirm the monophyly of each of the four taxa and indicate a basal position of *R. bieti*. After *R. bieti* split off, *R. brelichi* diverged as next, while the split between *R. roxellana* and *R. avunculus* represents the most recent divergence. Although the splitting events among the four species are resolved in the tree, the statistical support is not significant, so that the depicted scenario may not reflect the real relationships and are even in contrast to earlier results (Li Ming *et al.*, 2004; Roos, 2004).

Table 5. Pairwise differences (in %) within and between *Rhinopithecus* taxa

	1	2	3	4
(1) R. roxellana	0.9			
(2) R. brelichi	5.7-6.2	-		
(3) R. bieti	6.2-7.1	5.9-6.2	0.3-3.6	
(4) R. avunculus	6.4-6.9	6.2-6.6	6.8-7.5	0.2-0.3

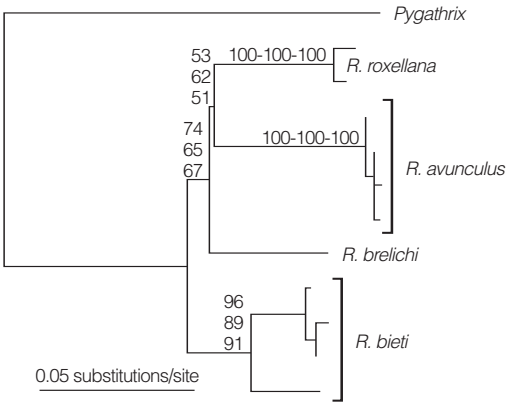

Fig. 5. Phylogenetic relationships among snub-nosed monkey (*Rhinopithecus*) haplotypes as obtained from 573bp long sequences of the mitochondrial cytochrome b gene. Branch lengths are based on the NJ tree with numbers on branches indicating internal support (first: ML, second: NJ, third: MP).

Based on these findings, we propose to recognise all four taxa as distinct species. The proposed subgenus *Presbyticus* for *R. avunculus* (Jablonski & Peng Yanzhang, 1993) does not resemble the evolutionary relationships among snub-nosed monkey and hence, should not be used. Further studies focusing on the phylogenetic relationships among the four snub-nosed monkey species are needed. The classification of the three *R. roxellana* subspecies (*R. r. roxellana*, *R. r. hubeiensis* and *R. r. qinlingensis*) needs also further molecular investigations.

Crested gibbons (*Nomascus*)

The complete mitochondrial cytochrome b gene was sequenced for 64 crested gibbons as well as from one individual of *Hylobates* lar used as the outgroup. Among the studied crested gibbons, 44 different haplotypes were detected. Pairwise differences (Table 6) among them range from 0.1-8.2%, with the lowest differences observed within taxa (0.1-1.1%). Between taxa, differences range from 1.2-8.2%. Largest differences within the genus ranging from 6.7-8.2% were detected between *N. nasutus*/*N. hainanus* and the remaining taxa, as well as between *N. nasutus* and *N. hainanus* (6.8%). *N. concolor* differs from the southern taxa in 4.5-6.2%, whereas *N. leucogenys* and *N. siki* vary from *N. gabriellae* only in 3.0-4.6%. Lowest differences between taxa

Table 6. Pairwise differences (in %) within and between *Nomascus* taxa

	1	2	3	4	5	6
(1) N. nasutus	0.2-0.5					
(2) N. hainanus	6.8	-				
(3) N. concolor	7.2-8.2	6.8-7.7	0.2-1.1			
(4) N. l. leucogenys	6.9-8.0	7.4-8.2	4.5-6.2	0.1-1.1		
(5) N. l. siki	6.7-7.4	7.4-7.7	4.6-5.8	1.2-1.8	0.2-0.8	
(6) N. gabriellae	7.1-7.8	7.4-7.9	4.8-6.0	3.3-4.6	3.0-3.9	0.1-1.1

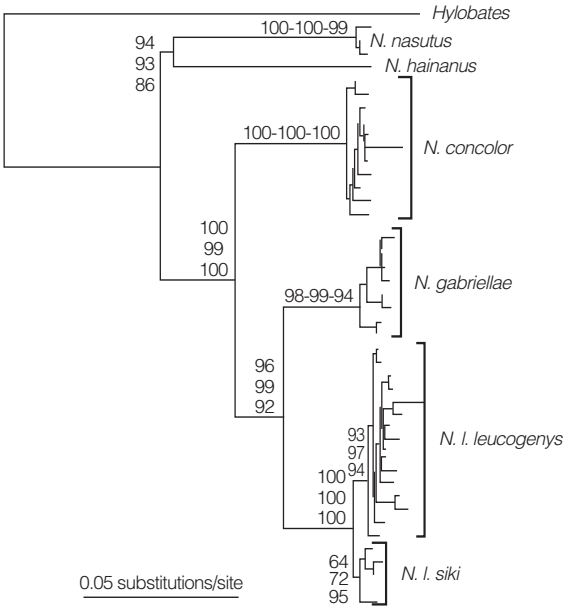

Fig. 6. Phylogenetic relationships among crested gibbon (*Nomascus*) haplotypes as obtained from complete mitochondrial cytochrome b sequence data. Branch lengths are based on the NJ tree with numbers on branches indicating internal support (first: ML, second: NJ, third: MP).

were observed between the northern and southern white-cheeked gibbon (1.2-1.8%), which is only slightly higher than intra-taxon variability.

The phylogenetic relationships among the different crested gibbon taxa are completely resolved and significantly supported (Fig. 6). With the exception of the *siki* clade, the monophyly of all the taxa is highly supported. Based on the tree topology, the two northernmost taxa *nasutus* and *hainanus* form a sister clade to the remaining taxa. Later, *N. concolor* split off, while *leucogenys* and *gabriellae* diverged later from each other. All *siki* representatives form a monophyletic sister clade to *leucogenys*. Within the *N. concolor* clade no subdivision was detected, and moreover the representatives of the different subspecies are not monophyletic. In principal, these results confirm earlier results (Roos, 2004; Takacs *et al.*, 2005), although those have not included the Hainan gibbon. Roos (2004) showed that *siki* representatives form two paraphyletic clades, with one related to *leucogenys* and the other to *gabriellae*. However, in contrast to this earlier study, in the recent analyses only karyotyped specimens were included, indicating that in the earlier study, incorrectly identified specimens may have been used.

Based on pairwise differences and phylogenetic relationships, *N. nasutus*, *N. hainanus*, *N. concolor*, *N. leucogenys* and *N. gabriellae* should be recognised as distinct species. The southern white-cheeked crested gibbon is closely related to *N. leucogenys* and hence should be classified as subspecies *N. l. siki*. The occurrence of possibly further taxa within the *gabriellae-leucogenys* complex as stated recently by Konrad & Geissmann (2006) has to be evaluated by further field and laboratory work. The different subspecies of *N. concolor* form no distinct clades in the tree and the pairwise differences among them fall in the range typical for within-taxon variability. Hence, *N. concolor* is regarded as monotypic here.

Conclusions

In the following, a summary on the genetically based systematic classification and distribution of Indochinese primates is given. Taxa with uncertain classification or which are recognised only as synonyms are mentioned in the text as well.

Pygmy loris *Nycticebus pygmaeus*

Distinct species status is supported. Distributed east of the Mekong River in Vietnam, Cambodia, Laos and in southernmost China. *N. intermedius* is recognised as synonym.

Bengal slow loris *Nycticebus bengalensis*

Genetic data support separation from all other slow loris forms on species level; distributed from north-eastern India into Indochina, and south to the northern parts of peninsular Thailand.

Long-tailed macaque *Macaca fascicularis fascicularis*

M. fascicularis is genetically distinct from all other macaque species. Hybridisation or introgression with/from *M. mulatta* however is common, so that the morphological based classification is sometimes contradicting with genetic data. Hence, further research is needed to definitively resolve the systematics of the different *M. fascicularis* subspecies. Distributed over wide ranges of Sunda land (Java, Borneo, Sumatra), north to Thailand, Cambodia and southern Vietnam.

Con Dao long-tailed macaque *Macaca fascicularis condorensis*

See also *M. f. fascicularis*. Based on genetic data, *M. f. condorensis* is distinct from *M. f. fascicularis*, hence a provisional subspecies status is supported. Further studies however are necessary to definitively settle the taxonomic position. Occurs only on some islands of the Con Dao Archipelago, Vietnam.

Rhesus macaque *Macaca mulatta mulatta*

Distinct species status proposed. Hybridisation or introgression with/from *M. fascicularis* however is common, so that the morphological based classification is sometimes contradicting with genetic data. In the response to SIV/HIV, rhesus macaques from India and China differ from each other. Hence, further research is needed to definitively resolve the systematics of the different *M. mulatta* subspecies. Distributed from India to China and Vietnam.

Assamese macaque *Macaca assamensis assamensis*

Distinct species status proposed. Distributed from Assam (India) throughout mainland Southeast Asia.

Stump-tailed macaque *Macaca arctoides*

Genetic data support distinct species status. This species is the result of ancient hybridisation between progenitors of *M. fascicularis* and *M. assamensis-M. thibetana*. Distributed from India throughout mainland Southeast Asia.

Northern pig-tailed macaque *Macaca leonina*

Distinct species status supported. Distributed from India through Bangladesh, Myanmar to Thailand and Indochina.

Indochinese grey langur *Trachypithecus crepusculus*

Genetic data support distinct species status. The species is the result of hybridisation or introgression of progenitors of the two species groups [*T. francoisi*] and [*T. obscurus*]. Distributed in northern Vietnam, southernmost China, Laos and northern Thailand.

Annamese silvered langur *Trachypithecus margarita*

Distinct species status is proposed. Distributed in Vietnam, Cambodia and southernmost Laos, with most likely the Mekong River as western barrier. The taxonomic status of *T. germaini caudalis* has to be evaluated by further studies.

Indochinese silvered langur *Trachypithecus germaini*

Genetic data support distinct species status. Distributed in southernmost Vietnam, Cambodia, Thailand and eastern Myanmar, with most likely the Mekong River as eastern barrier. The taxonomic status of *T. germaini caudalis* has to be evaluated by further studies.

Francois' langur *Trachypithecus francoisi*

Distinct species status supported. Distributed in northernmost Vietnam and southernmost China. More data are necessary to determine if the different populations form distinct "significant evolutionary units".

Cat Ba langur *Trachypithecus poliocephalus poliocephalus*

Genetic data support distinct subspecies status. Restricted to Cat Ba Island, Vietnam.

White-headed langur *Trachypithecus poliocephalus leucocephalus*

The closest relative of the white-headed langur is *T. p. poliocephalus* and hence a classification as subspecies of this taxon is proposed. Restricted to a small range in Guangxi Province, southernmost China.

Delacour's langur *Trachypithecus delacouri*

Genetic data support distinct species status; endemic to a restricted range in northern Vietnam.

Laos langur *Trachypithecus laotum laotum*

Genetic data indicate close relationship with the Hatinh langur and hence both are classified as subspecies of *T. laotum*; endemic to a small range in Laos.

Hatinh langur *Trachypithecus laotum hatinhensis*

Genetic data indicate close relationship with the Laos langur and hence classification as subspecies of *T. laotum*. Distributed in a restricted range in Vietnam and Laos. *T. auratus ebenus* is provisionally recognised as synonym of this subspecies, however further studies are necessary to definitively settle its taxonomic status.

Red-shanked douc langur *Pygathrix nemaeus*

A distinct species status is supported. Distributed in Vietnam and Laos.

Grey-shanked douc langur *Pygathrix cinerea*

Genetically distinct from *P. nemaeus* and *P. nigripes* and hence classified as distinct species. Distributed in the Central Highlands of Vietnam. Most likely also present in a small range in north-eastern Cambodia and south-eastern Laos.

Black-shanked douc langur *Pygathrix nigripes*

Genetic data support distinct species status. Distributed in southern Vietnam and Cambodia.

Tonkin snub-nosed monkey *Rhinopithecus avunculus*

Genetic data support species status; restricted to small patches in northern Vietnam.

Yunnan snub-nosed monkey *Rhinopithecus bieti*

Distinct species status supported; endemic to Yunnan Province, China.

Guizhou snub-nosed monkey *Rhinopithecus brelichi*

Genetically distinct on species level; occurs only in Fanjingshan National Nature Reserve, Guizhou Province, China.

Sichuan snub-nosed monkey *Rhinopithecus roxellana*

Distinct species status supported. The classification of the three subspecies *R. r. roxellana*, *R. r. hubeiensis* and *R. r. qinlingensis* however needs further investigations. Distributed in central and western China.

Hainan gibbon *Nomasus hainanus*

Genetically separated from all other crested gibbons and hence a distinct species status is proposed; endemic to Hainan Island, China.

Eastern black gibbon *Nomascus nasutus*

Distinct species status proposed. Currently only known from two locations one in north-east Vietnam, and another in the adjacent area in China.

Western black gibbon *Nomascus concolor*

Genetically distinct on species level. The described subspecies *N. c. lu*, *N. c. furvogaster* and *N. c. jingdongensis* are downgraded as synonyms of the nominate form here; distributed in northern Vietnam, south-western China and north-western Laos.

Northern white-cheeked gibbon *Nomascus leucogenys leucogenys*

Genetically separated from all other crested gibbons and hence a distinct species status is proposed. However, further studies are necessary to discriminate between *N. l. leucogenys* and *N. l. siki* and to elucidate the exact distribution area. Distributed in Vietnam and Laos.

Southern white-cheeked gibbon *Nomascus leucogenys siki*

The taxonomic status of this taxon is unclear and needs more research. Karyotyped specimens in captivity (with unknown origin) form a sister clade to *N. l. leucogenys* and hence a subspecies status of *N. leucogenys* is proposed. Distributed in Laos and Vietnam.

Yellow-cheeked gibbon *Nomascus gabriellae*

Distinct species status proposed. However, further studies are necessary to elucidate the exact distribution area. Distributed in Vietnam, Cambodia and possibly also southernmost Laos.

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