Evolutionary history and phylogenetic position of the Indochinese grey langur (Trachypithecus crepusculus)

Rasmus Liedigk¹, Van Ngoc Thinh², Tilo Nadler³, Lutz Walter⁴, and Christian Roos⁵

¹ Department of Primate Genetics, German Primate Center, Kellnerweg 4, 37077 Goettingen, Germany. <ossar@gmx.net>
² Department of Primate Genetics, German Primate Center, Kellnerweg 4, 37077 Goettingen, Germany. <vanthinhngoc@yahoo.com>
³ Frankfurt Zoological Society / Endangered Primate Rescue Center, Cuc Phuong National Park, Nho Quan District, Ninh Binh Province, Vietnam. <t.nadler@mail.hut.edu.vn>
⁴ Department of Primate Genetics, German Primate Center, Kellnerweg 4, 37077 Goettingen, Germany. <lwalter@gwdg.de>
⁵ Gene Bank of Primates and Department of Primate Genetics, German Primate Center, Kellnerweg 4, 37077 Goettingen, Germany. Corresponding author. <croos@dpz.eu>

Key words: Trachypithecus crepusculus, mitochondrial DNA, nuclear DNA, introgression, hybridization

Summary

Although traditionally classified as a subspecies of Trachypithecus phayrei, recent genetic studies using mitochondrial sequence data have shown that the Indochinese grey langur, T. crepusculus, represents a distant relative of the T. francoisi species group and not of the T. obscurus group or specifically of T. phayrei. To further elucidate the phylogenetic position of T. crepusculus and to uncover possible hybridization events in the evolutionary history of the species, we expanded earlier mitochondrial studies by sequencing complete mitochondrial genomes, and generated sequence data from five autosomal, one X chromosomal and six Y chromosomal loci. According to the depicted mitochondrial phylogeny, T. crepusculus is indeed closely related to the T. francoisi group. In contrast, nuclear sequence data, although providing only limited information due to the low number of polymorphic sites, support a sister grouping of T. crepusculus to the T. obscurus group. Hence, nuclear sequence data are in agreement with the traditional classification of T. crepusculus as member of the T. obscurus group. We explain the discordance between mitochondrial and nuclear phylogenies with ancient male introgression events from T. phayrei into a basal member of the T. francoisi group, which led after repeated introgression to a complete replacement of the original nuclear genome of the ancestral T. crepusculus form and thus to a phenotype similar to that of T. phayrei.

Lịch sử tiến hóa và vị trí chúng loài phát sinh của loài voọc xâm đong dương (Trachypithecus crepusculus)

Tóm tắt

Mắc dự lâu nay vẫn được xem là loài phu của Trachypithecus phayrei, nhưng các nghiên cứu di truyền sử dụng truy cứu gen học thấy đã cho thấy voọc xâm đong dương (Trachypithecus crepusculus) có quan hệ hoặc xa và với loài voọc dạng trắng (T. francoisi) và không có quan hệ với loài voọc xâm đong dương T. obscurus hoặc T. phayrei. Đ經濟 làm rõ vị trí chúng loài phát sinh của T. crepusculus và xác định sự liên quan với các loài khác ra trong quá trình tiến hóa của loài này, chúng tôi đã mở rộng nghiên cứu về hệ thống di truyền bằng cách giải...
Introduction

Within the Asian leaf monkey genus *Trachypithecus*, traditionally five species groups (*T. pileatus*, *T. vetulus*, *T. francoisi*, *T. cristatus* and *T. obscurus*) are recognized, mainly due to differences in fur colouration, behaviour, ecology and distribution (Groves, 2001). However, recent genetic investigations have shown that the *T. vetulus* group is actually a member of the genus *Semnopithecus* and that the *T. pileatus* group might be the product of ancestral hybridization between *Semnopithecus* and *Trachypithecus* (Geissmann et al., 2004; Karanth et al., 2008; Osterholz et al., 2008). Thus, only three species groups, *T. francoisi*, *T. obscurus* and *T. cristatus*, remain as true members of the genus *Trachypithecus* (Osterholz et al., 2008).

Each of these three species groups include taxa that are genetically closely related to each other (Geissmann et al., 2004; Osterholz et al., 2008; Roos, 2003; 2004; Roos et al., 2007; 2008), and which are also similar in fur colouration, behaviour and ecology (Brandon-Jones et al., 2004; Groves, 2001; Nadler et al., 2003). Accordingly, *T. francoisi*, *T. poliocephalus*, *T. delacouri* and *T. laotum* are combined in the *T. francoisi* group (Osterholz et al., 2008; Roos, 2003; 2004; Roos et al. 2007), *T. obscurus*, *T. phayrei* and *T. barbei* in the *T. obscurus* group (Geissmann et al., 2004; Osterholz et al., 2008; Roos et al., 2007), and *T. cristatus*, *T. auratus*, *T. mauritius*, *T. margarita* and *T. germaini* in the *T. cristatus* group (Nadler et al., 2005; Roos et al., 2008).

An additional taxon, the Indochinese grey langur (*T. crepusculus*), is distributed from central to north-east Thailand and south Yunnan, east to south-west Laos and northern Vietnam, and west of the Bay of Bengal, south of the range of *T. phayrei phayrei* (Groves, 2001). Although originally described as a distinct species, *Pithecus crepuscula* by Elliot (1909), this taxon is traditionally recognized as a subspecies of *T. phayrei* because of similar colouration (Corbet & Hill, 1992; Groves, 2001; Napier & Napier, 1967). Ignoring whether it is subspecies or a distinct species, based on general appearance the taxon is obviously a member of the *T. obscurus* group. Most prominent in this respect are the light eyerings and depigmented lips, which are present in all members of the *T. obscurus* group and also in *T. crepusculus* (Groves, 2001). Contradicting this view, however, are recent genetic studies, which depict *T. crepusculus* as a distant relative of the *T. francoisi* group and not as member of the *T. obscurus* group (Geissmann et al., 2004; Roos, 2003; 2004; Roos et al., 2007).

Discordance between phenotype and genetic data or even between phylogenies derived from different genes occurs relatively frequently (Avise, 2000). In addition to other reasons such as incomplete lineage sorting or insufficient data, hybridization has been gaining increasing acceptance as an explanation for such discordances (Avise, 2000; Funk & Omland 2003; Seehausen, 2004). However, for primates, information about hybridization is still scare compared to
that for fishes, birds or other mammals, but recent investigations have uncovered natural hybridization events for primates (for review see Arnold & Meyer, 2006; Arnold, 2008). These occurred mainly between species of the same genus (e.g., *Lepilemur* sp., Rumpler et al., 2008; *Alouatta* sp., Cortés-Ortiz et al., 2007; *Macaca* sp., Tosi et al., 2000; *Papio* sp., Zinner et al., 2009a; *Gorilla* sp., Thalmann et al., 2007) but also between genera (e.g., *Trachypithecus* × *Semnopithecus*, Osterholz et al., 2008; *Rungwecebus* × *Papio*, Zinner et al., 2009b), and even led to the formation of new species (e.g., *Macaca arctoides*, Tosi et al., 2000; *Macaca munzala*, Chakraborty et al., 2007; *Trachypithecus pileatus*, Osterholz et al., 2008). Even for the human lineage, hybridization was suggested as important evolutionary mechanism (Pääbo, 2003).

To uncover possible hybridization events in the evolutionary history of the Indochinese grey langur and to further settle its phylogenetic position, we analysed complete mitochondrial genome data as well as sequences from five autosomal (Alb3, IRBP3, TP2, TTR1, vWF11), one X chromosomal (Xq13.3) and six Y chromosomal (DBY5, SMCY7, SMCY11, SRY, UTY18, ZFY_LI) loci from one representative of each of the three *Trachypithecus* species groups as well as from *T. crepusculus*.

**Material and Methods**

Blood samples were obtained from *T. crepusculus* and *T. delacouri* (both from the Endangered Primate Rescue Center, Vietnam), *T. auratus* (Wilhelma, Germany), *T. obscurus* (Wuppertal Zoo, Germany) and *Presbytis melalophos fluviatilis* (Howletts Wild Animal Park, UK). *T. delacouri*, *T. auratus* and *T. obscurus* were chosen because they represent members of the three species groups *T. francoisi*, *T. cristatus* and *T. obscurus*, respectively.

Genomic DNA was extracted with the DNeasy Blood & Tissue Kit from Qiagen. To exclude the miss-amplification of nuclear pseudogenes, the complete mitochondrial genome (ca. 16,500 bp) was amplified via two overlapping fragments with a size of ca. 6,500 and 13,800 bp, respectively. Using these templates, PCR fragments with ca. 1,000-1,200 bp in length were amplified via nested PCRs. Nuclear loci were amplified with already published primers (DBY5, SMCY7, SMCY11: Hellborg & Ellegren, 2003; SRY: Tosi et al., 2000; vWF11: Chaves et al., 1999; Xq13.3: Tosi et al., 2005) or newly designed primers (Alb3, IRBP3, TTR1, TP2, UTY18, ZFY_LI). Primers and PCR programs are available from the authors. All PCR reactions were checked on 1% agarose gels, excised from the gel and purified with a standard silica method (Sambrook et al., 1989). Afterwards, PCR products were sequenced on an ABI 3730xl sequencer using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). Coding regions of the mitochondrial genome and the SRY gene were translated into amino acid sequences to check for unexpected stop codons. All other loci represent non-coding intronic sequences.

For phylogenetic analyses, sequences were aligned with ClustalW v1.7 (Thompson et al., 1994) and manually checked by eye. In all statistical analyses, *Presbytis melalophos fluviatilis* served as outgroup. Due to the low number of informative sites, nuclear sequence data were only inspected by eye and not subjected to complex phylogenetic reconstructions. For the mitochondrial genome data, we performed neighbor-joining (NJ), maximum-likelihood (ML) and Bayesian reconstructions. Before analysing the data, indels and poorly aligned positions were removed with G-blocks v0.91b (Castresana, 2000). As optimal nucleotide substitution model, the GTR + I + G model was selected under the Akaike Information Criterion by MODELTEST v3.7 (Posada & Crandall, 1998). NJ and ML reconstructions were performed in PAUP* v4.0b10 (Swofford, 2002) and GARLI v0.951 (Zwickl, 2006). For both calculations, only the model specification settings were adjusted according to the
data set; all other settings were left at their default value. NJ and ML bootstrap percentages were estimated by performing 10,000 and 500 replications, respectively. To calculate a majority-rule consensus tree to obtain ML bootstrap percentages, PAUP was used. Bayesian analyses were conducted with MrBayes v3.1.2 (Huelsenbeck et al., 2001; Ronquist & Huelsenbeck, 2003) using four Monte Carlo Markov Chains with the default temperature of 0.1. Four repetitions were run for 10,000,000 generations with tree and parameter sampling occurring every 100 generations. The first 25% of samples were discarded as burnin, leaving 75,001 trees per run. Posterior probabilities for each split were calculated from the posterior density of trees.

To evaluate the reliability of the depicted phylogenetic position of *T. crepusculus*, alternative tree topologies with *T. crepusculus* being closer related to either *T. obscurus* or *T. auratus* instead to *T. delacouri* were evaluated with the Kishino-Hasegawa (Kishino & Hasegawa, 1989) and Shimodaira-Hasegawa (Shimodaira & Hasegawa, 1999) tests with full optimization and 1,000 bootstrap replications in PAUP.

**Results**

We successfully amplified and sequenced the complete mitochondrial genome from *T. crepusculus*, *T. delacouri*, *T. auratus*, *T. obscurus* and *Presbytis melalophos fluviatilis*. The alignment comprised 16,590 bp, which was reduced to 16,452 bp after gaps and poorly aligned positions were removed. Among them, 2,353 sites were variable and 954 parsimony-informative. Phylogenetic reconstructions were performed with NJ, ML and Bayesian algorithms. For all of them, identical and strongly supported tree topologies were obtained. Accordingly, *T. crepusculus* clusters with *T. delacouri*, and *T. auratus* forms a clade together with *T. obscurus* (Fig. 1a). To test for the reliability of the depicted relationships we evaluated alternative relationships. However, trees, in which *T. crepusculus* clusters with either *T. obscurus* or *T. auratus* instead with *T. delacouri*, were significantly rejected (P<0.001).

The concatenated nuclear data set including sequence data from 12 loci generated from *T. crepusculus*, *T. delacouri*, *T. auratus*, *T. obscurus* and *Presbytis melalophos fluviatilis* comprised 13,994 bp. After the removal of gap positions, the alignment was 13,551 bp in length. Among them, 301 sites were polymorphic and only nine parsimony-informative. Hence, mutations were visually inspected and no detailed phylogenetic reconstructions were performed. All in all, we detected five mutations (SMCY11: position 150, TP2: position 875, TTR1: position 436, Xq13.3: positions 4886 and 4979), which supported a grouping of *T. crepusculus* and *T. obscurus* (Fig. 1b). No mutations were found which supported a close affiliation between *T. crepusculus* with either *T. delacouri* or *T. auratus*.

**Discussion**

The present study shows that nuclear data support a close relationship between *T. crepusculus* and the *T. obscurus* group, which is in agreement with phenotypical characteristics and the traditional classification of *T. crepusculus* as subspecies of *T. phayrei* (Corbet & Hill, 1992; Groves, 2001; Napier & Napier, 1967). However, our mitochondrial phylogeny which supports earlier studies (Geissmann et al., 2004; Roos, 2003; 2004; Roos et al., 2007) suggests a close affiliation of *T. crepusculus* to the *T. francoisi* group. Thus, discordance between mitochondrial and nuclear/phenotypical phylogeny concerning the phylogenetic position of *T. crepusculus* becomes obvious.
The observed discordance might be explained by introgressive hybridization or incomplete lineage sorting, since both can result in similar phylogenetic patterns, and hence, complicate the interpretation of phylogenetic reconstructions (Avise & Ball, 1990; Morando et al., 2004). Although we can not rule out completely that incomplete lineage sorting may have had an effect, the geographical pattern, i.e. sympatric occurrence of *T. crepusculus* and the *T. francoisi* group members, provides some evidence against incomplete lineage sorting, because lineage sorting is a random process and the paraphyletic relationships that result from the failure of haplotypes to sort during speciation events should be random with respect to geography (Avise, 2004). In contrast, in our mitochondrial phylogeny sympatric populations cluster together, which is a strong indication of reticulation (Funk & Omland, 2003). Thus, we conclude that introgressive hybridization rather than incomplete lineage sorting has resulted in the discordance between mitochondrial and nuclear/phenotype phylogeny in the case of *T. crepusculus*.

Male dispersal and female philopatry are the norm in langurs (Davies & Oates, 1994; Fleagle, 1999) and one can assume that this is the ancestral state. Therefore, male introgression would be the most likely introgression scenario, where males from one taxon, i.e. *T. phayrei*, invaded groups of a basal relative of the *T. francoisi* group and reproduced successfully. Extensive backcrossing of the hybrid offspring over generations with more invading males of *T. phayrei* would have resulted in nuclear swamping. This process finally led to the extinction of the distantly related *T. francoisi* group member and only its mitochondrial genome remained as the only vestige of its former existence in a population, which is phenotypically similar to the introgressing *T. phayrei* (Fig. 2,3).

The classification of hybrid taxa is highly disputed and no consensus has been reached yet. In the case of *T. crepusculus*, we find a nuclear genome and a phenotype, which is similar to that of the *T. obscurus* group. Hence, a classification of *T. crepusculus* as species of the *T. obscurus* group or even as subspecies of *T. phayrei* would be appropriate. However, *T. crepusculus* carries mitochondria of a species, which is extinct now and was originally closely related to the *T. francoisi* group. Hence, *T. crepusculus* can not be regarded as true member of the *T. obscurus* group. Due to this incongruence, we propose to recognize *T. crepusculus* besides *T. cristatus, T. obscurus* and *T. francoisi* as a fourth distinct species group in the genus *Trachypithecus*. 
Acknowledgements

We thank the zoos in Stuttgart, Wuppertal and Howletts for providing langur blood samples. This study was financially supported by the German Primate Center.

References


