A note on phenotypical and genetic differences of silvered langurs in Indochina (*Trachypithecus germaini* and *T. margarita*)

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**Key words:** Silvered langurs, *Trachypithecus germaini*, *Trachypithecus margarita*, fur coloration, DNA, mitochondrial cytochrome b gene, taxonomy

**Summary**

A genetic study using mitochondrial DNA sequences supports a separation of the silvered langurs from the South-east Asian mainland from the Sundaland species and further a species-level division of mainland silvered langurs into *T. germaini* (Indochinese silvered langur) and *T. margarita* (Annamese silvered langur). The exact distribution areas of the mainland silvered langur species are still unknown but the Mekong River is considered as zoogeographical barrier. Both species possess high phenotypical variability due to individual variation, which makes it difficult to diagnose species. However, the arrangement of the hair on the head shows a clear difference. In *T. germaini* the hairs on the head form a central occipital crest whereas in *T. margarita* a crest is missing and the hairs on the head form a cap or hood. Moreover, the skin of the face is completely black in *T. germaini*, whereas in *T. margarita* the space around the eyes is paler and forms a ring, sometimes in flesh colour. Further investigations are required and should include additional samples, in particular from populations along the Mekong.

Ghi nhận về sự khác biệt về kiểu hình và kiểu gen của những loài vọoc bạc ở Đông Dương (*Trachypithecus germaini* và *T. margarita*)

**Tóm tắt**

Nghiên cứu về tính phân tách về phần loại học của các loại vọoc bạc ở khu vực Đông Nam Á gồm hai nhóm, nhóm lục địa và nhóm đa đảo. Đồng thời, hai loại ở nhóm lục địa cũng đã được khẳng định gồm vọoc bạc Đông Dương (*T. germaini*) và vọoc bạc Annam (*T. margarita*). Hiện tại, vẹng phần bộ của các loại vọoc bác lục địa vẫn cần phải làm sáng tỏ, tuy nhiên sông Mê Kông được xem như là ranh giới địa động vật quan trọng. Cả hai loại vọoc bác lục địa đều có sự biến thiên về kiểu hình rất đa dạng. Do đó việc nhận dạng loại vọoc bác qua hình thái trở nên khó khăn. Nghiên cứu này cho thấy sự sắp xếp của chỏm lông trên đầu của hai loại vọoc bác có sự khác biệt. Đối với vọoc bác Đông Dương, phần lông ở chỏm đầu dựng lên ở đỉnh đầu tạo nên chỏm mào, trong khi đối với vọoc bác Annam không có chỏm mào mà tạo nên hình dạng giống chiếc mũ. Ngoài

Introduction

Langurs of the *Trachypithecus cristatus* species group (Napier & Napier, 1970; Groves, 2001; Nadler et al., 2005; Osterholz et al., 2008; Roos et al., 2008) are distributed over a large area of southeast Asia, from Borneo through Java to Sumatra, islands of the Riau and Natuna Archipelago, the west coast of the Malayan Peninsula, to Thailand and southern Indochina. All taxa of this species group closely resemble one another in external characters. While the six currently recognized species (Zinner et al., in press) are clearly distinguished from one another in mitochondrial DNA (Roos et al., 2008) there is variation in fur coloration and hair structure, in particularly on the head, within these species. For example, Elliot (1912) mentioned the conspicuous differences in the hair structure on the head for two silvered langurs from Thailand.

Brandon-Jones et al. (2004) divided the silvered langurs on the South-east Asian mainland into two subspecies (*T. villosus germaini* and *T. v. margarita*), along with a third subspecies from Sundaland (*T. v. villosus*). According to Brandon-Jones et al. (2004), *T. v. germaini* occurs in south Thailand, Cambodia and southwest Vietnam, and *T. v. margarita* in south Laos, northeast Thailand and south Vietnam. In contrast, Groves (2001) recognized villosus as a synonym of *cristatus* and separated silvered langurs from the mainland from all other members of the species group as *T. germaini*. Within *T. germaini*, he recognized two subspecies, *T. g. germaini* and *T. g. caudalis*, with the latter from unknown origin, and *margarita* as synonym of *T. g. germaini*. Based on a small number of individuals it was argued that the two mainland forms differ phenotypically (Nadler et al., 2005). A genetic study using mitochondrial DNA sequences further supported a separation of the silvered langurs from the mainland from the Sundaland species and further a species-level division of mainland silvered langurs into *T. germaini* (Indochinese silvered langur) and *T. margarita* (Annamese silvered langur) (Roos et al., 2008).

However, the exact distribution areas of the mainland silvered langur species and the zoogeographical barrier separating them is still unknown. Nadler et al. (2005) and Roos et al. (2008) considered the Mekong River, a well-known barrier for many primate species, as possible limit for both species, but highlighted the importance of further investigations. Moody et al. (2011) and Timmins et al. (2011) doubted the Mekong as barrier for silvered langurs, because species of this species group occur naturally in coastal and riverine forests throughout their range and are even found on islands in the Mekong in northern Cambodia (Bezuïjen et al., 2007; Timmins et al., 2011), thus, suggesting that they might be able to cross the Mekong.

Due to these uncertainties concerning taxonomy and distribution, further investigations are urgently required. Hence, to further shed light on the phenotypic and genetic variation in silvered langurs form the Asian mainland, we collected information on phenotypic features from field observations and photos, and by analysing a fragment of the mitochondrial cytochrome b gene of an expanded silvered langur data set.

Materials and Methods

Phenotypical features

There exists only a limited number of museum specimen for comparison of the silvered langurs,
in particular from the mainland species. Moreover, museum specimens are not very useful for the comparison of skin colour and the arrangement of hairs on the head. Thus, to study phenotypical features of silvered langurs from eastern and western parts of the Mekong we used field observations and photos taken primarily from individuals of known locations.

Molecular analysis

To expand our previous study on silvered langurs (Roos et al., 2008), we analysed faecal samples from nine additional silvered langurs. Samples were collected either during field surveys or from confiscated individuals from known origin (Table 1, Fig. 1) and stored in 80% ethanol before further processing. DNA was extracted with the QiAamp DNA Stool Mini Kit from Qiagen following protocols of the company. A 573bp fragment of the mitochondrial cytochrome b gene was amplified and sequenced according to methods outlined in Geissmann et al. (2004) and Roos et al. (2008). To prevent cross-sample contamination, laboratory procedures followed described protocols (Osterholz et al., 2008; Roos et al., 2008). PCR products were checked on 1% agarose gels and purified with a standard silica extraction method (Sambrook et al., 1989). Sequencing was performed on an ABI 3130xL sequencer using both amplification primers and the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems).

<table>
<thead>
<tr>
<th>Species</th>
<th>Origin</th>
<th>Number in Fig. 1</th>
<th>Number of samples</th>
<th>Haplotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. germaini</td>
<td>Kien Luong, Vietnam (10°19’N; 104°38’E)</td>
<td>1</td>
<td>7</td>
<td>gA, gB, gC, gG, gH</td>
</tr>
<tr>
<td></td>
<td>Cambodia</td>
<td>-</td>
<td>2</td>
<td>gB</td>
</tr>
<tr>
<td></td>
<td>Thailand</td>
<td>-</td>
<td>2</td>
<td>gB, gD</td>
</tr>
<tr>
<td></td>
<td>unknown</td>
<td>-</td>
<td>6</td>
<td>gB, gE, gF</td>
</tr>
<tr>
<td></td>
<td>Southern Cardamoms, Cambodia</td>
<td>2</td>
<td>1</td>
<td>gK</td>
</tr>
<tr>
<td></td>
<td>Battambang, Cambodia</td>
<td>3</td>
<td>2</td>
<td>gG, gJ</td>
</tr>
<tr>
<td></td>
<td>Siem Reap, Cambodia</td>
<td>4</td>
<td>1</td>
<td>gJ</td>
</tr>
<tr>
<td></td>
<td>Preah Vihear, Cambodia</td>
<td>5</td>
<td>1</td>
<td>gE</td>
</tr>
<tr>
<td>T. margarita</td>
<td>Kontum, Vietnam</td>
<td>6</td>
<td>1</td>
<td>mA</td>
</tr>
<tr>
<td></td>
<td>Veal Thom, Cambodia (14°02’N; 106°45’E)</td>
<td>7</td>
<td>2</td>
<td>mC, mD</td>
</tr>
<tr>
<td></td>
<td>Gia Lai, Vietnam</td>
<td>8</td>
<td>3</td>
<td>mA, mC</td>
</tr>
<tr>
<td></td>
<td>Koh Nhek, Cambodia (13°02’N; 107°10’E)</td>
<td>9</td>
<td>1</td>
<td>mA</td>
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<tr>
<td></td>
<td>Dak Mil, Vietnam (12°22’N; 107°40’E)</td>
<td>10</td>
<td>1</td>
<td>mA</td>
</tr>
<tr>
<td></td>
<td>Keo Seima, Cambodia (12°30’N; 106°50’E)</td>
<td>11</td>
<td>1</td>
<td>mB</td>
</tr>
<tr>
<td></td>
<td>Cat Tien, Vietnam (11°31’N; 107°19’E)</td>
<td>12</td>
<td>1</td>
<td>mA</td>
</tr>
<tr>
<td></td>
<td>Lo Go Sa Mat, Vietnam (11°35’N; 105°54’E)</td>
<td>13</td>
<td>1</td>
<td>mA</td>
</tr>
</tbody>
</table>
Sequences were aligned in SeaView 4 (Gouy et al., 2010) and corrected by eye. For statistical analysis, we added further orthologous sequences from T. germaini and T. margarita published earlier (Roos et al., 2008). The final dataset comprised 34 sequences including 22 T. germaini individuals, 11 T. margarita individuals and one T. cristatus individual, with the latter used as an outgroup. Phylogenetic trees were constructed with a Bayesian algorithm in MrBayes 3.1.2 (Huelsenbeck et al., 2001; Ronquist & Huelsenbeck, 2003). For the reconstruction, the HKY nucleotide substitution model was chosen as the best-fit model under the Bayesian information criterion (BIC) as implemented in jModeltest 0.1 (Posada, 2009). Four independent Markov Chain Monte Carlo (MCMC) runs with the default temperature of 0.2 were used. Four repetitions were run for 10 million generations with tree and parameter sampling occurring every 100 generations. Acceptance rates were in the optimal range of 10 - 70%. The first 25% of samples were discarded as burn-in, leaving 75,001 trees per run. The adequacy of this burn-in and convergence of all parameters was assessed by examining the uncorrected potential scale reduction factor (PSRF) (Gelman & Rubin, 1992) as calculated by MrBayes, which should approach 1 as runs converge and by visual inspection of the trace of the parameters across generations using the software TRACER 1.5 (Rambaut & Drummond, 2007). Posterior probabilities for each split and a phylogram with mean branch lengths were calculated from the posterior density of trees.

Results and Discussion

In contrast to the clear phenotypical differences between T. germaini and T. margarita presented by Nadler et al. (2005), the analysis of additional individuals revealed that both species possess high phenotypical variability due to individual variation (see also Timmins et al. (2011)). A century ago Elliot (1912) mention for T. margarita: “The colouring varies greatly according to the light which at certain times casts shadows on the gray that are almost black in their intensity”. Such variation occurs also in T. germaini (Fig 2, 3, 4, 5). The general colour of both species is silvery grey. Forearms, hands and feet are black. Chin, throat, under parts of the body, and inner side of arms and legs sparsely covered with white hairs. Flanks are paler silvery grey than the upper parts. The tail is above black, beneath silvery grey. The head is grey, the forehead covered by erect black hairs. However, the arrangement of the hair on the head shows a clear difference: in T. germaini the hairs on the head form a central occipital crest (Fig. 6, 7), whereas in T. margarita a crest is missing and the hairs on the head form a cap or hood, resembling T. auratus (Fig. 8, 9, 10). Moreover, the skin of the face is completely black in T. germaini, whereas in T. margarita, as noted by Elliot (1912) the space around the eyes is paler and forms a ring, sometimes in flesh colour. The eye rings appear very prominent if a flash is used to take photos, which is not the case in T. germaini (Fig. 11).
Fig. 2. The coloration of Annamese silvered langurs (*T. margarita*) shows individual variation (Zoo Saigon – origin of animals Dong Nai Province, Vietnam). Photo: Tilo Nadler.

Fig. 3. The Indochinese silvered langur (*T. germaini*) varies also in fur coloration, animal from southeast Cambodia. Photo: Tilo Nadler.

Fig. 4. Indochinese silvered langur (*T. germaini*) from Kien Giang Province, Vietnam. Photo: Hoang Minh Duc.

Fig. 5. Indochinese silvered langur (*T. germaini*) from Kien Giang Province, Vietnam. Photo: Hoang Minh Duc.

Fig. 6. Hair arrangement on the head of Indochinese silvered langur (*T. germaini*) shows the typical crest (animal from southeast Cambodia). Photos: Tilo Nadler.

Fig. 7. The extension of the black band on the forehead varies, and the hair of the cheeks is very long. Indochinese silvered langur (*T. germaini*) from southeast Cambodia. Photo: Tilo Nadler.
In contrast to phenotypic features, the extended genetic data set provides additional support for a clear distinction between these species and further suggests the Mekong as natural barrier (Fig. 12). It has to be noted here that in the extended genetic data set, samples from both *T. germaini* and *T. margarita* were included which derived from silvered langur populations closer to the Mekong than in the original data set (Roos et al., 2008). Thus, the Mekong might be indeed the zoogeographic barrier between both species, despite inconclusive phenotypic features. Since we analysed only mitochondrial DNA which is only maternally inherited (Melnick & Hoelzer, 1993) and most primate species show female philopatry (Pusey & Packer, 1987; but see Koenig & Borries (2012) for male philopatry in *Trachypithecus*), nuclear markers need to be studied as well. From the limited nuclear data available to date (Roos, unpublished), all silvered langur species, including *T. germaini* and *T. margarita*, can be distinguished, but confirmation of species-specificity of
polymorphisms on a population level is required. Moreover, future studies should include additional samples, in particular from populations along the Mekong. Limited gene flow between both forms on either or both sides of the Mekong cannot be excluded, but is most probably locally restricted and will not lead to panmixia of the whole South-east Asian mainland silvered langur population.

The validity of *T. margarita* and the status of taxa in the silvered langur species group in general was recently questioned (Tan et al., 2008; Moody et al., 2011; Timmins et al., 2011). However, in the silvered langur species group, all species are clearly diagnosable by mitochondrial DNA and to a lesser degree by phenotype, and thus, distinct species status for all taxa, including *T. germaini* and *T. margarita*, is supported by the Phylogenetic Species Concept (Cracraft, 1983, 1989), which is widely applied in primate systematics today (Groves, 2012).

Acknowledgements

We are grateful to the authorities in Vietnam and Cambodia for permissions, and to Marina Kenyon, Markus Handschuh, and Benjamin Rawson for samples. This study was financially supported by the German Primate Center. Field surveys conducted by Hoang Minh Duc and Herbert Covert were partly funded by Wenner-Gren Foundation, The John D. And Catherine T. MacArthur Foundation and Margot Marsh Foundation.

References


