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TAXONOMY AND PHYLOGENY OF LEAF MONKEYS (COLOBINAE) WITH FOCUS ON THE GENUS *PRESBYTIS* (ESCHSCHOLTZ, 1821)

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Summary

Leaf monkeys (Colobinea) constitute a very diverse group of primates with major radiations in Africa and Asia. The Asian colobines are tradionally divided into the odd-nosed group (*Simias*, *Nasalis*, *Pygathrix*, *Rhinopithecus*) and the langur group (*Semnopithecus*, *Trachypithecus*, *Presbytis*). Among the langur group *Presbytis* constitutes a particular diverse taxon, but the phylogenetic position of *Presbytis* among the Asian colobines, as well as the number of *Presbytis* taxa and their phylogenetic relationships remain controversial.

Previous molecular studies on leaf monkeys on the generic level based on incomplete sampling and revealed discordant gene trees for the Asian group. In particular the phylogenetic position of *Presbytis* was unclear. In a comperative genetic approach, we combined presence/absence analysis of mobile elements with autosomal, X chromosomal, Y chromosomal and mitochondrial sequence data from all recognized colobine genera. Our results could not clarify the phylogenetic position of *Presbytis*, but indicated an unidirectional gene flow from *Semnopithecus* into *Trachypithecus* via male introgression, rather than a previously proposed hybridization between *Presbytis* and *Trachypithecus*.

Regarding the genus *Presbytis*, almost all current classifications predominantly based on morphological traits, while the only molecular study relied heavily on captive animals of uncertain origin. Therefore during two extended field surveys on Java, Sumatra and the Mentawai Islands, fecal and acoustic samples of more than 30 wild *Presbytis* populations were collected and subsequently analysed. Phylogenetic reconstructions based on a 1.8 kb long fragment of the mitochondrial genome and revealed various well-supported terminal clades, which refer mainly to the described taxa. The *P.melalophos* group emerged as paraphyletic, which was strongly supported by a structural analysis of 100 male loud-calls. This enabled us to propose a revised classification of the *P.m.melalophos* group. Furthermore and in concordance with the com-

plex geographic distribution of *Presbytis*, we found a highly significant correlation between call structure and genetic similarity, and lesser significant correlations between call structure and geographic distance, and genetic similarity and geographic distance. Based on divergence time estimates we detected two periods of radiation, the first during the late Miocene and the second during the late Pliocene/early Pleistocene. Previous phylogeographic hypothesis based predominantly on a proposed basal position of *P.potenziani* from the Mentawai Islands. Our results however indicated that these morphological features might be pleisiomorphic. In our phylogenetic reconstruction *P.thomasi* is the sister to the remaining taxa. *P.thomasi* most likely evolved on the Asian mainland, while *P.potenziani* diverged during the second phase of radiation. The general sympatry of *Presbytis* on Borneo is at least to some extend the result of a second colonization of the island during the Pleistocene.

Ringkasan

Monyet daun (Colobinea) mewakili kelompok primata yang sangat beragam dengan radiasi/sebaran utama di Afrika dan Asia. Colobine Asia secara tradisional terdiri dari kelompok berhidung aneh [odd-nosed] (*Simias, Nasalis, Pygathrix, Rhinopithecus*) dan kelompok langur (*Semnopithecus, Trachypithecus, Presbytis*). Diantara kelompok langur, *Presbytis* mewakili sejumlah takson yang beragam, tetapi posisi filogenetik *Presbytis* diantara colobine asia maupun jumlah taksa *Presbytis* dan hubungan filogenetik mereka-hingga saat ini- masih kontroversial.

Studi molekuler terdahulu tentang monyet daun pada tingkatan generik berdasarkan sampling yang tidak lengkap dan mengungkapkan pohon gen yang tidak sesuai untuk kelompok Asia. Secara khusus adalah tidak jelasnya posisi filogenetik Presbytis. Dalam pendekatan genetis komparatif, kami menggabungkan kehadiran/ketidakhadiran analisa elemen bergerak dengan autosomal, kromosom X, kromosom Y dan data sekuensi mitokondrial dari semua genera colobine yang bisa dikenali. Hasil kami tidak dapat mengklarifikasi posisi filogenetik *Presbytis*, tetapi mengindikasikan aliran gen searah dari *Semnopithecus* ke *Trachypithecus* melalui introgesi jantan, berbeda dari usulan sebelumnya tentang hibridisasi (persilangan) antara *Presbytis* dan *Trachypithecus*.

Mengenai genus *Presbytis* sendiri, hampir semua pengelompokan terkini utamanya berdasarkan sifat morfologi, sementara satu-satunya studi molekuler sangat bergantung pada hewan tangkapan dari asal yang belum diketahui. Oleh karena itu, selama dua survey lapang lanjutan di Jawa, Sumatra dan Kepulauan Mentawai, contoh kotoran dan bunyi dari 30 populasi *Presbytis* liar telah dikumpulkan dan dianalisa secara berurutan. Rekonstruksi filogenetik berdasarkan pada fragmen sepanjang 1.8 kb dari genom mitokondrial dan mengungkapkan beragam terminal klad yang cukup didukung, yang umumnya mengacu pada takson yang telah digambarkan. Kelompok *P. melalophos* muncul sebagai parafiletik, yang didukung secara kuat oleh analisa struktural dari

100 suara panggilan keras jantan dewasa. Hal ini memungkinkan kami untuk mengajukan klasifikasi revisi dari kelompok *P. m. melalophos*. Lebih jauh dan berkaitan dengan penyebaran geografis kompleks *Presbytis*, kami menemukan korelasi yang sangat signifikan antara struktur panggilan dan kemiripan genetis, dan korelasi yang kurang signifikan antara struktur panggilan dan jarak geografis, dan kemiripan genetis dan jarak geografis. Berdasarkan perkiraan waktu divergen, kami mendeteksi dua periode radiasi, pertama pada masa akhir Miosen dan yang kedua pada akhir Pliosen/awal Pleistosen. Hipotesis filogeografi sebelumnya utamanya berdasarkan pada usulan posisi basal dari *P. potenziani* dari Kepulauan Mentawai. Namun hasil kami mengindikasikan bahwa fitur morfologis ini kemungkinan adalah plesiomorfis. Dalam rekonstruksi filogenetik kami *P. thomasi* adalah kerabat dari takson yang sudah ada. *P. thomasi* kemungkinan besar berevolusi di daratan Asia, sementara *P. potenziani* terdivergen saat fase kedua radiasi. Simpatri umum dari *Presbytis* di Borneo setidaknya merupakan hasil dari kolonisasi kedua dari pulau tersebut pada masa Pleistosen.

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"While wandering a deserted beach at dawn, stagnant in my work, I saw a man in the distance bending and throwing as he walked the endless stretch toward me. As he came near, I could see that he was throwing starfish, abandoned on the sand by the tide, back into the sea. When he was close enough I asked him why he was working so hard at this strange task. He said that the sun would dry the starfish and they would die. I said to him that I thought he was foolish. There were thousands of starfish on miles and miles of beach. One man alone could never make a difference. He smiled as he picked up the next starfish. Hurling it far into the sea he said, "It makes a difference for this one." I abandoned my writing and spent the morning throwing starfish."

— Loren Eiseley

Chapter 1 General Introduction

With more than 630 currently described taxa, the primate order is one of the most diverse and successful group of mammals (Groves 2004; Mittermeier et al. 2009; Rowe and Myers 2011). Over the course of their evolutionary history, non-human primates display great diversity of behavioral and morphological traits (Smuts 1987). With the exception of Antarctica, non-human primates have been documented on every major continent colonized by placental mammals (Rowe and Myers 2011).

1.1 Leaf monkeys (Colobinae)

According to Groves (2001) the Old World Monkey family Cercopithecidae is divided into two subfamilies, the Cercopithecinae (Cheek Pouch Monkeys) and the Colobinae (Leaf monkeys). In contrast to the Cercopithecinae, the Colobinae are particularly distinguished by the presence of a ruminant-like chambered stomach, unique among primates (Napier and Napier 1967; Oates and Davies 1994). The complexity of their stomach is partly a response to the chemical problems in digesting leaves, that contain much fiber and other secondary components, as well as to neutralize the effects of inhibitors and toxins (Chivers 1994; Strasser and Delson 1987). Other key anatomical characters are also related to food processing, for instance greatly enlarged salivary glands (Davies and Oates 1994), and the morphology of their teeth (Lucas and Teaford 1994). Colobines get their name from very short or absent thumbs of the African species (Greek kolobos, mutilated), whereas Asian colobines have a small thumb (Oates and Davies 1994). Typically the hindlimbs of colobines are much longer than the forelimbs (Strasser 1992) and almost all colobines have long tails (Oates and Davies 1994). These postcranial characters are proposed to be related to the colobine monkey commitment to arborality and leaping (Strasser 1992).

Colobines show a great diversity in the social organizations of their societies. Reported are matrilinial – harems (e.g. *Colobus guereza*, *Trachypithecus obscurus*, *Presbytis me-*

lalophos, Nasalis larvatus, Semnopithecus entellus), matriliniael – multi-male societies (e.g. *Colobus satanas, Nasalis larvatus, Semnopithecus entellus*), patrilineal – multi-male societies (e.g. *Piliocolobus badius, Procolobus verus*) and even monogamy (e.g. *Presbytis potenziani*) (Newton and Dunbar 1994; Tilson 1976b; Watanabe 1981). Variations in other aspects of colobine social life, such as grooming patterns, sexual swellings, flamboyant natal coats, nurtureing behavior and infanticide are probably linked to this variation in social organization (Clutton Brock and Harvey 1977; Newton and Dunbar 1994).

Tradionally behavioral patterns, in particular anatomical traits were used to propose working classifications. Taxonomy always was, and still is a very dynamic and controversially discussed scientific discipline. With increasing knowledge over the past decades and since molecular genetic methods were applied more often in order to draw taxonomic and phylogenetic conclusions, it is not surprising that colobine monkeys have a long history of taxonomic revisions. They have been grouped into between four and ten genera (Delson et al. 1982; Groves 2001; Napier and Napier 1967; Napier 1985; Oates et al. 1994; Szalay and Delson 1979; Thorington Jr and Groves 1970).

I will follow the classification of Groves (2001), who further divided the colobines themselves into two groups: an African group with the genera *Colobus, Procolobus* and *Piliocolobus*, and an Asian group comprising the langur genera *Semnopithecus*, *Trachypithecus* and *Presbytis,* and the odd-nosed monkey genera (which were named after their unusual nose morphology) *Rhinopithecus*, *Pygathrix*, *Nasalis* and *Simias* (Figure 1.1).

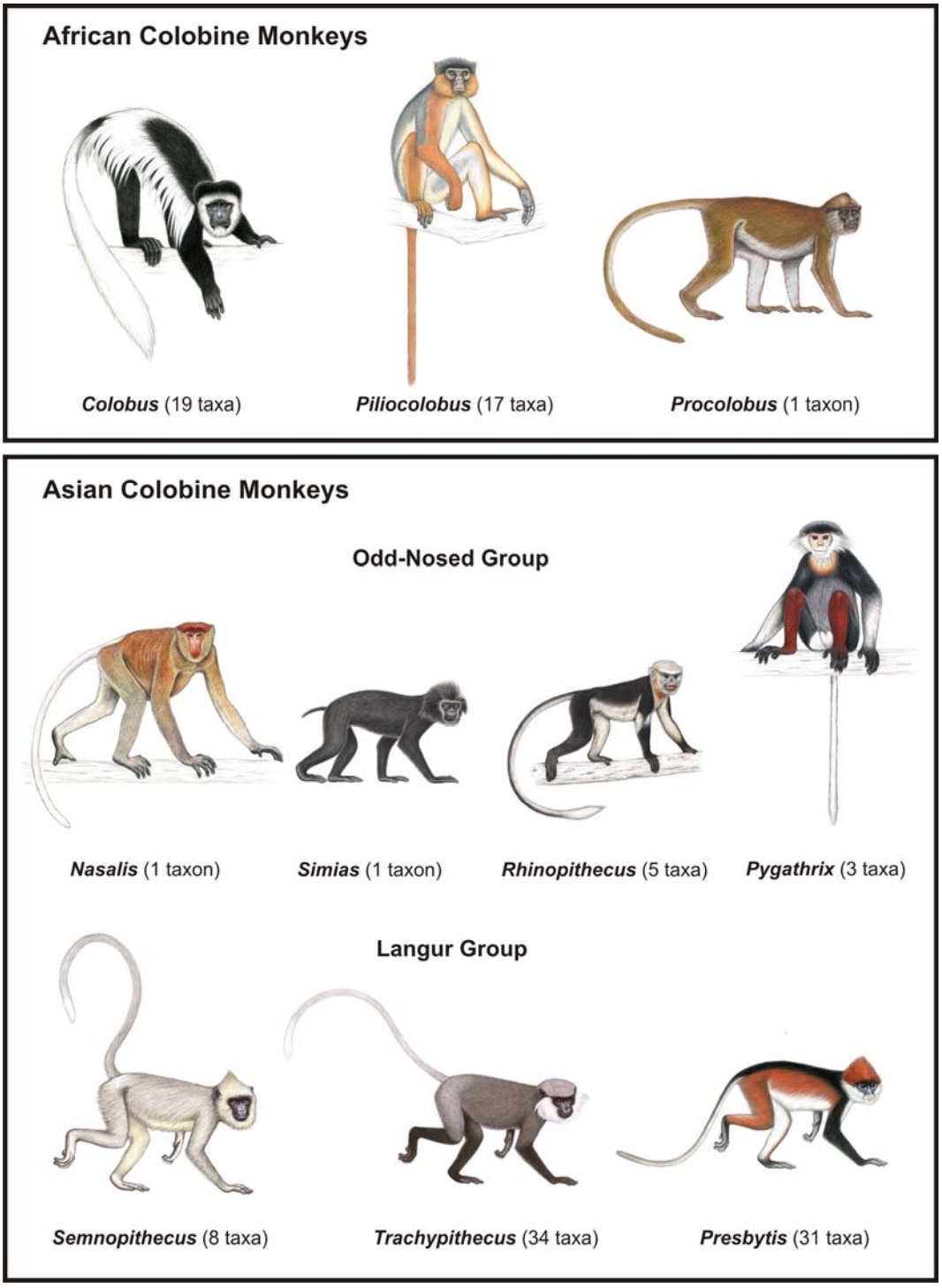

Figure 1.1: Illustrations of colobine monkey genera (classification based on Groves 2001; Illustrations used with permission by Stephen D. Nash / Conservation international. Copyright 2011)

The monophyly of the Asian and African groups is supported by molecular genetic data, but based on incomplete sampling (Osterholz et al. 2008; Sterner et al. 2006; Ting et al. 2008).

Within the Asian group, genetic studies indicate conflicting phylogenetic relationships between langur genera in relation to mitochondrial and nuclear data. Mitochondrial data either support a sister grouping of *Presbytis* and *Trachypithecus* (Sterner et al. 2006) or do not resolve langur relationships in general (Osterholz et al. 2008). X-chromosomal data however place *Presbytis* as sister to all Asian colobines, and support a sister grouping of *Trachypithecus* and *Semnopithecus*. This could be explained by introgressive hybridization (Ting et al. 2008).

1.2 Surilis (genus *Presbytis*, Eschscholtz 1821)

Compared to *Trachypithecus, Presbytis* has longer hind limbs, leaps more, uses less quadrupedalism (Fleagle 1977; Strasser 1992) and has a smaller stomach (Chivers 1994). Leaves contribute less than 40% in some *Presbytis* diet (i.e. in *rubicunda, siamensis, hosei*) (Chivers 1994) which mainly consists of seeds and fruits (Bennett and Davies 1994), whereas foliage nutrition constitutes more than 60% in *Trachypithecus* (Chivers 1994). Neonates of *Trachypithecus* are orange, while newborn *Presbytis* are white or whitish and as the coat darkens during development they pass through a stage that displays a cruciform pattern on the back and upper head (Pocock 1928).

The majority of *Presbytis* live in matrilineal (or female bonded) uni-male groups (Newton and Dunbar 1994). Only *P.potenziani* is reported to live partly monogamous (Tilson 1976b; Watanabe 1981) and *P.thomasi* forms matrilineal uni-male or multi-male groups (Newton and Dunbar 1994).

The geographical range of *Presbytis* is confined to Sundaland, which includes the Malay peninsular and the western Indo-Malay archipelago consisting of Borneo, Sumatra, Java, the Natuna Islands and the Mentawai Islands (Brandon-Jones et al. 2004; Groves 2001)(Figure 1.2).

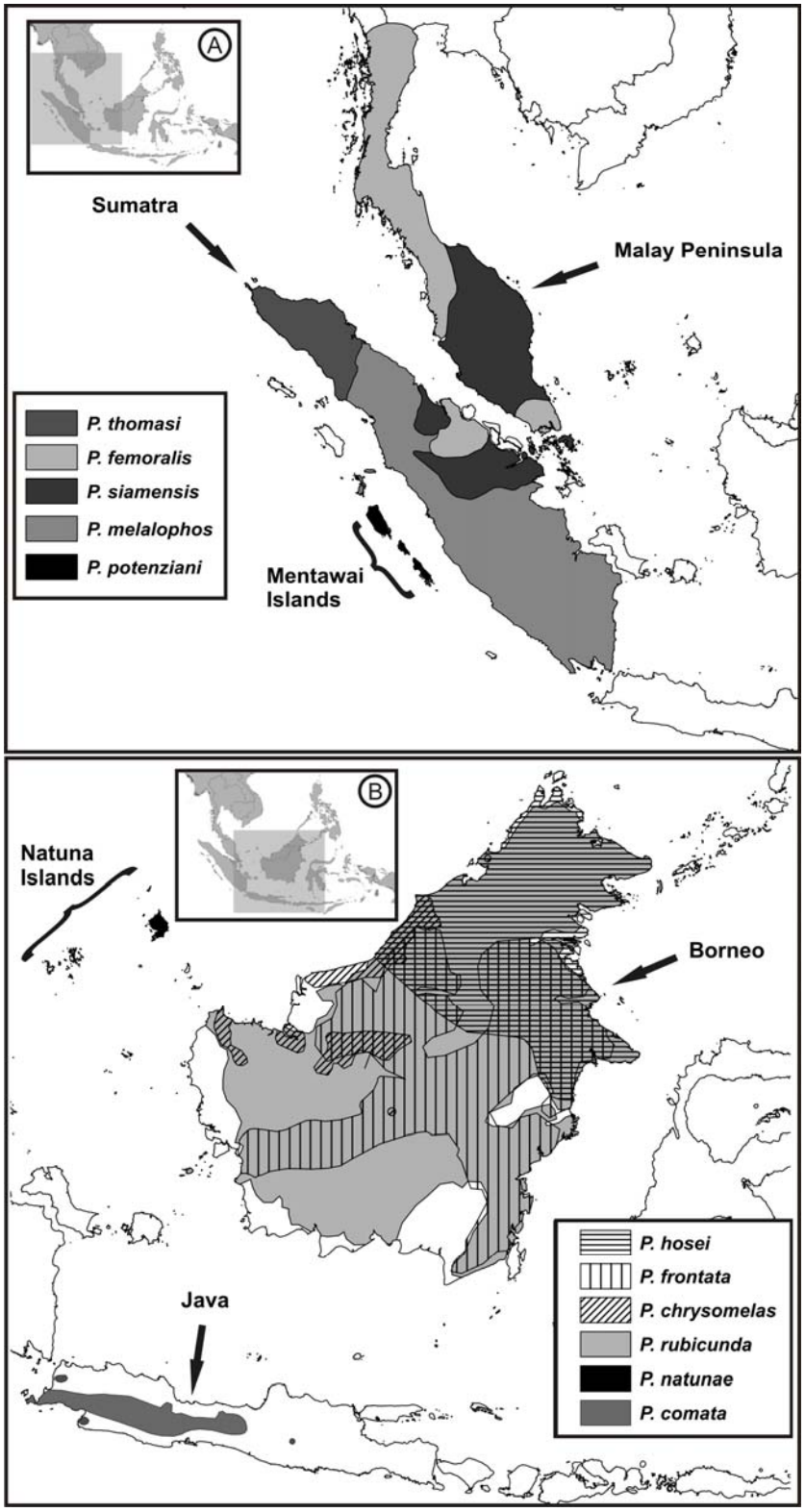

Figure 1.2: Present distribution range of *Presbytis* on the Asian Mainland, Sumatra and the Mentawai Islands (A) and on Borneo, Java and the Natuna Islands (B) (Groves 2001).

1.2.1 *Presbytis* taxonomy

Presbytis is one of the most diverse primate genera. Since 1821 more than 50 *Presbytis* color-morphs have been described (Brandon-Jones et al. 2004; Groves 2001). Based on morphological features, i.e. natal pelage color, body weight or body length Napier & Napier (1967) proposed 4 *Presbytis* species groups, with 14 species. Currently however, only seven are still considered as members of the genus (Brandon-Jones et al. 2004; Groves 2001).

The taxonomy of *Presbytis* continued to be unclear with subsequent studies dividing the genus into either four (Wolfheim 1983), seven (Brandon-Jones 1984; Groves 1970; Medway 1970; Oates et al. 1994) or eight (Corbet and Hill 1992; Weitzel et al. 1988) species according to various interpretations of anatomical, ecological and behavioral data. A few studies also used phonetic descriptions of male vocalizations as a tool to propose the separation of respective taxa or phylogenetic relationships. Based on one-phrase, two-phrase or three-phrase vocalizations, Sumatran langurs were divided into distinct species (Aimi and Bakar 1992; Wilson and Wilson 1976; Wilson and Wilson 1975) and a close affiliation of the Mentawai langur (*P.potenziani*) with the Thomas's langur (*P.thomasi*) was suggested (Wilson and Wilson 1976).

Most recent studies led Groves (2001) and Brandon-Jones et al. (2004) to propose a revised classification resulting in 11 and 10 species respectively. Although the authors agree on the species status of *P.melalophos*, *P.thomasi*, *P.potenziani*, *P.siamensis*, *P.femoralis*, *P.comata*, *P.frontata*, *P.hosei* and *P.rubicunda*, major differences in their classifications still exist (Table 1.1). These differences exist on the level of species and subspecies, even the validity of some taxa is disputed (synonyms). Furthermore, although Brandon-Jones et al. (2004) divided *P.comata*, *P.thomasi* and *P.hosei* as single species (which stands in accordance with Groves 2001), the authors still refer to a polytypic species concept (Brandon-Jones 1978, 1996c). According to this concept *thomasi* and *hosei* are conspecifics of *P.comata*.

To date, the only molecular genetic study addressing the *Presbytis* taxonomy and phylogeny came out by Md Zain (2001). Using mitochondrial, Y-chromosomal and auto-

somal markers, he suggested that *P.melalophos* might be a single highly polytypic species with numerous subspecies, including *P.femoralis*, *P.siamensis* and *P.natunae*.

Table 1.1: Arrangement of the classifications of Groves (2001) and Brandon-Jones et al. (2004). Bold letters indicate species. * possibly referable to *Presbytis comata* (Brandon-Jones 1978, 1996c).

Taxon	Groves (2001)	Brandon-Jones et al. (2004)
potenziani	***P.potenziani potenziani***	***P.potenziani potenziani***
siberu	*P.p.siberu*	*P.p.siberu*
thomasi	***P.thomasi***	***P.thomasi****
melalophos	***P.m.melalophos***	***P.m.melalophos***
mitrata	*P.m.mitrata*	*P.m.mitrata*
bicolor	*P.m.bicolor*	*P.m.bicolor*
sumatrana	*P.m.sumatrana*	*synonym of melalophos*
nobilis	*synonym of melalophos*	*P.m.nobilis*
siamensis	***P.siamensis siamensis***	***P.siamensis siamensis***
paenulata	*P.s.paenulata*	*P.s.paenulata*
cana	*P.s.cana*	*P.s.cana*
rhionis	*P.s.rhionis*	*P.s.rhionis*
natunae	***P.natunae***	*P.s.natunae*
femoralis	***P.femoralis femoralis***	***P.femoralis femoralis***
robinsoni	*P.f.robinsoni*	*P.f.robinsoni*
percura	*P.f.percura*	*P.f.percura*
batuana	*synonym of sumatrana*	*P.f.batuana*
chrysomelas	***P.chrysomelas chrysomelas***	*P.f.chrysomelas*
cruciger	*P.c.cruciger*	*P.f.cruciger*
comata	***P.comata comata***	***P.comata***
fredericae	*P.c.fredericae*	***P.fredericae***
frontata	***P.frontata***	***P.frontata***
hosei	***P.hosei hosei***	***P.hosei hosei****
sabana	*P.h.sabana*	*P.h.sabana **
everetti	*P.h.everetti*	*P.h.everetti **
canicrus	*P.h.canicrus*	*P.h.canicrus **
rubicunda	***P.rubicunda rubicunda***	***P.rubicunda rubicunda***
chrysea	*P.r.chrysea*	*P.r.chrysea*
carimatae	*P.r.carimatae*	*P.r.carimatae*
ignita	*P.r.ignita*	*P.r.ignita*
rubida	*P.r.rubida*	*synonym of carimatae*

In summary, therefore the taxonomy of the genus *Presbytis* remains unresolved. Many different classifications have been proposed over the past 40 years and the recent trend is that the taxon has become more and more speciose, but no final agreement

on *Presbytis* taxonomy could be reached to date – neither on the level of species nor subspecies.

1.2.2 *Presbytis* phylogeny and phylogeography

Previous divergence time estimates suggest that the *Presbytis* ancestor have colonized Sundaland at the end of the Miocene (Sterner et al. 2006). During that geological period and also subsequently during the Pliocene and Pleistocene, Sundaland was affected by dramatic changes in geology (Barry et al. 1985; van Bemmelen 1970), fluctuation in ocean levels (Haq et al. 1987; Miller et al. 2005) and changes in vegetation patterns (Cerling et al. 1997), which gave rise to a high degree of radiation (Meijaard 2004). As a result *Presbytis* represents one of the most diverse genera within the Old World Monkeys. Almost all evolutionary models on *Presbytis* that have been proposed so far, are predominantly based on anatomical features (Brandon-Jones 1978; Brandon-Jones 1996a; Groves 1989; Meijaard and Groves 2004) and only one model is derived from molecular genetic analysis (Md Zain 2001).

According to Md Zains' (2001) phylogeographic model proto-*Presbytis* underwent two cladogenic events on Borneo. The first separated the proto-*Presbytis* into a *hosei* like form and an intersectional *Presbytis*. The latter underwent a further subdivision into a *melalophos* like - and a *comata* / *thomasi* / *rubicunda* like form. This dispersal pattern is opposite to the direction of events proposed by previous studies of Meijaard and Groves (2004), Groves (1989) and Brandon-Jones (1978; 1996a).

In Brandon-Jones's (1978; 1996a) reconstruction, *P.potenziani* represents the most primitive form with its dark coat coloration. *P.potenziani* evolved during the middle Miocene where it was restricted to the Mentawai Islands during a severe glaciation. After this glaciation stage *P.comata* splitted from *P.potenziani*. During a subsequent glacial period *P.comata,* with a greyish pelage colour, was restricted to glacial refugias in northern Sumatra (*thomasi*), West Java (*comata*) and Borneo (*hosei*). From the Sumatran population two branches dispersed. One branch dispersed to the southern tip of the island (*femoralis* and *melalophos*) and another migrated to the Malay Peninsular

(*siamensis*) and Northwest Borneo (*frontata*). Furthermore Brandon-Jones (1996b) proposed a close affinity between *P.rubicunda* and *P.melalophos* in terms of behavioral characters. Thus *P.melalophos*, *P.femoralis*, *P.frontata and P.rubicunda* represent the most derived forms in Brandon-Jones's model of coat coloration, all with red/brown pelage coloration.

According to the principles of the metachromism model of Hershkovitz (1968), where coat colours advance from a primitive agouti pattern via blackish / brown to more derived colours like red or white (but see Lawlor 1969), Groves (1989) proposed his centrifugal speciation hypothesis for *Presbytis*. With regards to the basal position of *P.potenziani* he agreed with Brandon-Jones. In Groves' model, the greyish taxa *comata, thomasi* and *hosei*, which evolved from the black *P.potenziani*, are found at the periphery of the present geographical range of the genus. The derived red / brown forms are central in distribution (*melalophos, femoralis, frontata, rubicunda*).

Based on the models of Brandon-Jones and Zain, while additionally analysing craniometrical data, Meijaard and Groves (2004) proposed that an ancestral species crossed Sumatra, Java and Borneo during an early/middle Pleistocene glacial period. In contrast to the model of Brandon Jones, Meijaard and Groves' hypothesis claims that the ancestral *potenziani* evolved on Sumatra and spread to the Mentawai islands. *P.potenziani* subsequently became extinct on mainland Sumatra. Furthermore they suggested an early split of *thomasi* from a previous form. In addition to that and as a direct result of the dispersal of its ancestor, *P.melalophos* evolved exclusively on Sumatra in their model.

In summary, Meijaard, Groves and Brandon-Jones agree about the basal phylogenetic position of *P.potenziani* based on craniometrical data and on coat colouration respectively. In marked contrast to the other models, Zains' results show a dispersal pattern of the genus which is opposite to the direction of all other reconstructions.

1.3 Taxonomy and species concepts

Species are the units of classification, biogeography and conservation; as they must be defined as objectively as possible (De Queiroz 2005; Groves 2004). Every classification is a hypothesis, which relies not only on the type and quantity of data available, but also on a framework, called "species concept" (Groeneveld 2008). The question "What is a species?" is maybe one of the most controversial discussed issues in biology. It is not the aim of this study to discuss pros and cons of certain species concepts, but we had to decide for a framework based on which we could draw our taxonomic conclusions. The tradional Biological Species Concept (BSC) by Mayr (1963) is proven to be insufficient (De Queiroz 2007). If two populations are sympatric and maintain their separateness, this is of course evidence that they are distinct species and the BSC applies well. But as mentioned above, many populations of *Presbytis* are allopatric and this is the critical argument, where the BSC leaves the vast majority of the natural world unclassifiable (Groves 2004). Therefore we will apply the so called "Phylogenetic Species Concept" (PSC) proposed by Cracraft (1983, 1989), which defines a species as "*the smallest cluster of individual organisms within there is a parental pattern of ancestry and descent and that is diagnosbly distinct from other such clusters by a unique combination of fixed character states*". The major advantages are 1) that the PSC is completely objective and therefore falsifiable, 2) it is free of speculations about mechanisms of speciation, even in the case of species of hybrid origin and 3) the recognition of species depends on whether the differences are fixed, not on the degree or amount of differences (Groves 2004). This might be the reason why the PSC is widely applied in most recent studies on primate taxonomy, for instance in *Callicebus* (van Roosmalen et al. 2002), Saguinus (Matauschek et al. 2011), *Microcebus* or *Lepilemur* (Russell et al. 2010; Tattersall 2007).

1.4 Objectives and specific questions

With eight species listed among the 25 most endangered primates in the world, almost one third are colobine monkeys (Mittermeier et al. 2009), hence it is suprising, that

colobines still receive much less scientific attention as opposed to other primates, especially their close cercopithecine relatives and the apes (Davies and Oates 1994). Their neglect relative to cercopithecines since the early 1960s is probably partly a consequence of some of their ecological differences. In contrast to cercopithecines, the great majority of colobines live in moist forests, in which observational studies are more difficult than in more open woodland or savanna habitats. Additionally many colobine populations occur in countries that have presented serious political or logistical obstacles to field research (Davies and Oates 1994).

Regarding *Presbytis* phylogeny and taxonomy, there is a particular lack of knowledge. As mentioned above, almost all previous studies are mainly based on anatomical features, while molecular genetic studies are extremely limited and relied heavily on samples of captive animals. Furthermore the majority of *Presbytis* taxa live in Indonesian territories and were to date not adequately addressed. For instance genetic data on key taxa such as *P.potenziani*, which are crucial for the reconstruction and understanding of *Presbytis* phylogeny, are completely lacking. Therefore, and from the above outlined state of art the present study was designed with the overall goal to clarify colobine monkey taxonomy, phylogeny and phylogeography with particular emphasis on the genus *Presbytis*. I conducted two comprehensive field surveys on Java, Sumatra and the Mentawai Islands. During a period of 13 month I collected fecal samples for genetic analysis and recorded male loud-calls for an acoustic study of wild non-habituated *Presbytis* populations. Additionally I collected fecal samples from captive animals and tissue samples of museum specimen. In total I achieved samples of more than 30 locations representing eight of the ten species (Groves 2001). The following main questions arose and are addressed in this dissertation: newly collected data from the field were used to answer the following questions:

1. **What can be concluded from the re-examination of the leaf monkey phylogeny regarding *Presbytis*?**

- What is the phylogenetic position of *Presbytis* among the Asian colobines?

- Does the colobine monkey phylogeny support a possible hybridization between *Presbytis* and *Trachypithecus*, or between *Semnopithecus* and *Trachypithecus* (Ting et al. 2008)?

2. Are previous classifications of *Presbytis* supported by new acoustic and genetic data from the field?

- Is *P.comata* a polytypitc species group (Brandon-Jones 1978, 1996c)?
- Is *P.melalophos* a polytypic species group (Md Zain 2001)?
- Is there a conspecific relationship between *P.potenziani* and *P.thomasi* (Wilson and Wilson 1976)?

3. Can existing phylogeographic hypothesis of *Presbytis* be supported by divergence time estimates and a reassessed phylogeny of the genus?

- When did the genus *Presbytis* evolve?
- Do our results support an east/west (Brandon-Jones 1978; Brandon-Jones 1996a; Meijaard and Groves 2004) or a west/east dispersal pattern of *Presbytis* (Md Zain 2001)?
- Are there any explanations for *Presbytis'* allopatry on Sumatra and sympatry on Borneo?

The following chapters address the above mentioned main questions:

Chapter 2 gives insights into the phylogenetic relationships between colobine monkey genera on a broader scale. To address the first main question, a presence/absence analysis of mobile elements is combined with the analysis of autosomal, X-chromosomal, Y-chromosomal and complete mitochondrial genome sequence data derived from samples of all recognized colobine monkey genera. Additionally divergence time estimates of all taxa are calculated. The following Chapters focus on the genus *Presbytis*. To address the second and third main questions, two different ap-

proaches are used: A molecular genetic approach (chapter 3) and an acoustic approach (chapter 4). **Chapter 3** deals with the mitochondrial phylogeny of *Presbytis* based on a sequence data analysis of the cytochrome b and the hypervariable region I. Aditionally divergence time estimates were calculated for all taxa. In **Chapter 4** acoustic data in combination with genetic data is used to reassess our findings of chapter 3, based on a detailed structural analysis of male loud-calls and correlations between acoustic, genetic and geographic distances.

Chapter 2 Nuclear versus mitochondrial DNA: evidence for hybridization in Colobine monkeys

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Nuclear versus mitochondrial DNA: evidence for hybridization in colobine monkeys

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Abstract

Background: Colobine monkeys constitute a diverse group of primates with major radiations in Africa and Asia. However, phylogenetic relationships among genera are under debate, and recent molecular studies with incomplete taxon-sampling revealed discordant gene trees. To solve the evolutionary history of colobine genera and to determine causes for possible gene tree incongruences, we combined presence/absence analysis of mobile elements with autosomal, X chromosomal, Y chromosomal and mitochondrial sequence data from all recognized colobine genera.

Results: Gene tree topologies and divergence age estimates derived from different markers were similar, but differed in placing *Piliocolobus/Procolobus* and langur genera among colobines. Although insufficient data, homoplasy and incomplete lineage sorting might all have contributed to the discordance among gene trees, hybridization is favored as the main cause of the observed discordance. We propose that African colobines are paraphyletic, but might later have experienced female introgression from *Piliocolobus/Procolobus* into *Colobus* . In the late Miocene, colobines invaded Eurasia and diversified into several lineages. Among Asian colobines, *Semnopithecus* diverged first, indicating langur paraphyly. However, unidirectional gene flow from *Semnopithecus* into *Trachypithecus* via male introgression followed by nuclear swamping might have occurred until the earliest Pleistocene.

Conclusions: Overall, our study provides the most comprehensive view on colobine evolution to date and emphasizes that analyses of various molecular markers, such as mobile elements and sequence data from multiple loci, are crucial to better understand evolutionary relationships and to trace hybridization events. Our results also suggest that sex-specific dispersal patterns, promoted by a respective social organization of the species involved, can result in different hybridization scenarios.

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2.1.1 Ringkasan

Latar Belakang: Monyet colobine terdiri atas kelompok berbagai jenis primata yang memiliki radiasi-radiasi utama di Afrika dan Asia. Namum denikian, hubungan filogenetik antara marga masih diperdebatkan, dan studi-studi molekuler dengan penarikan contoh taxon yang tidak lengkap mengungkapkan adanya pohon gen yang tidak harmonis. Untuk menjelaskan sejarah evolusi marga colobine dan menentukan penyebab dari kemungkinan adanya ketidaksamaan pohon genetik, kami mengombinasikan analisis presence/absence dari unsur-unsur begerak dengan autosomal, X chromosomal, Y chromosomal dan data sekuens mitokondria dari semua marga colobine yang sudah dikenal.

Hasil: Topologi pohon genetik dan dugaan umur divergensi diduga dari berbagai penanda yang sama, tetapi berbeda dalam penempatan *Piliocolobus/Procolobus* serta marga lutung di antara colobine. Meskipun data yang ada tidak mencukupi, homoplasi dan pemilahan garis keturunan kemungkinan berperan dalam ketidaksesuaian antara pohon genetik, hibridisasi dipilih sebagai penyebab utama ketidaksesuaian yang tampak. Menurut kami, colobine Afrika adalah paraphyletik, tetapi mungkin selanjutnya terjadi penggabungan gen betina dari *Piliocolobus/Procolobus* menjadi Colobus. Pada akhir Miosen, colobine menginvasi Eurasia dan memecah menjadi beberapa garis keturunan. Di antara colobine Asia, *Semnopithecus* yang pertama kali terpisah, menunjukkan parafili lutung. Namun, aliran gen searah dari *Semnopithecus* menjadi *Trachypithecus* melalui penggabungan gen jantan dengan nuklir swamping mungkin telah terjadi hingga awal Pleistosen

Kesimpulan: Secara umum, studi kami menyajikan bahasan yang paling komprehensif sampai saat ini tentang evolusi colobine dan menekankan bahwa analisis berbagai penanda molekuler, misalnya unsur-unsur bergerak dan data sekuens dari loci ganda, sangalah penting untuk lebih memahami hubungan evolusioner dan untuk melacak peristiwa hibridisasi. Hasil kami juga menunjukkan bahwa pola-pola dispersal yang

khas menurut jenis kelamin, didukung oleh organisasi sosial dari suatu jenis dapat berakibat pada skenario hibridisasi yang berbeda untuk jenis tersebut.

2.2 Introduction

With more than 50 species and due to some ecological adaptations, such as a ruminant-like chambered stomach to digest food rich in fiber, the Old World monkey subfamily Colobinae represents a diverse and enigmatic group of primates (Groves 2001; Oates and Davies 1994). Colobines are predominantly arboreal and occur in forest and woodland habitats. They have experienced two major radiations, one in Africa with the genera *Procolobus*, *Piliocolobus* and *Colobus*, and a second in South and Southeast Asia comprising the langur genera *Semnopithecus*, *Trachypithecus* and *Presbytis,* and the odd-nosed monkey genera *Rhinopithecus*, *Pygathrix*, *Nasalis* and *Simias* (Groves 2001). However, their phylogenetic relationships are disputed (Groves 1989; Jablonski 1998; Jablonski et al. 1999; Stewart and Disotell 1998; Zhang and Ryder 1999), and recent molecular studies detected substantial gene tree discordance (Osterholz et al. 2008; Sterner et al. 2006; Ting et al. 2008).

Traditionally, African and Asian genera are believed to form reciprocally monophyletic groups (Groves 2001; Napier and Napier 1967; Oates et al. 1994; Szalay and Delson 1979), though paraphyly has also been proposed (Groves 1989; Jablonski 1998; Jablonski et al. 1999). Molecular investigations clearly confirm a common origin of Asian colobines and the odd-nosed monkey group (Osterholz et al. 2008; Sterner et al. 2006; Ting et al. 2008), but evidence for monophyly of the langur group as well as for African colobines is still lacking. Moreover, nuclear and mitochondrial data indicate conflicting relationships among langur genera, and between langurs and the odd-nosed monkeys (Osterholz et al. 2008; Sterner et al. 2006; Ting et al. 2008). While nuclear data consistently link *Semnopithecus* and *Trachypithecus* to the exclusion of all other Asian colobines (Osterholz et al. 2008; Ting et al. 2008), mitochondrial data either do not resolve

these relationships (Osterholz et al. 2008) or suggest a clade consisting of *Presbytis* and *Trachypithecus* (Sterner et al. 2006).

Incongruent phylogenetic relationships among genes, like those detected among colobines are common in phylogenetic studies and could be explained by homoplasy, insufficient data, nucleotide composition, differential lineage sorting, or hybridization (Avise 2004; Barton 2001; Funk and Omland 2003; Koblmüller et al. 2007; McCracken and Sorenson 2005; Nichols 2001; Philippe and Laurent 1998; Pollard et al. 2006; Seehausen 2004). To ascertain which of these possibilities are responsible for the incongruence, information from various independent molecular loci can be helpful (Petit and Excoffier 2009). To date, only mitochondrial and X chromosomal data as well as presence/absence information of mobile elements, all based on an incomplete taxon sampling, are available for comparative phylogenetic studies in colobines (Osterholz et al. 2008; Sterner et al. 2006; Ting et al. 2008; Xing et al. 2005). Among all marker systems, mobile element insertions are a promising tool to uncover phylogenetic relationships among colobine genera. Compared to sole sequence data, mobile elements such as Short Interspersed Elements (SINEs) and Long Interspersed Elements (LINEs) exhibit advantages which make them ideal markers for phylogenetic reconstructions (for review see (Batzer and Deininger 1991; Okada 1991; Ray et al. 2006; Salem et al. 2005; Schmitz et al. 2005; Shedlock and Okada 2000; Van de Lagemaat et al. 2005)). Accordingly, mobile elements are successfully applied in numerous primate phylogenetic studies (Herke et al. 2007; Li et al. 2009; Osterholz et al. 2008, 2009; Ray et al. 2005; Roos et al. 2004; Salem et al. 2003; Schmitz et al. 2001; Schmitz et al. 2005; Xing et al. 2007a; Xing et al. 2007b).

In our study, we examined the presence/absence pattern of mobile elements and compared the inferred phylogeny with those derived from mitochondrial and nuclear sequence data (in total ~30,000 bp per genus). We extended available X chromosomal and mitochondrial genome data, and sequenced de novo five autosomal loci that map to different human chromosomes, and six Y chromosomal loci from all ten colobine genera. By combining results from different marker systems, we provide detailed in-

sights into the evolutionary and biogeographic history of colobine monkeys, and show that different hybridization mechanisms might have been involved during the colobine radiation.

2.3 Methods

2.3.1 Sample collection and DNA extraction

Blood, tissue or fecal samples from representatives of all ten colobine genera (*Colobus*, *Piliocolobus, Procolobus, Presbytis, Trachypithecus, Semnopithecus, Rhinopithecus, Pygathrix, Nasalis, Simias*) and several non-colobine taxa (*Macaca, Papio, Theropithecus, Chlorocebus, Pongo, Pan*) were obtained from specimens kept in zoos or breeding facilities, or collected in the field (Table 2.1). Sample collection was conducted according to relevant German and international guidelines, including countries where we collected samples. Fecal samples were collected in a non-invasive way without disturbing, threatening or harming the animals. Blood samples were taken by veterinarians for diagnostic reasons to check the health status of the respective individuals, and tissue samples were obtained only from deceased specimens. Total genomic DNA was extracted with the DNeasy Blood & Tissue or QIAamp DNA Stool Mini kits from Qiagen following standard procedures.

2.3.2 Analysis of mobile elements

Due to their high copy number (~one million) and relatively small size (~300 bp), the primate specific *Alu* elements were selected as molecular-cladistic markers. The presence or absence of mobile elements in different colobines at specific loci was tested via PCR using primers occupying the flanking region of the insertion site. Details on analyzed loci, primers and presence/absence pattern of mobile elements in studied species are listed in Additional File 1. For most loci, sequencing was neglected, but in relevant cases the insertion orthology was confirmed by sequencing, and direct repeats flanking the insertion as well as the original target site prior to transposition were traced.

Table 2.1: Origin and sample type of studied species

species	origin	sample type
Colobus guereza	Cologne zoo, Germany	tissue
Piliocolobus badius	Taï National Park, Ivory Coast	tissue
Procolobus verus	Taï National Park, Ivory Coast	tissue
Semnopithecus entellus	Dresden zoo, Germany	blood
Trachypithecus obscurus	Wuppertal zoo, Germany	blood
Presbytis melalophos	Howletts Wild Animal Park, Great Britain	tissue
Pygathrix nemaeus	Cologne zoo, Germany	tissue
Rhinopithecus avunculus	Endangered Primate Rescue Center, Vietnam	tissue
Nasalis larvatus	Wilhelma Stuttgart, Germany	blood
Simias concolor	Siberut Conservation Programme, Indonesia	feces
Macaca sylvanus	Nuremberg zoo, Germany	blood
Papio hamadryas	Munich zoo, Germany	blood
Theropithecus gelada	Duisburg zoo, Germany	blood
Chlorocebus aethiops	Paul-Ehrlich-Institute, Germany	blood
Pongo abelii	Nuremberg zoo, Germany	blood
Pan troglodytes	Munich zoo, Germany	blood

In our study, we included published markers (Herke et al. 2007; Osterholz et al. 2008; Xing et al. 2005), which were further examined in previously untested genera, and newly detected integration loci (Additional File 1). Therefore, we performed subtractive hybridizations following described methods (Osterholz et al. 2008). To avoid biased hybridization results, various species combinations were used as tracer and driver (hybridization 1: tracer *Nasalis/Pygathrix*, driver *Presbytis*; hybridization 2: tracer *Nasalis/Pygathrix*, driver *Semnopithecus*; hybridization 3: tracer *Trachypithecus/Presbytis*, driver *Pygathrix*; hybridization 4: tracer *Presbytis*, driver *Semnopithecus*; hybridization 5: tracer *Piliocolobus/Colobus*, driver *Pygathrix*). Besides *Alu* insertions, a LINE present in *Piliocolobus* and *Procolobus* in the studied Xq13.3 fragment was additionally applied as marker (Additional File 1).

Phylogenetic reconstructions using the MP algorithm were conducted in PAUP v4.0b10 (Swofford 2003). Presence of an integration was coded as 1, its absence as 0, and missing data as '?'. Internal node support was obtained via a heuristic search with 10,000 bootstrap replications. To evaluate the reliability of the depicted relationships among colobines, various alternative tree topologies were assessed with the Kishino-Hasegawa test (Kishino and Hasegawa 1989) with full optimization and 1,000 bootstrap replications in PAUP.

2.3.3 Amplification and sequencing of nuclear loci

Inter-exonic intron and exonic sequences were generated for six single-copy genes of the Y chromosome, five autosomal loci, and a fragment of the X chromosomal Xq13.3 region. With exception of the *SRY* gene (sex-reversal, Y chromosome), all other Y chromosomal loci (*DBY5*: Dead Box, intron 5; *SMCY7*: SMC mouse homologue, intron 7; *SMCY11*: SMC mouse homologue, intron 11; *UTY18*: ubiquitous TPR motif, intron 18; *ZFYLI*: Zinc finger, last intron) have homologues on the X chromosome (X degenerate). As autosomal loci, we selected intron 11 of the von Willebrand Factor (*vWF11*), located on human chromosome 12, intron 3 of the serum albumin gene (*ALB3*, human chromosome 4), intron 3 of the interstitial retinol-binding protein (*IRBP3*, human chromosome 10), intron 1 of the transition protein 2 (*TNP2*, human chromosome 16) and intron 1 of the transthyretin gene (*TTR1*, human chromosome 18). *SRY, DBY5, SMCY7, SMCY11, UTY18, vWF11* and a ~4,300 bp fragment of the Xq13.3 region were amplified using primers and PCR conditions as described (Chaves et al. 1999; Hellborg and Ellegren 2003; Ting et al. 2008; Tosi et al. 2005; Whitfield et al. 1993) (Additional File 2). For the amplification of *ZFYLI, ALB3, IRBP3, TNP2* and *TTR1*, new primers (Additional File 2) were designed on the basis of available primate sequences in GenBank. PCR conditions for the latter comprised a pre-denaturation step at 94°C for 2 min, followed by 40 cycles each with denaturation at 94°C for 1 min, annealing at varying temperatures (Additional File 2) for 1 min, and extension at 72°C for 2 min. At the end, a final extension step at 72°C for 5 min was added. The results of all PCR amplifications were checked on 1% agarose gels. PCR products were cleaned with the Qiagen PCR Purifica-

tion kit and subsequently sequenced on an ABI 3130xl sequencer using the BigDye Terminator Cycle Sequencing kit. Alignments and sequences are available in TreeBASE (http://purl.org/phylo/ treebase/phylows/study/TB2:S11179) and GenBank, respectively (for GenBank accession numbers see Additional File 3).

2.3.4 Amplification and sequencing of mitochondrial genomes

To reduce the likelihood of amplifying nuclear pseudogenes (numts), complete mitochondrial genomes from four colobine genera (*Rhinopithecus*, *Pygathrix*, *Nasalis*, *Procolobus*) were generated following an approach in which two overlapping ~10,000 bp long fragments were amplified via long-range PCR (Raaum et al. 2005; Sterner et al. 2006). Due to degradation of DNA extracted from faeces, the mitochondrial genome of *Simias* was amplified via five overlapping fragments, each with a size of ~5,000 bp. All long-range PCRs were performed with the SuperTaq Plus polymerase from Ambion following protocols of the supplier and primers as described (Raaum et al. 2005; Sterner et al. 2006). Long-range PCR amplicons were separated on 1% agarose gels, excised from the gel, purified with the Qiagen Gel Extraction kit and used as template for nested PCRs. PCR conditions for all nested PCR amplifications were identical and comprised a pre-denaturation step at 94°C for 2 min, followed by 30 cycles each with denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1.5 min. At the end, a final extension step at 72°C for 5 min was added. Nested PCR products (900-1,200 bp in length) were cleaned with the Qiagen PCR Purification kit and sequenced on an ABI 3130xl sequencer. Sequences were assembled with Geneious v4.6.1 (Drummond et al. 2008). No inconsistent positions in overlapping regions were detected and all protein-coding genes were correctly translated. Annotation of mitochondrial genomes was conducted with the online program DOGMA (Wyman et al. 2004) and manually inspected. Alignment and sequences are available in TreeBASE (http://purl.org/phylo/treebase/phylows/ study/TB2:S11179) and GenBank, respectively (for GenBank accession numbers see Additional File 3).

2.3.5 Statistical analysis of sequence data

For phylogenetic reconstructions, all datasets comprised 17 sequences including each one representative of the ten colobine genera (*Colobus*, *Piliocolobus*, *Procolobus*, *Trachypithecus*, *Semnopithecus*, *Presbytis*, *Rhinopithecus*, *Pygathrix*, *Nasalis*, *Simias*), four cercopithecine genera (*Papio*, *Theropithecus*, *Macaca*, *Chlorocebus*), and three hominoid genera (*Homo*, *Pan*, *Pongo*), which were used as outgroup taxa. To complete datasets, we partly implemented sequences from GenBank (Additional File 3). Alignments for individual loci were generated with MAFFT v6 (Katoh et al. 2005) and corrected by eye. In all alignments, poorly aligned positions and indels were removed with Gblocks v0.91b (Castresana 2000) using default settings. For the mitochondrial dataset, also the D-loop region was excluded (dataset mtDNA1) and a second alignment, generated in Mesquite v2.6 (Maddison and Maddison 2009), included solely protein-coding genes (dataset mtDNA2). For all datasets, uncorrected pairwise differences were estimated in PAUP. Nucleotide composition for all and only parsimony-informative positions for the combined nuclear and both mitochondrial alignments was also estimated in PAUP. To test whether datasets can be combined, we performed partition homogeneity tests in PAUP with 10,000 replications.

Phylogenetic trees were constructed with MP and NJ algorithms as implemented in PAUP as well as with ML and Bayesian algorithms, using the programs GARLI v0.951 (Zwickl 2006) and MrBayes v3.1.2 (Huelsenbeck et al. 2001; Ronquist and Huelsenbeck 2003). 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, ML and Bayesian reconstructions, the optimal nucleotide substitution models for each locus and concatenated datasets were chosen using AIC as implemented in MODELTEST v3.7 (Posada and Crandall 1998). Relative support of internal nodes was assessed by bootstrap analyses with 10,000 (MP, NJ) or 500 replications (ML). In GARLI, only the model specification settings were adjusted according to the respective concatenated dataset, while all other settings were left at their default value. ML majority-rule consensus trees were calculated in PAUP.

For Bayesian reconstructions, the datasets were partitioned treating each locus separately and each with its own substitution model. The solely protein-coding alignment of the mitochondrial genome (mtDNA2) was partitioned into codon positions. We used four independent Markov Chain Monte Carlo (MCMC) runs 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. PPs for each split and a phylogram with mean branch lengths were calculated from the posterior density of trees.

To evaluate the reliability of obtained relationships among colobines, various alternative tree topologies were tested with the Shimodaira-Hasegawa test (Shimodaira and Hasegawa 1999) with full optimization and 1,000 bootstrap replications in PAUP.

2.3.6 Divergence age estimation

A Bayesian MCMC method, which employs a relaxed molecular clock approach (Drummond et al. 2006), as implemented in BEAST v1.4.8 (Drummond and Rambaut 2007), was used to estimate divergence times. Therefore, a relaxed lognormal model of lineage variation and a Yule prior for branching rates was assumed. Divergence times were calculated for each locus separately and for the combined nuclear dataset. The latter was partitioned treating each locus as distinct unit. The mitochondrial alignment comprising solely protein-coding genes (mtDNA2) was partitioned into codon positions and the substitution model, rate heterogeneity and base frequencies were unlinked across codon positions. Optimal nucleotide substitution models were chosen using AIC in MODELTEST.

As calibrations we used the fossil-based divergence between *Homo* and *Pan*, which has been dated at 6-7 mya (Brunet et al. 2005; Lebatard et al. 2008; Vignaud et al. 2002), the separation of *Pongo* from the *Homo*/*Pan* lineage ~14 mya (Kelley 2002), the split

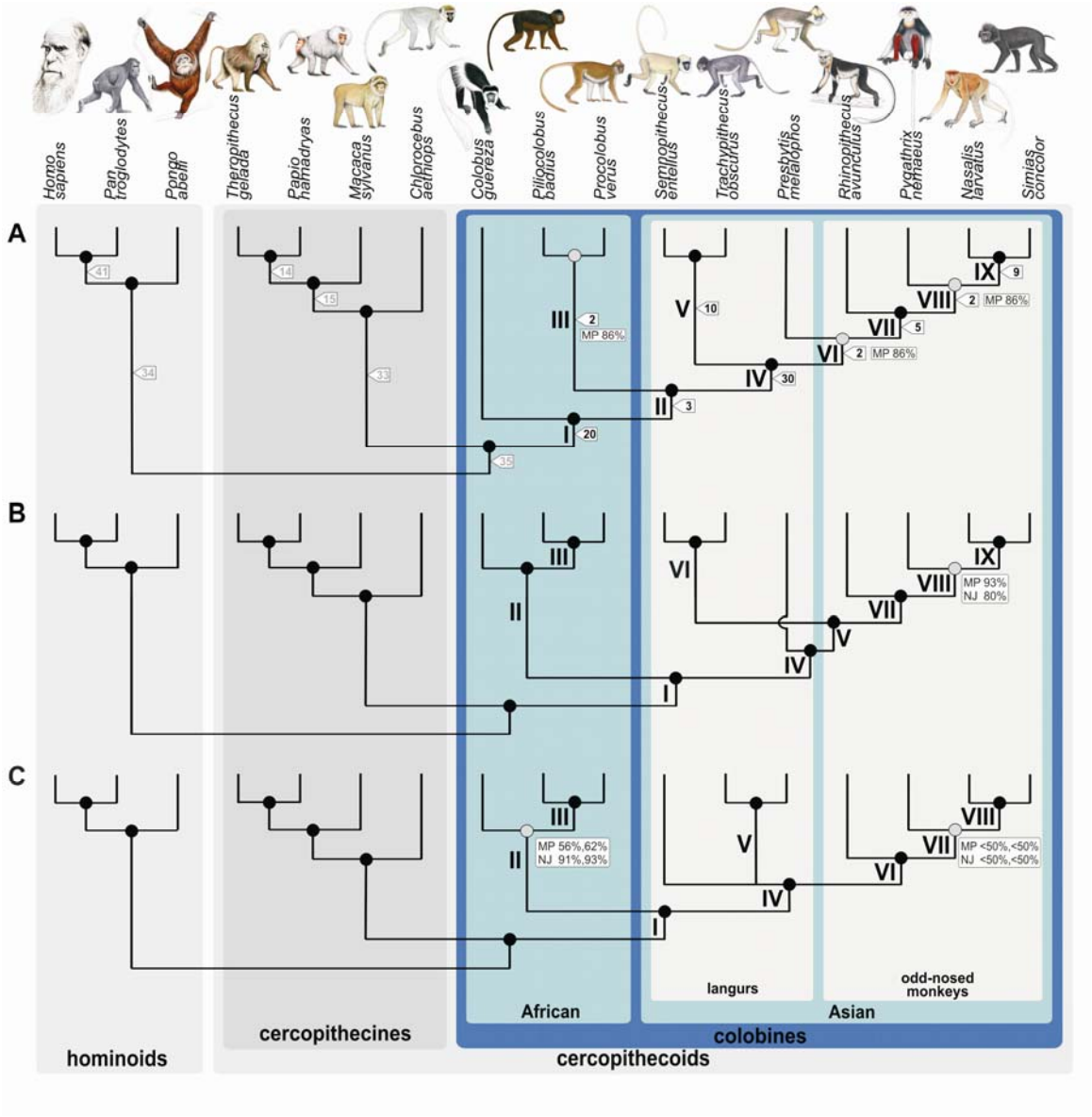

Figure 2.1: Phylogenetic relationships among colobine and outgroup genera as inferred from different datasets. Panels refer to insertions of mobile elements (A), combined nuclear sequence data (B), and mitochondrial genome data (C). Roman numbers are used as branch identifiers and are discussed in the text. In A, numbers in flags represent the number of available mobile elements (black: colobine markers, grey: non-colobine markers). In B and C, all nodes are significantly supported by ML and Bayesian reconstructions (≥95%, 1.0). Black and grey dots on nodes indicate high (≥95%) and lower (<95%) branch support as obtained from MP (in A-C) and NJ (in B and C) reconstructions, respectively. Bootstrap values <95% are presented at respective nodes. In C, first and second values refer to those obtained from reconstructions using datasets mtDNA1 and mtDNA2, respectively.

between *Theropithecus* and *Papio* ~4 mya (Delson 2000; Leakey 1993), and the divergence of hominoids and cercopithecoids ~24 mya (Benefit and McCrossin 2002; Young and MacLatchy 2004; Zalmout et al.). Instead of hardbounded calibration points, we used the published dates as a normal distribution prior for the respective node.

For the *Homo* - *Pan* divergence, this translates into a normal distribution with a mean of 6.5 mya and a standard deviation (SD) of 0.5 mya, for the separation of *Pongo* from the *Homo*/*Pan* clade into a mean of 14.0 mya and a SD of 1.0 mya, for the *Theropithecus* - *Papio* split into a mean of 4.0 mya and a SD of 0.5 mya, and for the hominoid - cercopithecoid divergence into a mean of 24 mya and a SD of 2 mya.

Since the estimation of phylogenetic relationships was not the main aim of this analysis, we used an apriori fixed tree topology as obtained from mobile elements (Figure 2.1 A) for the calculation from nuclear sequence data. Four replicates were run for 10,000,000 generations with tree and parameter sampling occurring every 100 generations. The adequacy of a 10% burnin and convergence of all parameters were assessed by visual inspection of the trace of the parameters across generations using TRACER v1.4.1 (Rambaut and Drummond 2007). Subsequently, the sampling distributions were combined (25% burnin) using the software LogCombiner v1.4.8 and a consensus chronogram with node height distribution was generated and visualized with TreeAnnotator v1.4.8 and FigTree v1.2.2 (Rambaut 2008).

2.3.7 Inferring hybridization in the presence of incomplete lineage sorting

Statistical support for putative hybridization scenarios was assessed with the method proposed by Kubatko (2009), in which statistical model selection techniques (e.g., AIC) are used to compare species trees that may or may not include hybridization scenarios. For our data, we hypothesized two possible hybridization events (for details see Results). The estimated gene trees used as input were those derived from single locus tree reconstructions (Additional File 4) and branch lengths as estimated in BEAST. To estimate evolutionary rates for individual loci, we followed the suggestion of Yang

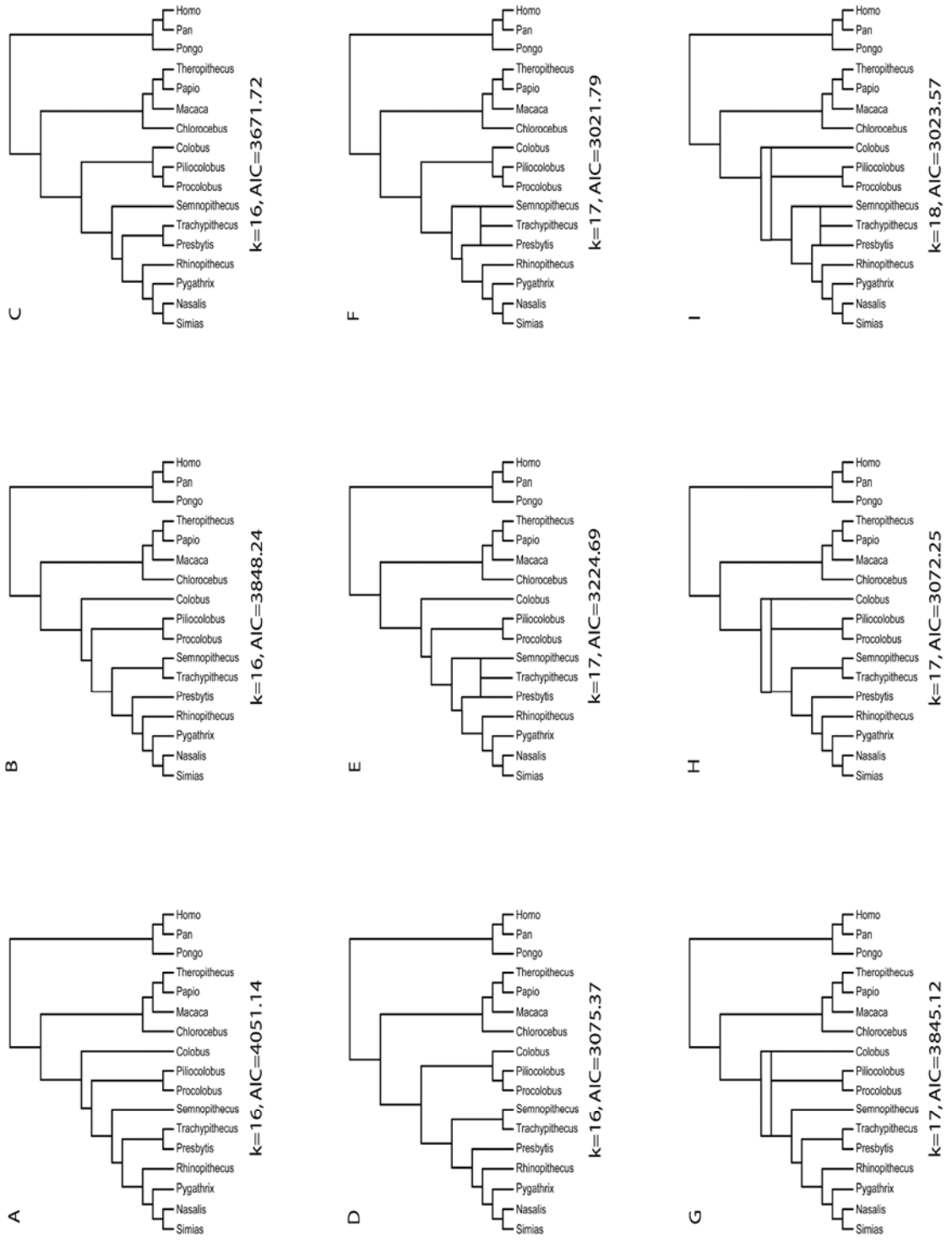

Figure 2.2: The nine alternative hybridization scenarios compared in the coalescent framework. Beneath each tree, the number of parameters in the model (k) is given as well as the AIC. The lowest AIC values are observed for trees F and I, which indicate a similar fit fort hese scenarios.

(2002) (see also (Kubatko et al. 2009)) and computed for each gene the average pairwise sequence divergence of each ingroup (colobine) sequence to the outgroup (non-colobine) taxa.

We then assigned to each locus a rate that was calculated by dividing the mean pairwise divergence for that locus by the median of the entire set of pairwise divergences. To convert gene tree branch lengths to coalescent units, we considered two effective population sizes, 50,000 and 100,000, and used a generation time of 5 years. Since the results were identical in terms of the trees preferred, we show here the results only for effective population size 50,000. For haploid loci (mitochondrial genome, Y chromosomal loci), we additionally divided the rate by 2 (see (Kubatko et al. 2009)). We compared a total of nine species trees (four corresponding to no hybridization, four corresponding to single hybridization events, and one that included both hybridization scenarios, Figure 2.2). The AIC was computed for each tree using the STEM software (Kubatko et al. 2009). Models with AIC values within 2 of one another were regarded as providing similar fit to the data (Burnham and Anderson 2002).

2.4 Results

2.4.1 Nuclear phylogeny

Eighty-three mobile elements are phylogenetically informative for colobines (Figure 2.1 A, Additional File 1). Each of the following clades is strongly supported by at least five integrations: all colobines (clade I [A-I]), Asian colobines (A-IV), odd-nosed monkeys (A-VII), *Trachypithecus* and *Semnopithecus* (A-V), and *Nasalis* and *Simias* (A-IX). Three integrations were found in *Piliocolobus* and *Procolobus* and all Asian colobines (A-II), but not in *Colobus*. Two insertions suggested a sister grouping of *Procolobus* and *Piliocolobus* (A-III), *Presbytis* and the odd-nosed monkeys (A-VI), and a basal position of *Rhinopithecus* among the latter (A-VIII). Based on maximum-parsimony (MP) bootstrap analysis, most relationships were strongly supported (≥95%). Only the *Pilio-*

colobus/*Procolobus* (A-III), *Presbytis*/odd-nosed monkey (A-VI), and *Pygathrix*/*Nasalis*/*Simias* (A-VIII) clades gained relatively weak bootstrap values (86%). Based on alternative tree topology tests, different positions of the *Piliocolobus/Procolobus* clade and *Presbytis* among colobines were not rejected ($P > 0.05$), while relationships other than the most likely one were significantly rejected for all other taxa ($P < 0.001$, $P < 0.05$).

Next, we performed phylogenetic analyses based on the concatenated nuclear sequence dataset, including five autosomal loci, six Y chromosomal loci and a fragment of the X chromosomal Xq13.3 region. We combined all nuclear sequence data, because heuristic search methods for individual loci produced no conflicting relationships (Additional File 4), and partition homogeneity tests revealed no significant difference in their evolutionary history (Y chromosomal loci combined: $P = 0.2939$; autosomal loci combined: $P = 0.1543$; all nuclear loci combined: $P = 0.3559$). Nucleotide composition of studied species was similar. Phylogenetic reconstructions yielded identical and significantly supported branching patterns irrespectively of the applied algorithm (MP, neighbor-joining [NJ], maximum-likelihood [ML], Bayesian) (Figure 2.1 B). Only the *Pygathrix*/*Nasalis*/*Simias* (B-VIII) clade had lower support values (MP: 93%, NJ: 80%, but ML: 98%, Bayesian posterior probabilities [PP]: 1.0). The resultant tree topology was mainly congruent with the mobile element-based phylogeny, but two cases of incongruence were obvious. First, in the nuclear sequence-based phylogeny, African (B-II) and Asian (B-IV) colobine genera formed reciprocally monophyletic clades and second, *Presbytis* represented a sister lineage to the other Asian genera (B-V). According to alternative tree topology tests, paraphyly of African colobines with *Piliocolobus*/*Procolobus* being closer related to Asian colobines than to *Colobus* as well as various alternative positions of *Presbytis* among Asian colobines were not rejected ($P > 0.05$). However, affiliations of *Presbytis* to either *Semnopithecus* or *Trachypithecus* were rejected ($P < 0.001$).

Estimated divergence ages from the combined nuclear dataset (Table 2.2) and single loci (Additional File 5), both based on an a-priori fixed tree topology as obtained from

mobile elements, differed slightly, most likely due to the general low variability in the studied loci. However, estimates were in the same range suggesting that loci evolve at similar evolutionary rates. According to our nuclear estimates, *Colobus* and *Piliocolobus/Procolobus* successively split off from Asian genera 10.93 million years ago (mya) and 10.73 mya, respectively (for 95% highest posterior densities see Table 2.2). The latter two separated 6.92 mya. In Asia, an initial split occurred 8.12 mya and led to a clade consisting of *Trachypithecus* and *Semnopithecus,* and a group containing *Presbytis* and the odd-nosed monkeys. Among the latter, *Presbytis* diverged 7.96 mya and the odd-nosed monkeys began differentiating 6.43 mya. The most recent splits among Asian genera occurred between *Trachypithecus* and *Semnopithecus* (2.56 mya) and between *Nasalis* and *Simias* (1.06 mya).

2.4.2 Mitochondrial phylogeny

Mitochondrial and nuclear datasets were not combined, because the partition homogeneity test suggested that both track different evolutionary histories (P = 0.0002). Thus, mitochondrial sequence data were analyzed separately. For both alignments (mtDNA1, mtDNA2; for details about alignments see Materials and methods), we observed a major shift in nucleotide composition between colobine and non-colobine representatives. Both alignments produced identical and significantly supported branching patterns among genera (Figure 2.1 C). Only the *Pygathrix*/*Nasalis*/*Simias* (C-VII) and African colobine (C-II) clades gained low MP (<50%, <50%, 56%, 62%) and NJ (<50%, <50%, 91%, 93%) bootstrap values, but ML and Bayesian reconstructions provided strong support for both nodes (96%, 100%; 1.0, 1.0). In principal, the tree topology was identical to those obtained from mobile elements and nuclear sequence data. However, as in the nuclear sequence tree, mitochondrial data suggested African (C-II) and Asian (C-IV) colobines as reciprocal monophyletic clades. Moreover, Asian colobines further diverged into a lineage leading to the odd-nosed monkeys (C-VI), a lineage comprising *Trachypithecus* and *Presbytis* (C-V), and finally a lineage with solely

Semnopithecus, while the relationships among these three lineages remained unresolved.

According to alternative tree topology tests, paraphyly of African colobines with *Piliocolobus/Procolobus* being closer related to Asian colobines than to *Colobus* was rejected ($P < 0.001$). Among Asian colobines, relationships in which *Trachypithecus* and *Presbytis* do not form a monophyletic clade were also rejected ($P < 0.001$, $P < 0.05$), as well as a close relationship of *Trachypithecus* and *Semnopithecus* ($P < 0.01$). In contrast, different positions of *Semnopithecus* among Asian colobines were similarly likely ($P > 0.05$).

Divergence age estimates from mitochondrial data were similar to nuclear estimates in case where identical branching patterns were obtained (Table 2.2). According to mitochondrial data, African and Asian colobine lineages were separated 10.90 mya. In Africa, *Colobus* represents the first split (8.47 mya), followed by the divergence of *Piliocolobus* and *Procolobus* (6.58 mya). The major Asian split leading to the three lineages *Semnopithecus, Trachypithecus/Presbytis* and the odd-nosed monkeys occured 8.91 mya.

Trachypithecus diverged from *Presbytis* 7.45 mya. The diversification of odd-nosed monkeys into genera started 6.91 mya and ended with the split between *Nasalis* and *Simias* 1.88 mya.

Table 2.2: Estimation of divergence ages in mya (95% highest posterior density)

node	nuclear DNA	mitochondrial DNA
cercopithecoids – hominoids	24.39 (22.44-26.47)	23.73 (21.88-25.94)
Pongo – Homo/Pan	13.89 (12.80-14.95)	13.58 (12.51-14.64)
Homo – Pan	6.39 (5.85-7.01)	6.18 (5.62-6.70)
cercopithecines – colobines	15.50 (14.45-16.56)	15.92 (14.11-17.79)
Cholorocebus – other cercopithecines	9.47 (7.52-11.57)	10.56 (8.78-12.29)
Macaca – Papio/Theropithecus	6.59 (5.12-8.27)	8.55 (6.82-10.03)
Papio – Theropithecus	3.80 (3.20-4.38)	3.97 (3.39-4.46)
Colobus – other colobines (A-I)	10.93 (9.60-12.31)	-
Piliocolobus/Procolobus – Asian colobines (A-II)	10.73 (9.38-12.04)	-
African – Asian colobines (C-I)	-	10.90 (9.34-12.44)
Colobus – Piliocolobus/Procolobus (C-II)	-	8.47 (6.83-9.88)
Piliocolobus – Procolobus (A-III, C-III)	6.92 (4.38-9.35)	6.58 (4.99-8.04)
Asian colobines (A-IV, C-IV)	8.12 (7.14-9.16)	8.91 (7.43-10.23)
Trachypithecus – Semnopithecus (A-V)	2.56 (1.25-4.22)	-
Presbytis – odd-nosed monkeys (A-VI)	7.96 (6.93-8.95)	-
Presbytis – Trachypithecus (C-V)	-	7.45 (5.88-8.86)
odd-nosed monkeys (A-VII, C-VI)	6.43 (5.03-7.75)	6.91 (5.60-8.20)
Pygathrix – Nasalis/Simias (A-VIII, C-VII)	5.66 (4.22-7.01)	6.23 (5.11-7.38)
Nasalis – Simias (A-IX, C-VIII)	1.06 (0.44-1.81)	1.88 (1.21-2.45)

2.4.3 Inferring hybridization in the presence of incomplete lineage sorting

To assess the possible reasons for the incongruence between the nuclear and mitochondrial trees, we applied the method proposed by Kubatko (2009). The method assumes that incomplete lineage sorting (ILS) explains observed gene tree incongruence to some extent, and seeks to determine whether all variation in observed gene trees can be explained by ILS alone, as modeled by the coalescent process, or whether hybridization helps to explain significantly more the observed variation. Then, the Akaike information criterions (AIC) in each model (may or may not include hybridization scenarios) were compared to determine the best-fit model. For our data, two possible hybridization events were hypothesized. The first involved *Trachypithecus*, with parental taxa *Semnopithecus* and *Presbytis*, while the second involved the clade containing *Piliocolobus* and *Procolobus*, *Colobus* and the ancestor of Asian colobines.

By comparing the results from models with or without the hybridization events, the best-fit model (AIC = 3021.79, Figure 2.2) was a tree in which *Trachypithecus* is the result of hybridization between *Presbytis* and *Semnopithecus*. The second best-fit model (AIC = 3023.57, Figure 2.2 I) comprised the tree that includes both tested hybridization events. AIC values for all seven other models were considerably higher (3072.25 - 4051.14). Since AIC values for the scenarios presented in Figure 2.2 F and Figure 2.2 I were the lowest and were within 2 of one another, both were considered plausible explanations for the observed gene tree discordances (Burnham and Anderson 2002).

It is worth pointing out that the model used here to compute the AIC assumes that ILS is a possible source of gene trees incongruence. Since the two best-fit models include at least one hybridization event, it is clear that ILS alone does not adequately describe the extent of incongruence in the observed gene trees.

2.5 Discussion

By combining presence/absence analysis of mobile elements with autosomal, X chromosomal, Y chromosomal and mitochondrial sequence data, the present study provides comprehensive insights into the evolutionary history of colobines. Most relationships are resolved and strongly supported by mobile elements and sequence data. Moreover, relationships and estimated divergence ages as obtained from different datasets are mainly congruent and in agreement with earlier studies (Goodman et al. 1998; Karanth et al. 2008; Osterholz et al. 2008; Raaum et al. 2005; Sterner et al. 2006; Ting 2008; Ting et al. 2008; Xing et al. 2005). Our study, however, also reveals significant discrepancies among gene trees. First, mitochondrial and nuclear sequence data suggest a monophyletic African colobine clade, while mobile elements provide evidence for a closer connection of the *Piliocolobus*/*Procolobus* clade to Asian genera than to *Colobus*. Second, mobile elements indicate close relationships between *Semnopithecus* and *Trachypithecus,* and between *Presbytis* and the odd-nosed monkeys. Nuclear sequence data support the former clade, but suggest *Presbytis* as basal among Asian colobines. In contrast, in the mitochondrial phylogeny, *Presbytis* and *Trachypithecus* are displayed as sister lineages, while the position of *Semnopithecus* remains ambiguous.

2.5.1 Possible explanations for gene tree discordance

Inadequate data, homoplasy, nucleotide composition, ILS or hybridization could be potential explanations for the observed differences (Avise 2004; Barton 2001; Funk and Omland 2003; Koblmüller et al. 2007; McCracken and Sorenson 2005; Nichols 2001; Philippe and Laurent 1998; Pollard et al. 2006; Seehausen 2004). For the mitochondrial dataset, at least for the African and *Presbytis*/*Trachypithecus* clades, incorrect branching patterns due to inadequate data or homoplasy are unlikely, since sufficient phylogenetic resolution with long internal branches is obtained. Likewise, a shift in nucleotide composition and differential sorting of ancestral mitochondrial lineages is implausible. Since the major shift in nucleotide composition was detected between

colobines and non-colobines, it cannot be responsible for gene tree discordances among colobines. If the African and *Presbytis*/*Trachypithecus* clades are indeed the result of incomplete sorting of mitochondrial lineages, the mitochondrial divergence between respective genera should predate the nuclear splitting times, which is not the case (African colobines: 10.93 mya nuclear vs. 8.47 mya mitochondrial; *Presbytis* - *Trachypithecus*: 8.12 mya nuclear vs. 7.45 mya mitochondrial). However, the unresolved position of *Semnopithecus* among Asian colobines might have been affected by one or several of the above mentioned factors, or alternatively, might be the result of a true radiation-like divergence of lineages. For nuclear data, these factors are unlikely explanations as well for the branching of *Trachypithecus* and *Semnopithecus*, because ten independent insertions and sequence data from 12 nuclear loci clearly confirm their close relationship. More challenging are explanations for the discordant positions of *Presbytis* and the African genera among colobines in phylogenies revealed by mobile elements and nuclear sequence data. Mixed genomes due to differentially selected genes cannot be excluded, but interestingly, both mobile elements and nuclear sequence data (as revealed from single locus analysis) show no conflicting phylogenies themselves. Most prominent, however, the mobile element-based phylogeny is not rejected by nuclear sequence data, indicating that insufficient informative sites, as also suggested by the low resolution of phylogenetic relationships in single-locus analysis, in the latter dataset might display incorrect relationships. For the integration of mobile elements, homoplasy is typically regarded as minimal (Okada 1991; Ray et al. 2006; Schmitz et al. 2005), but ILS has been reported (Li et al. 2009; Xing et al. 2007a). Only two and three integrations support the branching of *Presbytis* with odd-nosed monkeys and the paraphyly of African colobines, and alternative relationships cannot be rejected statistically. However, no inconsistent elements were detected and subtractive hybridizations specifically set up to screen for African colobine and *Trachypithecus*/*Presbytis* monophyly markers revealed no equivalent insertions. Accordingly, ILS seems to be an unreasonable explanation for our findings. Since the mobile element-based phylogeny is not rejected by nuclear sequence data and due to their reliability as molecular-cladistic markers, the phylogeny suggested by mobile elements is assumed

to reflect the true nuclear phylogeny of colobines, although we explicitly note that mosaic genomes cannot be excluded.

Because all above-mentioned factors provide no sufficient explanation for the herein detected discordances between mitochondrial and nuclear phylogenies, we favor ancestral hybridization as the main reason for the discordant pattern. Furthermore, comparisons of models with and without hybridization in a model selection framework strongly support hybridization in the presence of ILS over models of ILS alone. In other words, even after ILS was taken into account as a factor in the observed incongruence among gene trees, we still found support for hybridization in the evolutionary history of these taxa. This refers at least to Asian colobines, but hybridization among African colobines cannot be excluded either by the method we applied here.

2.5.2 Hybridization hypothesis

Although bidirectional hybridization, which would be indicated by mixed genomes, cannot be excluded with our data, a female introgression event is hypothesized for African colobines. The direction of gene flow remains obscure due to the rapid diversification of the colobine ancestor in Africa, but female introgression from *Piliocolobus/Procolobus* into *Colobus* is indicated and gains further support by some biological data (Groves 2001; Newton and Dunbar 1994). In contrast to *Colobus*, females in *Piliocolobus* and *Procolobus* tend to leave their natal groups, which was most likely also the case in their ancestor (Newton and Dunbar 1994), and *Colobus* males are on average larger than *Piliocolobus* and *Procolobus* males (Oates et al. 1994), thus increasing the chance of hybridization between *Colobus* males and *Piliocolobus/Procolobus* females. Moreover, hybridization between both ancestral lineages is in principal possible, because (at least nowadays) they occur in sympatry over wide ranges of their distribution (Groves 2001; Oates et al. 1994). Accordingly, after the successive separation of *Colobus* and *Piliocolobus/Procolobus* from the Asian colobine ancestor, *Piliocolobus/Procolobus* females might have entered *Colobus* populations and hybridized with their males. Backcrossing of hybrid females with resident *Colobus* males might has led to the fixation of the *Piliocolobus/Procolobus* mitochondrial lineage in the hy-

brid population, while the original nuclear genome of *Colobus* increased again in every generation.

For Asian langurs, we propose male introgression from *Semnopithecus* into *Trachypithecus* followed by nuclear swamping. Both genera are similar in their morphology and general appearance (Brandon-Jones 1984; Groves 2001; Strasser and Delson 1987), but males in *Semnopithecus* are larger than in *Trachypithecus* (Oates et al. 1994). Moreover, hybridization events due to (at least nowadays) partially overlapping ranges are generally possible (Groves 2001; Oates 1994). Accordingly, after an initial separation, *Semnopithecus* males, which leave their natal group like most other primate males (Newton and Dunbar 1994; Pusey and Packer 1987), might have invaded *Trachypithecus* populations and hybridized successfully with the resident females. By backcrossing with further invading *Semnopithecus* males over a longer period, the *Trachypithecus* population might have accumulated nuclear material of *Semnopithecus* (nuclear swamping), while the mitochondrial genome remained *Trachypithecus*-like.

2.5.3 Biogeographic implications

By combining the available information, we develop the following extended dispersal scenario for colobines (Figure 2.3). The origin of the subfamily is most likely in Africa, which is in agreement with earlier suggestions (Delson 1994; Stewart and Disotell 1998). On the African continent, *Colobus* split off first from the main stem ~10.93 mya, followed shortly afterwards by the progenitor of *Piliocolobus* and *Procolobus.* After this initial separation, hybridization between both lineages might have lasted until finally both mitochondrial lineages diverged (~8.47 mya). Presumably, respective splitting and hybridization events took place in western Africa, because all three genera occur there in sympatry (Groves 2001; Oates 1994), and the most ancient splits among *Piliocolobus* and *Colobus* species are also found there (Ting 2008). The Asian colobine ancestor most likely invaded Eurasia via an emerging land bridge connecting Africa and the Arabian Peninsula in the late Miocene (Stewart and Disotell 1998; Whybrow 1992).

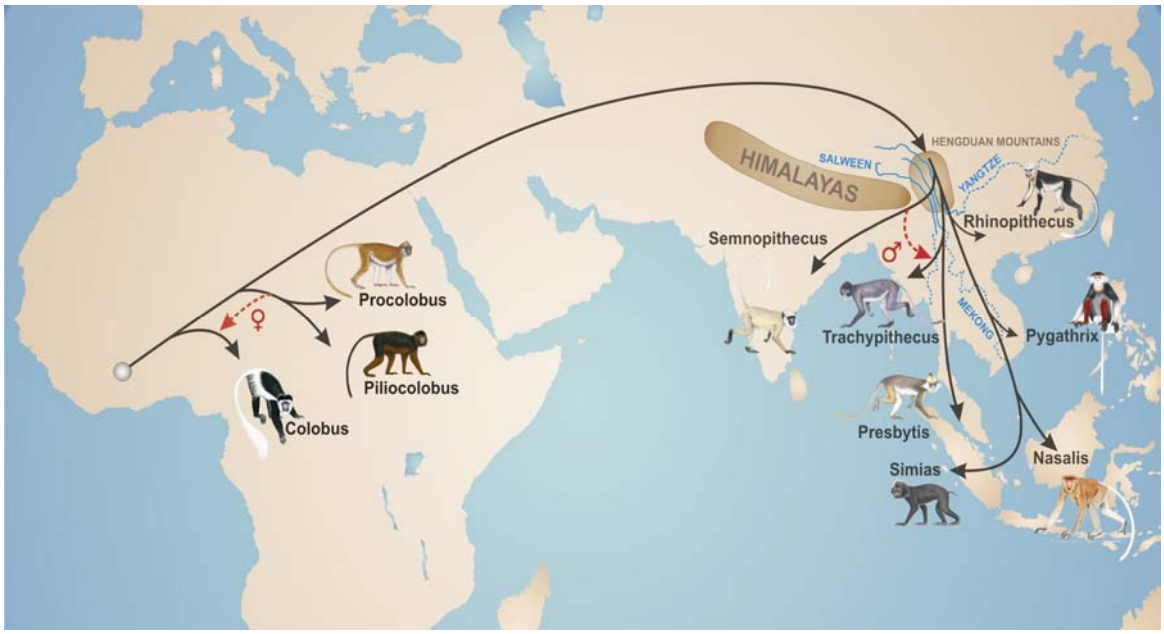

Figure 2.3: Dispersal scenario for colobine monkeys. Colobines most likely originated in western Africa. After the successive split of *Colobus* (~10.9 mya) and a progenitor of *Piliocolobus/Procolobus* (~10.7 mya) from the ancestor of Asian colobines, gene flow between both African lineages via female introgression from the *Piliocolobus/Procolobus* progenitor into *Colobus* occurred until ~8.5 mya (displayed by red-dashed arrow). During the late Miocene, colobines invaded eastern Asia most likely via a route north of the Himalayas. After their arrival at the Hengduan Mountains, Asian colobines diversified into a lineage comprising a progenitor of the odd-nosed monkeys and *Trachypithecus/Presbytis*, and of *Semnopithecus*, which later colonized the Indian subcontinent. Shortly afterwards, *Trachypithecus/Presbytis* split off from odd-nosed monkeys, and migrated to southern mainland Asia, before finally both genera diverged from each other. In the region of today's Burma, Bangladesh and India, *Semnopithecus* and *Trachypithecus* came into secondary contact and hybridized until ~2.6 mya (displayed by red-dashed arrow). In the latest Miocene, odd-nosed monkeys migrated from China to the south and expanded their range into Indochina and Sundaland. *Nasalis* and *Simias* finally separated from each other 1.1-1.9 mya.

Whether a route into eastern Asia north or south of the Himalayas was chosen is a matter of speculation, but north of the Himalayas, on the Tibetan plateau, colobine fossils from the late Miocene were found, which is not the case south of the Himalayas (Delson 1994). Although not confirmed, the Hengduan Mountains in the border region of today's Burma, India and China might have been a possible diversification hotspot (Jablonski 1998; Peng et al. 1993; Thinh et al. 2010a). In the region, all the larger Southeast Asian rivers (Mekong, Salween, Yangtze) rise, which are all well-known as

barriers for arboreal primates (Meijaard and Groves 2006) and are all known to exist since at least the early Miocene (Hallet and Molnar 2001). *Semnopithecus* might have diverged as first lineage and invaded the Indian subcontinent. Subsequently, the progenitor of *Presbytis* and *Trachypithecus* separated from the odd-nosed monkey ancestor and migrated into southern mainland Asia. Afterwards, *Presbytis* diverged from *Trachypithecus* and entered first the Malaysian peninsular and later on Sundaland during periods of lowered sea levels (Miller et al. 2005). *Trachypithecus* and *Semnopithecus* came into secondary contact and might have hybridized until the earliest Pleistocene. A potential contact zone could be the region of today's Bangladesh, Burma and the northeast of India, which is suggested as hybridization area for several primate species (Chakraborty et al. 2007; Karanth et al. 2008; Osterholz et al. 2008). On the Asian mainland, odd-nosed monkeys successively migrated from China to the south and expanded their range into Indochina and Sundaland in the latest Miocene. The migration into Sundaland was probably via land bridges connecting the mainland with Sundaland islands during periods of lowered sea levels (Miller et al. 2005). Finally, *Nasalis* on Borneo and *Simias* on the Mentawai islands west of Sumatra diverged in the Pleistocene. Due to the dating discrepancy (mitochondrial data: 1.88 mya, nuclear data: 1.06 mya), further gene flow between both genera after the initial separation cannot be excluded, especially considering that migration was repeatedly possible via land bridge connections during the Pleistocene (Miller et al. 2005).

2.6 Conclusion

Our study gives new and most comprehensive insights into the evolutionary history of colobine monkeys, and suggests hybridization among ancestral lineages as the most likely cause for the observed phylogenetic incongruences. Only the combination of maternally, paternally and bi-parentally inherited markers as well as the combination of sequence data with presence/absence patterns of mobile elements proved to be an adequate and reliable phylogenetic approach, particularly in revealing hybridization events. However, data from additional nuclear loci and a broader taxonomic sampling is required to fully understand hybridization mechanisms in colobines.

Hybridization among taxa is traditionally recognized as a factor leading to limited diversification, reproductive isolation and lowered fitness (Darwin 1859; Mayr 1963), whereas our and earlier studies clearly indicate that hybridization played a prominent role in diversification and speciation of primates (for review see (Arnold and Meyer 2006; Zinner et al. 2011)). Hybridization events are genetically confirmed within all major primate lineages, mainly among species (Chakraborty et al. 2007; Cortes-Ortiz et al. 2007; Merker et al. 2009; Rumpler et al. 2008; Thalmann et al. 2007; Zinner et al. 2009b) but also between genera (Karanth et al. 2008; Osterholz et al. 2008; Zinner et al. 2009a). Even for the human lineage, hybridization has been suggested as an important evolutionary mechanism (Green et al.; Pääbo 2003; Patterson et al. 2006).

Since male dispersal and female philopatry predominates in primates (Pusey and Packer 1987), male introgression, and if intensive backcrossing of hybrids with more invading males occurs, followed by nuclear swamping would be the most likely hybridization scenario. In fact, the hybridization among Asian langur genera is most likely the result of such an event. However, as proposed for African colobines, alternative mechanisms (e.g. female introgression) could also occur, promoted by a respective social organization, where female migration predominates.

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Chapter 3 Mitochondrial phylogeny of leaf monkeys (genus *Presbytis*, Eschscholtz, 1821) with implications for taxonomy and conservation

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Mitochondrial phylogeny of leaf monkeys (genus *Presbytis*, Eschscholtz, 1821) with implications for taxonomy and conservation

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ABSTRACT

The langurs of the genus *Presbytis* inhabit tropical rainforests of Sundaland, and with more than 50 color variants grouped in up to eleven species, *Presbytis* is one of the most diverse Old World monkey genera. The number of taxa and their phylogenetic relationships however remain controversial. To address these issues, we analyzed a 1.8 kb long fragment of the mitochondrial genome, including the cytochrome b gene, the hypervariable region I of the D-loop and the intermediate tRNAs, from individuals representing nine species. Based on our data, we obtained various well-supported terminal clades, which refer mainly to described taxa. Relationships among these clades are not fully resolved, suggesting at least two radiations in the evolutionary history of the genus. According to divergence age estimates, radiations occurred in the late Miocene and the early to middle Pleistocene. Our findings support the revision of the current classification of the genus *Presbytis* and enable us to discuss implications for conservation. However, further studies including nuclear sequence data are necessary to completely understand the evolutionary history of the genus, and to address possible hybridization events among taxa.

3.1 Abstract

The langurs of the genus *Presbytis* inhabit tropical rainforests of Sundaland, and with more than 50 color variants grouped in up to eleven species, *Presbytis* is one of the most diverse Old World monkey genera. The number of taxa and their phylogenetic relationships however remain controversial. To address these issues, we analyzed a 1.8 kb long fragment of the mitochondrial genome, including the cytochrome b gene, the hypervariable region I of the D-loop and the intermediate tRNAs, from individuals representing nine species. Based on our data, we obtained various well-supported terminal clades, which refer mainly to described taxa. Relationships among these clades are not fully resolved, suggesting at least two radiations in the evolutionary history of the genus. According to divergence age estimates, radiations occurred in the late Miocene and the early to middle Pleistocene. Our findings support the revision of the current classification of the genus *Presbytis* and enable us to discuss implications for conservation. However, further studies including nuclear sequence data are necessary to completely understand the evolutionary history of the genus, and to address possible hybridization events among taxa.

3.1.1 Ringkasan

Lutung dari marga *Presbytis* menghuni hutan hujan tropis wilayah Sunda, yang memiliki lebih dari 50 variasi warna yang dikelompokkan ke dalam 11 jenis. *Presbytis* merupakan salah satu marga monyet Dunia Lama yang paling beragam. Walaupun demikian jumlah taksa dan kekerabatan filogenetiknya masih kontroversial. Untuk membahas isu ini, kami menganalisis satu fragmen genom mitokondria, meliputi gen b cytochrome, region hypervariabel I dari D-loop dan tRNA intermediate, dari individu-individu yang mewakili sembilan jenis. Berdasarkan data kami, kami memperoleh berbagai clade terminal yang didukung dengan baik, yang terutama mengacu pada taksa yang telah dideskripsikan. Hubungan antara clade ini tidak sepenuhnya terjelaskan, menunjukkan bahwa sedikitnya ada dua radiasi dalam sejarah evolusi marga. Menurut divergensi dugaan umur, radiasi terjadi pada akhir Miosen dan awal

Pleistosen. Temuan kami mendukung revisi klasifikasi yang ada dari genus Presbytis dan memungkinkan kita untuk mendiskusikan implikasi-implikasi bagi konservasi. Namun, studi-studi lanjutan termasuk data sekuens nuklir diperlukan untuk dapat memahami secara sepenuhnya sejarah evolusi genus, dan untuk membahas kemungkinan terjadinya hibridisasi di antara taksa.

3.2 Introduction

Langurs of the Asian colobine genus *Presbytis* are exclusively arboreal animals, which inhabit tropical rainforest habitats of Sundaland, i.e., the Malay Peninsula and the western Indo-Malay archipelago (Oates et al. 1994) (Figure 3.1). Most *Presbytis* species live in unimale matrilineal or female bonded social systems, where males leave their natal troops at puberty (Bennett and Davies 1994; Newton and Dunbar 1994), but female dispersal is also reported (Sterck et al. 1997; Sterck et al. 2005). Mainly driven by Sundaland's dramatic geological and climatic changes during the past million years, the genus has undergone an extensive radiation (Meijaard 2004). With more than 50 described color variants, currently grouped into ten (Brandon-Jones et al. 2004) or eleven species (Groves 2001), *Presbytis* is one of the most diverse primate genera among Old World monkeys.

The classification of contemporary taxa and the evolutionary history of the genus, however, are poorly understood. Since the seminal work of Napier and Napier (1967), the genus *Presbytis* has been subject to a long history of frequent taxonomic revisions. Almost all proposed taxonomies and phylogenies for the genus are based on behavioral and anatomical features, largely coat coloration (Brandon-Jones 1978, 1996b, c; Brandon-Jones et al. 2004; Chasen 1940; Groves 1989, 2001; Hooijer 1962; Napier and Napier 1967; Wilson and Wilson 1976), while molecular genetic approaches are limited to a single study (Md Zain 2001). Unfortunately, the conclusions arising from these studies are at best inconsistent and often contradictory. In particular, the taxonomic status of the Sumatran langurs of the *melalophos* group (Brandon-Jones 2004; Groves

2001; Md Zain 2001; Md Zain et al. 2002; SAMD 2006) and the Javanese *comata* group (Brandon-Jones 1995; Brandon-Jones 1996a; Groves 2001; Nijman 1997, 2001) remains to be resolved.

Similarly, the phylogenetic relationships among taxa and the biogeographic history of the genus have yet to be clarified. For example, current reconstructions of the evolutionary history of the genus have yielded entirely conflicting scenarios, one proposing that either the Mentawai langur, *P. potenziani* (Brandon-Jones 1978, 1996c; Meijaard and Groves 2004) off the west coast of Sumatra, or the Hose's langur, *P. hosei* (Md Zain 2001), from Borneo is the sister to all *Presbytis* congeners. Unfortunately, Md Zain (2001) did not sample *P. potenziani*.

In the present study, we analyze a 1.8 kb long fragment of the mitochondrial genome, including the cytochrome b (cytb) gene, the hypervariable region I (HVI) of the D-loop and the intermediate transfer RNAs (tRNA), from 31 individuals representing nine species. Based on analysis of an extensive range of samples derived predominantly from wild living animals of known location, our results enable us to a) provide the most complete phylogeny of *Presbytis* available to date, b) estimate divergence times between lineages, c) provide a reliable basis for their taxonomic classification, and d) discuss implications for conservation.

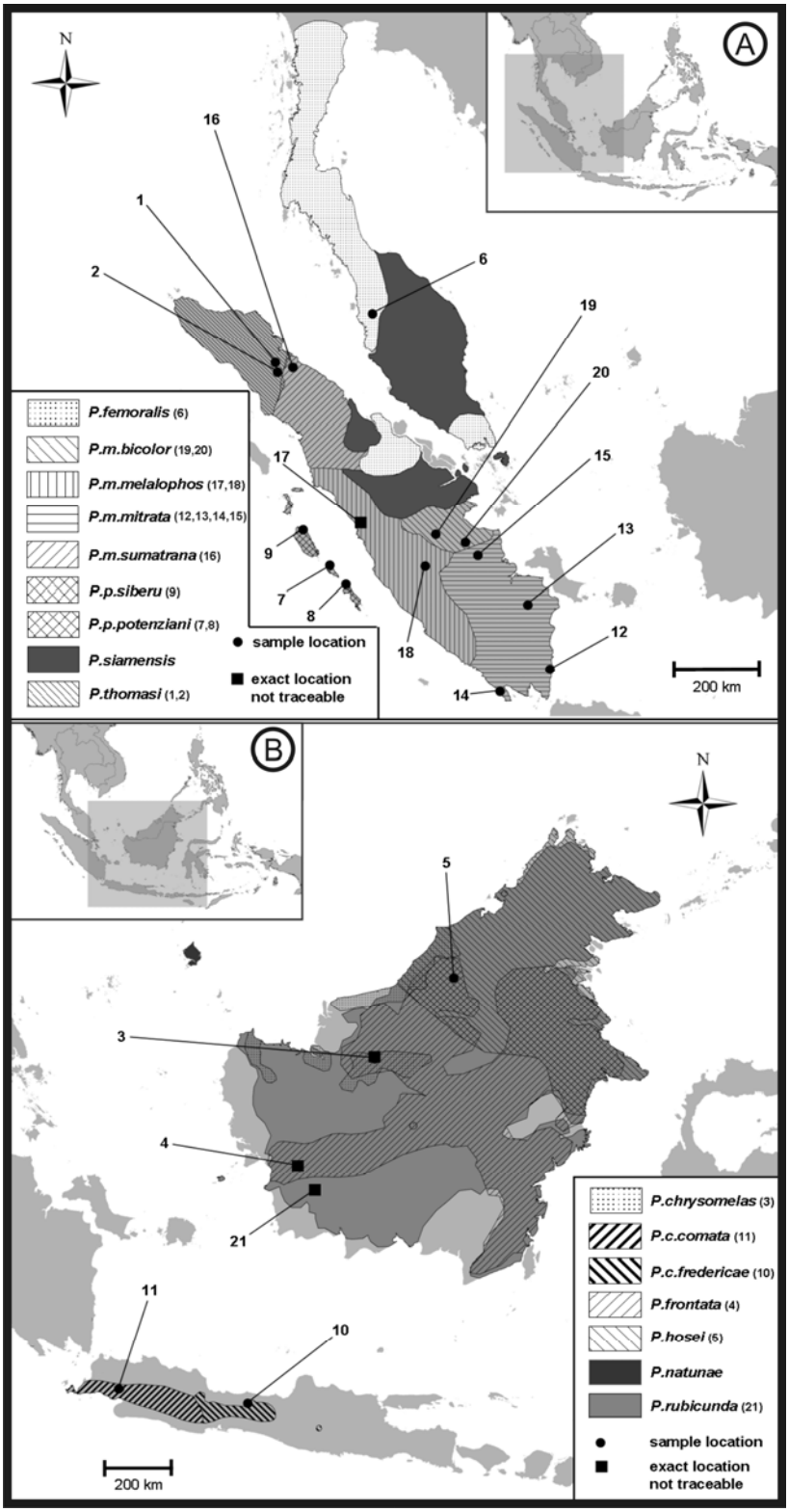

Figure 3.1: Present distribution range of *Presbytis* on the Asian Mainland, Sumatra and the Mentawai Islands (A) and on Borneo, Java and the Natunae Islands (B). Numbers indicate the origin of samples (see Table 3.1).

3.3 Methods

3.3.1 Sample collection

Fecal samples from 15 wild *Presbytis* populations were collected during two field surveys in September to October 2007 and June to November 2008. The localities spanned the range of *P. comata* (Java), *P. melalophos* (Sumatra), *P. thomasi* (Sumatra), *P. potenziani* (Mentawai Islands) and *P. hosei* (Borneo) (Figure 3.1). Animals were tracked in the early morning and followed until they defecated. Taxon identity of individuals was ascertained by pelage coloration, morphology, vocalization and geographic origin. The geographical position of the sampling sites was determined by GPS coordinates. Only fresh fecal samples were collected and preserved following the two-step ethanol-silica method described by (Nsubuga et al. 2004). We additionally included two fecal samples from captive animals (*P. m. mitrata*, Schmutzer Primate Centre of the Ragunan Zoo, Jakarta; *P. m. melalophos*, Howletts Wild Animal Park, Port Lympne) and four dry tissue samples from museum specimens preserved at the Bavarian State Collection of Zoology (*P. frontata*, coll. no. 1909/1394; *P. rubicunda*, coll. no. 1909/784; *P. chrysomelas*, coll. no. 1909/832; *P. m. sumatrana*, coll. no. 1921/226).

The skins of the museum specimens were checked for their taxonomic affiliation by examining morphological features particularly pelage coloration and their origin. Tissue samples were stored in plastic bags without any additive. For details about sampling sites see Table 3.1 and Figure 3.1.

3.3.2 Laboratory work

Genomic DNA from feces was extracted using the QIAamp™ Stool Mini Kit from Qiagen following the procedures recommended by the supplier, with the exception that the DNA was diluted in HPLC quality water and stored at -20 °C before further processing.

Table 3.1: Details on the *Presbytis* samples used in this study (for locations see also Figure 3.1)

Taxon	Location (Number)	Source	Sample Code	Accession Number
P. thomasi	Bukit Lawang, Sumatra (1)	Wild	Blaw	JF295124
P. thomasi	Tangkahan, Sumatra (2)	Wild	Tang	JF295125
P. chrysomelas	Kuna, Borneo (3)	Museum	Kun	JF295112
P. frontata	Paian, Borneo (4)	Museum	Pai	JF295113
P. hosei	Bukit Nakan, Sarawak (5)	Wild	Msa	JF295114
P. f. robinsoni	Redang Panjang, Malaysia (6)	GenBank	Mno	DQ355299
P. p. potenziani	Sipora, Mentawai Islands (7)	Wild	Sip	JF295122
P. p. potenziani	North Pagai, Mentawai Islands (8)	Wild	NPag	JF295123
P. p. siberu	Pungut, Menatwai Islands (9)	Wild	Pun1	JF295121
P. p. siberu	Pungut, Mentawai Islands (9)	Wild	Pun2	JF295119
P. p. siberu	Pungut, Mentawai Islands (9)	Wild	Pun3	JF295120
P. c. fredericae	Mt. Slamet, Java (10)	Wild	Sit1	JF295115
P. c. fredericae	Mt. Slamet, Java (10)	Wild	Sit2	JF295116
P. c. comata	TN Gunung Halimun, Java (11)	Wild	Hal1	JF295118
P. c. comata	TN Gunung Halimun, Java (11)	Wild	Hal2	JF295117
P. m. mitrata	TN Way Kambas, Sumatra (12)	Zoo	Rag	JF295098
P. m. mitrata	TN Way Kambas, Sumatra (12)	Wild	Wk1	JF295097
P. m. mitrata	TN Way Kambas, Sumatra (12)	Wild	Wk2	JF295096
P. m. mitrata	Riding, Sumatra (13)	Wild	Rid	JF295099
P. m. mitrata	Way Canguk, Sumatra (14)	Wild	Wc1	JF295100
P. m. mitrata	Way Canguk, Sumatra (14)	Wild	Wc2	JF295101
P. m. mitrata	Sungai Gelam, Sumatra (15)	Wild	Gel1	JF295103
P. m. mitrata	Sungai Gelam, Sumatra (15)	Wild	Gel2	JF295102
P. m. sumatrana	Deli (Medan), Sumatra (16)	Museum	Med	JF295110
P. m. melalophos	West Sumatra, Sumatra (17)	Zoo	How	JF295105
P. m. melalophos	Bangko, Sumatra (18)	Wild	Ban	JF295104
P. m. bicolor	Bukit Tigapuluh, Sumatra (19)	Wild	Btp1	JF295109
P. m. bicolor	Bukit Tigapuluh, Sumatra (19)	Wild	Btp2	JF295106
P. m. bicolor	Bukit Tigapuluh, Sumatra (19)	Wild	Btp3	JF295108
P. m. bicolor	Sengeti, Sumatra (20)	Wild	Seng	JF295107
P. rubicunda	Paun, Borneo (21)	Museum	Pau	JF295111

DNA from tissue material was extracted with the QIAamp™ DNA Mini Kit from Qiagen. The complete 1.8 kb fragment of the mitochondrial genome, which spans the complete cytb gene (1,140 bp), the tRNAs (ca. 140 bp) for Threonin (tRNA-Thr) and Prolin (tRNA-Pro), and the HVI region of the D-loop (ca. 533 bp), was amplified via four overlapping fragments using primers listed in Table 3.2. For all amplifications, standard wax-mediated hot-start PCRs were performed. The reactions were carried out in a total volume of 30µl containing a final concentration of 0,33 µM of each primer, 3 mM $MgCl_2$, 0,166 mM dNTPs, 1 x buffer and 1 U Taq DNA polymerase (Biotherm, Genecraft). PCR conditions for all amplifications were identical and consisted of a predenaturation step at 94°C for 2 min., followed by 40 cycles each with denaturation at 94°C for 1 min., annealing at variable temperatures (Table 3.2) for 1 min., and elongation at 72°C for 1 min. At the end, a final elongation step at 72°C for 5 min. was added. Aliquots of all PCR amplifications were checked on 1 % agarose gels and subsequently cleaned with the Qiagen PCR Purification Kit. Forward and reverse sequences of the PCR products were analyzed on an ABI 3730xl DNA Analyzer (Applied Biosystems) using the BigDye™ Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems).

To prevent cross-species contamination, laboratory methods followed described standards (Osterholz et al. 2008; Roos et al. 2008; Thinh et al. 2010a; Thinh et al. 2010b). Generated sequences were assembled and edited using Geneious Pro 4.7 (Drummond et al. 2008) and manually checked by eye. The cytb sequences were further checked for their potential to be correctly transcribed. All newly generated sequences were deposited in GenBank and are available under the accession numbers JF295096 – JF295125 (see also Table 3.1).

Table 3.2: Information about primers used in this study. The complete cytb was obtained by amplifying overlapping fragments (primer pairs 6405/6232 and 6435/2068 or 6405/6719 and 6720/6721). The HVI locus was amplified with primer pairs 2067/6234 and 6722/6723. AT: Annealing temperature.

Locus	Primer (ID)	Primer sequence 5'-3'	AT
tRNA-Glu	H-45 uni F (6405)	AAT GAT ATG AAA ARY CAT CGT TG	58
tRNA-Thr	15510 R (6232)	TGT CCG TTT CCA GTT TAC AAG	60
cytb	PresCytbR1 (6719)	TTR TCT GGG TCG CTY AAA AG	58
cytb	Pre945-F (6435)	TCG CCC AYT TAG CCA ATT CC	58
cytb	PresCytbF2 (6720)	CTR TTT CTA CAC GAA ACA GG	58
tRNA-Pro	PresCytbR2 (6721)	AAT ACA GAA AGT AGT TTA AAT AG	54
HVI	2068R (2068)	ATT GAT TTC ACG GAG GAT GGT	56
tRNA-Pro	2067 F (2067)	CTG GCA TTC TAT TTA AAC TAC TT	58
HVI	16220 R (6234)	TGA TAG ACC CGT GAT CCA TC	58
tRNA-Thr	PresLoopF (6722)	AAA TAC ACC AGT CTT GTA AAC	54
HVI	PresLoopR (6723)	TTT AAG GGG AAC GTG TGA G	52

3.3.3 Statistical analysis

To expand our dataset, we further incorporated orthologous sequences available at GenBank from one *P. melalophos* (DQ355299) which actually refers due to locality to *P. femoralis robinsoni* (Redang Panjang, Malaysia, pers. comm. Nelson Ting) and one *Trachypithecus obscurus* (AY863425), which was used as an outgroup. Sequences were aligned with the ClustalW program as implemented in Geneious and manually checked by eye. A computerized method was applied to eliminate poorly aligned positions and divergent regions using Gblocks 0.91b (Castresana 2000). Therefore, a relaxed selection of blocks was selected (Talavera and Castresana 2007). Phylogenetic trees were constructed with neighbor-joining (NJ), maximum-likelihood (ML) and Bayesian approaches. Data were divided into three partitions (cytb, tRNAs, HVI). As the optimal nucleotide substitution model for each partition, the GTR + I + G model was chosen by the Bayesian Information Criterion (BIC) with jModelTest 0.1.1 (Posada 2008). ML analyses were conducted using Garli v0.951 (Zwickl 2006). In Garli, only the model specifications settings were adjusted, while all other settings were left at their default

values. ML bootstrap percentages were estimated in Garli by performing 500 pseudoreplicate runs. 10 replicates were run to verify consistency in log likelihood scores and tree topologies. A 50% majority rule consensus tree was calculated with Paup* v4.0b10 (PPC) (Swofford 2003). Phylogenetic relationships based on the NJ algorithm were calculated in Paup. Relative support of internal nodes was performed by bootstrap analyses with 10,000 replications. For the Bayesian analysis in MrBayes v3.1.2 (Huelsenbeck et al. 2001; Ronquist and Huelsenbeck 2003), the dataset was partitioned into the three portions. Four Markov Chain Monte Carlo (MCMC) runs with a default temperature of 0.2 and a chain length of 10,000,000 generations were carried out. Trees and parameters were sampled every 100 generations. Flat priors were assumed for the model parameters including the proportion of invariable sites and the gamma shape parameter of rate variation among sites. The first 25% of samples were discarded as burnin, leaving 75,001 trees per run. The adequacy of this burnin and convergence of all parameters was assessed by examining the uncorrected potential scale reduction factor (PSRF) (Gelman and Rubin 1992), which should approach 1 as runs converge, and by visually inspecting the trace of the parameters across generations using the software Tracer v1.3 (Rambaut and Drummond 2005). Posterior probabilities for each split and a phylogram with mean branch lengths were calculated from the posterior density of trees. Phylogenetic trees were visualized with FigTree v1.3.1 (Rambaut 2006).

To estimate divergence times, we included sequences from another 13 primate species, which derived from GenBank (*Pongo pygmaeus*, NC001646; *Pan troglodytes*, D38113; *Homo sapiens*, AY339522; *Chlorocebus aethiops*, NC007009; *Macaca sylvanus*, AJ309865; *Papio hamadryas*, EU885446; *Theropithecus gelada*, EU885487; *Colobus guereza*, NC00690; *Semnopithecus entellus*, EU004478; *Rhinopithecus avunculus*, EU004480; *Nasalis larvatus*, EU004476). Due to the high mutation rate in the HVI region, the calculation was performed solely on the cytb sequence data. For the estimation, we applied a Bayesian MCMC method, which employs a relaxed molecular clock approach (Drummond et al. 2006), as implemented in the BEAST v1.5beta2 package

(Drummond and Rambaut 2007). A relaxed lognormal model of lineage variation and a Yule prior for branching rates was assumed. The alignment was partitioned according to 1+2 and 3 codon positions. The substitution model, rate heterogeneity and base frequencies were unlinked across codon positions ((1+2), 3). As calibration points, we selected the divergence between Hominoidea and Cercopithecoidea (C1), which was dated between 24 and 29 million years ago (Ma) (Zalmout et al. 2010), the divergence between Ponginae and Homininae (C2) ~14 Ma (Kelley 2002; Raaum et al. 2005), the split between *Homo* and *Pan* (C3) 6-7 Ma (Brunet et al. 2002; Steiper and Young 2006; Vignaud et al. 2002), and the separation of *Theropithecus* from *Papio* (C4) ~4 Ma (Delson 2000; Leakey 1993; Ting 2008). Instead of hardbounded calibration points, we used the published dates as a normal distribution prior for the respective node. For C1 this translates into a normal distribution with a mean of 26.5 Ma and a standard deviation (SD) of 1.36 Ma (95% credibility interval [CI]: 24-29 Ma), for C2 into a mean of 14.0 Ma and a SD of 0.60 Ma (CI: 13.0-15.0 Ma), for C3 into a mean of 6.5 Ma and a SD of 0.31 Ma (CI: 6-7 Ma), and for C4 into a mean of 4.0 Ma and a SD of 0.31 Ma (CI: 3.5-4.5 Ma). For the analysis, two replicates were run for 25 million generations with tree and parameter sampling occurring every 2,500 generations. The adequacy of a 10% burnin and convergence of all parameters were assessed by visual inspection of the trace of the parameters across generations using the software Tracer. Subsequently, the sampling distributions of multiple independent replicates were combined with the software LogCombiner v1.4.6 and then summarized and visualized with TreeAnnotator v1.4.6. Both programs are part of the Beast package (Drummond and Rambaut 2007).

3.4 Results

In this study, we successfully sequenced a ca. 1.8 kb fragment of the mitochondrial genome from a total of 30 *Presbytis* individuals, of which 29 were from known origins. By including an additional sequence from *P. femoralis* from GenBank and following the classification of Groves (2001), our data set comprises nine species and 14 subspecies. Nuclear pseudogenes could not be detected, given that overlapping fragments of dif-

ferent sequences from the same individual were identical, and that the cytb gene was correctly transcribed. Moreover, the amplification of nuclear pseudogenes (numts) is reduced since we used faecal and museum material in which nuclear DNA is highly degraded (Hofreiter et al. 2003; Thalmann et al. 2004).

The original alignment had a length of 1,816 bp. Poorly aligned positions and divergent regions were solely detected within t-RNAs and the HVI. After discarding these positions, the alignment was reduced to 1,745 bp. Among them, 172 sites were parsimony-uninformative and 402 parsimony-informative. All sequences represented unique haplotypes.

Phylogenetic reconstructions based on NJ, ML and Bayesian algorithms (Figure 3.2) revealed several strongly supported clades, which mainly referred to species and subspecies. However, although the branching pattern among various lineages gained only weak support, all algorithms showed similar tree topologies.

According to the phylogenetic reconstructions, the base of the tree indicates a separation of *P. thomasi* as the sister species to its congenerics. However, since this initial split is only weakly supported (Bayesian: 77; NJ: 77; ML: 59), we interpret the base of the tree as a polytomy between *P. thomasi* (clade A), clade B, and the remaining *Presbytis* taxa (clade C). Clade B includes the Bornean species *P. chrysomelas*, *P. frontata* and *P. hosei*, but relationships among them remain unresolved.

In clade C, *P. femoralis* represents the sister lineage to the remaining taxa (clade D), of which *P. potenziani* (clade E) is separated from the taxa on Sumatra and Java, and also from the Bornean *P. rubicunda* (clade F). Within *P. potenziani* (clade E), both subspecies, *P. p. potenziani* and *P. p. siberu,* form reciprocally monophyletic clades. Clade F is further divided into subclades G and H. The former segregates into two strongly supported clades (I and J). The Javanese *P. comata* falls into clade I, which further diverges into two reciprocally monophyletic clades represented by the two subspecies, *P. c. comata* and *P. c. fredericae.* Clade J consists solely of South Sumatran *P. m. mitrata* individuals.

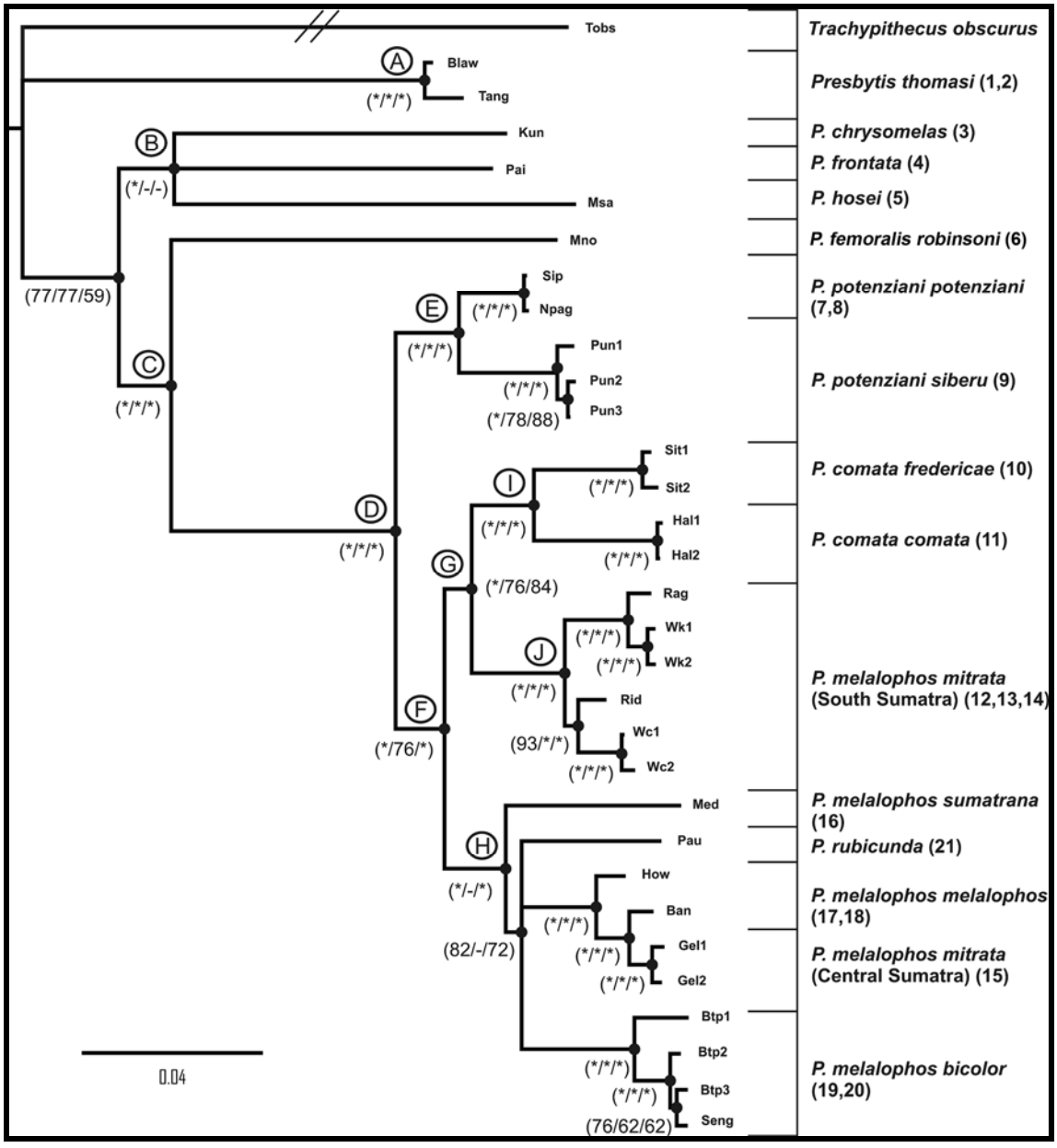

Figure 3.2: Phylogenetic reconstruction derived from Bayesian, NJ and ML algorithms based on 1.8 kb of the mitochondrial genome (for individual sequence codes see Table 3.1).

Clade H contains an unresolved polytomy with four highly supported clades or lineages: *P. m. sumatrana, P. rubicunda, P. m. bicolor*, and a clade formed by *P. m. melalophos* and *P. m. mitrata* from Central Sumatra. Thus, *P. m. mitrata* is mitochondrially paraphyletic.

Based on divergence time estimates (Figure 3.3, Table 3.3), Cercopithecidae separated from Hominidae (C1) 26.57 (24.26-29.09) Ma. Within Hominidae, *Pongo* branched off first (C2) 13.70 (12.54-14.79) Ma, followed by *Homo* and *Pan* (C3), which separated from each other 6.50 (5.92-7.09) Ma. The split between Colobinae and Cercopithecinae (N1) occurred 19.41 (15.81-23.65) Ma. Within the latter, Papionini separated from Cercopithecini (*Chlorocebus aethiops*) 12.04 (8.97-15.30) Ma (N2). Among Papionini, *Macaca* diverged first (N3) 10.56 (7.71-13.46) Ma, followed by the differentiation of *Papio* and *Theropithecus* (C4) 4.02 (3.44-4.57) Ma. The African colobine genus *Colobus* separated from Asian colobines 15.39 (12.29-18.98) Ma (N4). Among the latter, the split between *Semnopithecus/Nasalis/Rhinopithecus* and *Trachypithecus/Presbytis* took place 12.31 (9.72-14.86) Ma (N5). *Semnopithecus* separated from *Nasalis* and *Rhinopithecus* 10.24 (7.56-12.93) Ma (N6) and latter two 7.51 (5.00-13.10) Ma (N7). The split between *Presbytis* and *Trachypithecus* (N8) occurred 11.46 (9.01-14.09) Ma. Within a relative short time period of only 1.43 (3.91-8.53) Ma (N9-N11), four major *Presbytis* lineages emerged. The first leads to *P. thomasi*, the second to a clade containing *P. chrysomelas, P. frontata* and *P. hosei*, the third to *P. femoralis*, and the fourth to a clade including all remaining taxa.

In the latter, a subsequent radiation (N12-N19) leading to species and subspecies started with the separation of *P. potenziani* 2.62 (1.94-3.38) Ma (N12). Shortly afterwards, 2.22 (1.65-2.85) Ma (N13), South Sumatran *P. m. mitrata* and *P. comata* separated from Central Sumatran *P. m. mitrata, P. m. melalophos, P. m. sumatrana, P. m.*

bicolor and *P. rubicunda*. In the former, *P. m. mitrata* diverged 1.8 (1.28-2.37) Ma (N16), before finally *P. comata* split into its two subspecies 1.06 (0.63-1.51) Ma (N19).

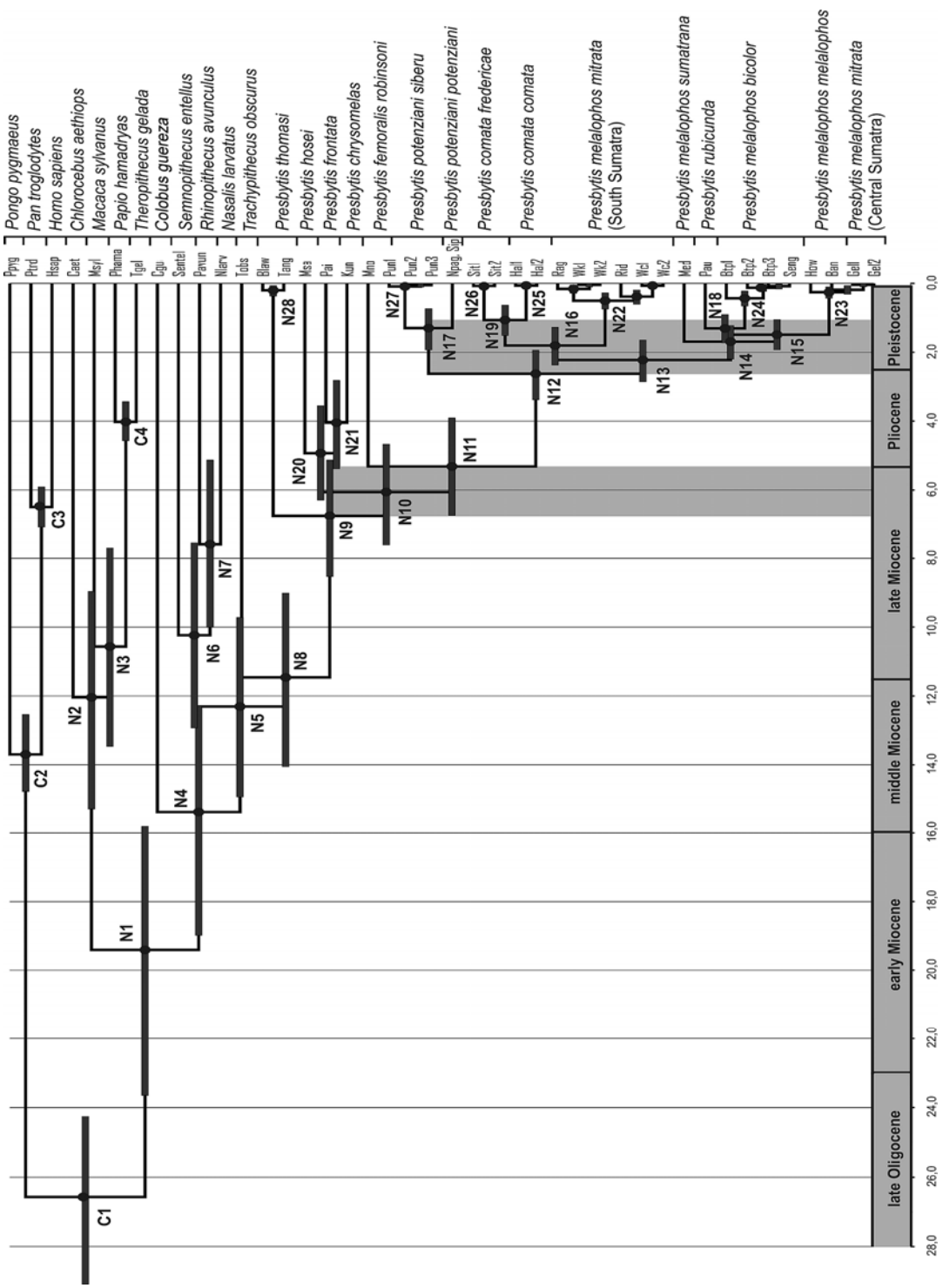

Figure 3.3: Ultrametric tree showing phylogenetic relationships and estimated divergence ages among studied Presbytis individuals based on the complete mitochondrial cytb sequences (for individual codes see Table 3.1). A time scale in million years and the geological periods are given. Nodes of interest are arbitrarily numbered (N1-N28). C1-C4 refer to nodes used for calibration. Light gray bars indicate the two radiations.

In the latter clade, *P. m. sumatrana* diverged first (1.69 [1.22-2.20] Ma) (N14), followed by the *P. m. melalophos*/Central Sumatran *P. m. mitrata* clade 1.40 (0.91-1.93) Ma (N15), before finally *P. m. bicolor* separated from *P. rubicunda* 1.31 (0.91-1.72) Ma (N18).

3.5 Discussion

We report here on the analysis of mitochondrial sequences from 31 *Presbytis* individuals, representing nine of the eleven currently recognized species. Accordingly and since we mainly used only individuals from known locations, this study is the most complete and reliable one up to date. Moreover, calculated divergence ages between various lineages are in similar ranges as earlier estimates (Chatterjee et al. 2009; Raaum et al. 2005; Sterner et al. 2006), although in general slightly older. According to our divergence age estimates, radiations within the genus occurred in two phases, one in the late Miocene and the other in the early to middle Pleistocene.

Interestingly, the tree topology depicted in the only other available molecular phylogeny (Md Zain 2001) differs from our one, although both investigations are based on large segments of the mitochondrial genome, albeit with different samples and loci. In the study by Md Zain (2001), data rely on samples from wild populations from Malaysia, while samples from Indonesia came mainly from captive animals with unknown origin. Unfortunately, sequence data from the earlier study are not available for reanalysis and therefore a discussion of disagreements would remain speculative. However, due to our extensive sampling from clearly identified wild populations, we regard our data as more reliable.

Although *P. siamensis* and *P. natunae* are not represented, the results allow the most comprehensive evaluation of the evolutionary history of the genus to date including material from key taxa such as *P. potenziani* that have not been analyzed before and thus provide a sound basis for a revised taxonomic classification of the *Presbytis* genus.

3.5.1 Phylogenetic relationships

Based on the grey coat coloration, *P. thomasi*, *P. hosei* and *P comata* have previously been combined in *P. comata* with a tripartite geographic distribution on West Java (*P. comata*), North Sumatra (*P. thomasi*) and North Borneo (*P. hosei*) (Brandon-Jones 1978, 1996b, c; Chasen 1940; Hooijer 1962). Our present data, however, do not support a polytypic *P. comata* species, but instead indicate an early separation of *P. thomasi* and *P. hosei* in respective lineages and a late differentiation of *P. comata*. Concerning *P. potenziani* our data reveal interesting results. Since the discovery of *potenziani* there have been conflicting statements on its taxonomic position relative to other Asian colobines (Tilson 1976a). Based on postcranial data (Washburn 1944) or infant coloration , *potenziani* was grouped together with *Trachypithecus*, while a morphological study by Groves (1970) proposed an intermediate position between *Trachypithecus* and *Presbytis*. Subsequent studies of infant coloration(Tilson 1976a) and male vocalization (Wilson and Wilson 1975) grouped *potenziani* together with *Presbytis*. The similarity in the adult vocalization between *P. potenziani* and *P. thomasi* led Wilson and Wilson (1976) to conclude that both species are closely related and that a subspecific affiliation might be indicated. Nevertheless, *P. potenziani* is currently considered as a distinct species (Brandon-Jones et al. 2004; Groves 2001).

Table 3.3: Bayesian divergence date estimates Means and 95% credibility intervals (CI) are given for 31 nodes in Ma. Nodes used as calibration points are labeled with a "C", all others with an "N". MRCA denotes the most recent common ancecestor. [a] = Mean of divergence times of nodes that belong to unresolved polytomies (see also Figure 3.2; Figure 3.3).

Node	mean (Ma)	95% CI (Ma)
C1 Hominoidea - Cercopithecoidea	26.57	24.26 – 29.09
C2 *Pongo – Pan + Homo*	13.70	12.54 -14.79
C3 *Homo - Pan*	6.50	5.92 – 7.09
C4 *Papio - Theropithecus*	4.02	3.44 – 4.57
N1 Cercopithecinae - Colobinae	19.41	15.81 – 23.65
N2 Cercopithecini - Papionini	12.04	8.97 – 15.30
N3 *Macaca - Papio + Theropithecus*	10.56	7.71 – 13.46

Table 3.3 continued

Node	mean (Ma)	95% CI (Ma)
N4 *Colobus* - Asian colobines	15.39	12.29 – 18.98
N5 *Semnopithecus* + *Nasalis* + *Pygathrix* – *Trachypithecus* + *Presbytis*	12.31	9.72 – 14.86
N6 *Semnopithecus* – *Nasalis* + *Rhinopithecus*	10.24	7.56 -12.93
N7 *Nasalis* - *Pygathrix*	7.51	5.00 – 13.10
N8 *Trachypithecus* - *Presbytis*	11.46	9.01 – 14.09
N9 *P. thomasi* - remaining *Presbytis* taxa	6.75	5.13 – 8.53
N10 *P. hosei* + *P. chrysomelas* + *P. frontata* - remaining taxa	6.06	4.67 – 7.61
N11 *P. femoralis* - remaining taxa	5.32	3.91 – 6.74
N12 *P. p. potenziani* + *P. p. siberu* - remaining taxa	2.62	1.94 – 3.38
N13 *P.c.comata* + *P.c.fredericae* + *P.m.mitrata* (South Sumatra) - remaining taxa	2.22	1.65 – 2.85
N14 *P. m. sumatrana* - *P. m. melalophos* + *P. m. mitrata* (Central Sumatra) + *P. m. melalophos* + *P. m. bicolor* + *P. rubicunda*	1.69	1.22 – 2.20
N15 *P. m. melalopho* + *P. m mitrata* (Central Sumatra) - *P. rubicunda* + *P. m. bicolor*	1.49 (1.40 [a])	1.06 – 1.93 (0.91 – 1.93 [a])
N16 *P. c. comata* + *P. c. fredericae* - *P. m. mitrata* (South Sumatra)	1.80	1.28 – 2.37
N17 *P. p. siberu* - *P. P. potenziani*	1.30	0.74 – 1.93
N18 *P. rubicunda* - *P. m. bicolor*	1.31	0.91 – 1.72
N19 *P. c. comata* - *P. c. fredericae*	1.06	0.63 – 1.51
N20 *P. hosei* - *P. chrysomelas* + *P. frontata*	4.93 (4.49 [a])	3.56 - 6.29 (2.82 – 6.29 [a])
N21 *P. chrysomelas* - *P. frontata*	4.05	2.82 – 5.38
N22 MCRA *P. m. mitrata* (South Sumatra)	0.50	0.28 – 0.74
N23 MRCA *P. m. melalophos* - *P. m mitrata* (Central Sumatra)	0.26	0.10 – 0.43
N24 MRCA *P. m. bicolor*	0.43	0.23 – 0.65
N25 MRCA *P. c. comata*	0.06	0.01 – 0.16
N26 MRCA *P.c. fredericae*	0.07	0.00 – 0.14
N27 MRCA *P. p. siberu*	0.09	0.02 – 0.17
N28 MRCA *P. thomasi*	0.20	0.07 – 0.36

Based on its morphological and behavioral characters described above, *P. potenziani* is proposed to be the most "primitive" member of the genus and thus the sister species to all congenerics (Brandon-Jones 1978, 1993; Meijaard and Groves 2004). Our molecular data support the monophyly of *P. potenziani*, but neither an early separation, nor a sister grouping with *P. thomasi*.

P. femoralis and *P. chrysomelas* were originally recognized as subspecies of *P. melalophos* (Napier and Napier 1967; Oates et al. 1994), but later, *P. femoralis* was separated from *P. melalophos* at the species level, with *P. chrysomelas* being included as a subspecies (Brandon-Jones, 1984). More recently, Groves (2001) proposed species status for *P. chrysomelas* as well, while Brandon-Jones et al. (2004) kept *P. chrysomelas* as subspecies of *P. femoralis*. Our data do not support a conspecific relationship between any of these three taxa, since they descend from distinct lineages with ancient divergences.

Of the remaining species, *P. comata* and *P. rubicunda* are nested within *P. melalophos*. Additionally, the possible paraphyletic origin of *P. m. mitrata*, with the central Sumatran populations being closely related to *P. m. melalophos* and the South Sumatran populations forming a sister lineage to *P. comata,* reflects a puzzling situation of this polyphyletic group. We can not rule out that incomplete lineage sorting might have had an effect. But in the case of incomplete lineage sorting, paraphyletic relationships, like in *P. m. mitrata*, result from the failure of haplotypes to sort during speciation events and should be random with respect to geography (Avise 2004). This is not the case in our phylogenetic reconstruction, where geographically close populations cluster together. Another explanation might be hybridization between *P. m. melalophos* and *P. m. mitrata*. Such a scenario is highly likely, since both taxa are found south of the Batang Hari river (Aimi and Bakar 1992, 1996; Groves 2001) eastwards from Bangko, where we identified *P. m. melalophos*. In addition to our mitochondrial data, the pale reddish coat coloration of the *P. m mitrata* population from Central Sumatra seems to be intermediate between the red *P. m. melalophos* and the grayish-white Southern *P. m. mitrata* populations, which is in accordance with the observations of

Aimi and Bakar (1996). Alternatively, the pale reddish Central Sumatran population might be a further color variant of *P. m. melalophos*. To test these hypotheses, the analysis of nuclear markers is mandatory.

Although the sample size (n=1) of *P. rubicunda* is low, and incomplete lineage sorting might be possible, our data support previous hypotheses, proposing a close affiliation of *P. rubicunda* and *P. melalophos* based on the red coat coloration (Brandon-Jones 1996b) or in some aspects of behavior and vocalization (Wilson and Wilson 1975).

3.5.2 Taxonomic implications

The taxonomic confusion within the genus *Presbytis* - and also in other primate genera - is associated with much controversial discussion about the recognition on the degree or amount of certain characters, in particular pelage coloration, to recognize species or phylogenetic relatedness. To apply a more objective and falsifiable approach, Groves (2001, 2004) suggests the phylogenetic species concept (PSC) to delimit species. The PSC defines a species as *the smallest cluster of individual organisms within there is a parental pattern of ancestry and descent and that is diagnosable distinct from other such clusters by a unique combination of fixed character states* (Cracraft 1983). At present, the PSC is widely applied in recent studies on primate taxonomy, for example in *Mico* (Groves 2001), *Callicebus* (van Roosmalen et al. 2002), *Saguinus* (Matauschek et al. 2011), *Lepilemur* (Craul et al. 2007), *Microcebus* (Louis Jr. et al. 2008), *Trachypithecus* (Roos et al. 2008) or *Nomascus* (Thinh et al. 2010b).

Among *Presbytis*, we detect several highly supported terminal clades or lineages, mainly formed by taxa, which are all also clearly distinguishable in their pelage coloration, thus in agreement with the PSC. Moreover, most of the examined *Presbytis* taxa differentiated on a similar time scale as did other Asian primates. For example, Tosi et al. (2003) and Ziegler et al. (2007) estimated the divergence between Sundaic and Continental pig-tailed macaques at 1.4 (1.6-1.2) Ma, which afterwards differentiated into respective species. In *Trachypithecus,* Roos et al. (2008) calculated the divergence between *T. germaini* and *T. margarita* 0.95 (1.04-0.86) Ma. Considering estimated di-

vergence ages and differences in mtDNA and pelage coloration, species status for all examined *Presbytis* taxa might be appropriate. An overview to the proposed revision in comparison with previous studies is given in Table 3.4.

3.5.3 Conservation implications

The rainforests of the Malay Peninsula and the western Indo-Malay archipelago belong to the biodiversity hotspots of our planet, but also to hotspots of environmental degradation. For instance, Indonesia is among the ten countries with the highest number of threatened species (FWI/GFW 2002; World-Bank 2004). The langurs of the genus *Presbytis* also suffer from this ongoing situation and population sizes of all taxa are still decreasing (IUCN 2010). Reasons for the decline of langurs are manifold, but habitat loss due to forest clearance for agricultural use or timber production, as well as illegal hunting for food, pet trade or traditional medicine, like bezoar stones (visceral secretions used in traditional medicine; (Nijman 2004)), are major threats to wild *Presbytis* populations.

In particular forest destruction often leads to isolated populations due to fragmentation and consequently to limited or disturbed gene flow. Recent evaluations of the conservation status rank various *Presbytis* taxa as vulnerable, endangered or even as critically endangered (Table 3.4).

For conservationists it is of great interest whether any population within a taxon is sufficiently differentiated genetically to warrant separate management and also to maintain genetic diversity. In our study we detected several monophyletic clades, which should all be regarded as independent management units (Moritz 1994). Thus, conservation actions are needed for all *Presbytis* taxa, but especially for the critically endangered ones *P. chrysomelas* and *P. p. potenziani*.

Threats to both taxa are exemplary for most members of the genus. The main threat to *P. chrysomelas* has been conversion of habitat into agricultural land, resulting in its disappearance from most of its former range and leading to five isolated populations.

In recent years, the species has in particular been affected by expanding plantations, especially oil palm (IUCN 2010). The population decline of *P. p. potenziani* was estimated at more than 80% over the past 40 years due to hunting and loss of habitat (Whittaker 2006).

Table 3.4: Proposed classification compared to earlier classifications along with information about type localities, authors and conservation status. [a] According to IUCN (2010) *P. c. fredericae* is a synonym of *P. comata* ; CS = conservation status (IUCN, 2010), DD = data deficient, LC = least concern, NT = near threatened, VU = vulnerable, EN = endangered, CR = critically endangered.

Groves (2001)	Brandon-Jones et al. (2004)	Proposed classification	CI	Author	Type locality
P. p. potenziani	*P. p. potenziani*	*P. potenziani*	CR	Bonaparte, 1856	Sipora Island, Mentawai Islands
P. p. siberu	*P. p. siberu*	*P. siberu*	EN	Chasen & Kloss, 1927	Siberut Island, Mentawai Islands
P. c. comata	*P. comata*	*P. comata*	EN	Desmarest, 1822	Java
P. c. fredericae [a]	*P. fredericae*	*P. fredericae*	EN	Sody, 1930	Mount Slamet, Central Java
P. m. melalophos	*P. m. melalophos*	*P. melalophos*	NT	Raffles, 1821	Bengkulu, Sumatra
P. m. mitrata	*P. m. mitrata*	*P. mitrata*	EN	Eschscholtz, 1821	Sumatran mainland opposite Zutphen Island
P. m. bicolor	*P. m. bicolor*	*P. bicolor*	DD	Aimi & Bakar, 1992	Batang Kering, Sumatra
P. m. sumatrana	*P. m. sumatrana*	*P. sumatrana*	EN	Müller & Schlegel, 1841	Mount Talamau, Sumatra
P. chrysomelas	*P. femoralis chrysomelas*	*P. chrysomelas*	CR	Müller, 1838	Pontianak, Borneo
P. thomasi	*P. thomasi*	*P. thomasi*	VU	Collett, 1892	Langkat, Aceh, Sumatra
P. hosei	*P. hosei*	*P. hosei*	VU	Thomas, 1899	Niah, Sarawak, Borneo
P. rubicunda	*P. rubicunda*	*P. rubicunda*	LC	Müller, 1838	Mount Sekumbang, South Kalimantan, Borneo
P. frontata	*P. frontata*	*P. frontata*	VU	Müller, 1838	Southeastern Borneo
P. femoralis	*P. femoralis*	*P. femoralis*	NT	Martin, 1838	Singapore

If *P. c. fredericae* is considered to be a separate species it undoubtedly can be ranked among the rarest and most endangered primate species in the world. It would be restricted to four isolated forest areas, none of which are adequately protected and two of them are situated on an active volcano (Nijman 2001).

To protect langurs in the future, urgent actions are required to prevent ongoing habitat destruction and hunting activities. Often captive animals offer the opportunity to conserve and build up a viable gene pool for later release purposes, but in *Presbytis*, it is of particular interest to protect wild populations since they are difficult to be kept in captivity. However, it is also crucial to confirm the taxon identity and if possible the geographical origin of confiscated animals in general.

3.6 Conclusions

We showed that accurate taxonomic identification of *Presbytis* taxa based on behavioral or morphological data alone is sometimes contradictory or misleading. In this respect, mtDNA analysis is a promising tool, which additionally should be used to answer taxonomic affiliations. This is also important for practical issues in conservation management in nature (in situ) and in captivity (ex situ). As shown in our study, *Presbytis* taxa can be diagnosed through mtDNA, and, hence, a secure identification of the taxon, even the population, can easily be obtained. In our study for example, we were able to confirm Way Kambas as the origin of the *P. m. mitrata* sample from the Ragunan Zoo in Jakarta. Yet since mtDNA is only maternally inherited, possible hybrids can not be detected in such analysis. Thus, for the identification of captive hybrids and also to trace possible natural hybridization events as it might be the case between *P. m. melalophos* and the Northern population of *P. m. mitrata*, nuclear markers should be studied as well. Moreover, to fully understand the evolutionary history of the genus, further studies should also include *P. natunae* and *P. siamensis*, as well as a broader sampling set of the Bornean taxa.

3.7 Acknowledgments

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Chapter 4 Acoustic structure of male loud-calls support molecular phylogeny of Sumatran and Javanese leaf monkeys (genus *Presbytis*)

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Acoustic structure of male loud-calls support molecular phylogeny of Sumatran and Javanese leaf monkeys (genus *Presbytis*)

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Abstract

Background: The degree to which loud-calls in nonhuman primates can be used as a reliable taxonomic tool is the subject of ongoing debate. A recent study on crested gibbons showed that these species can be well distinguished by their songs; even at the population level the authors found reliable differences. Although there are some further studies on geographic and phylogenetic differences in loud-calls of nonhuman primate species, it is unclear to what extent loud-calls of other species have a similar close relation between acoustic structure, phylogenetic relatedness and geographic distance. We therefore conducted a field survey in 19 locations on Sumatra, Java and the Mentawai islands to record male loud-calls of wild surilis (*Presbytis*), a genus of Asian leaf monkeys (Colobinae) with disputed taxanomy, and compared the structure of their loud-calls with a molecular genetic analysis.

Results: The acoustic analysis of 100 surili male loud-calls from 68 wild animals confirms the differentiation of *P. potenziani, P.comata, P.thomasi* and *P.melalophos*. In a more detailed acoustic analysis of subspecies of *P. melalophos*, a further separation of the southern *P.m.mitrata* confirms the proposed paraphyly of this group. In concordance with their geographic distribution we found the highest correlation between call structure and genetic similarity, and lesser significant correlations between call structure and geographic distance, and genetic similarity and geographic distance.

Conclusions: In this study we show, that as in crested gibbons, the acoustic structure of surili loud-calls is a reliable tool to distinguish between species and to verify phylogenetic relatedness and migration backgrounds of respective taxa. Since vocal production in other nonhuman primates show similar constraints, it is likely that an acoustic analysis of call structure can help to clarify taxonomic and phylogenetic relationships.

4.1 Abstract

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4.1.1 Ringkasan

Latar Belakang: Tingkatan dimana suara panggilan keras pada primata bukan manusia sebagai alat taksonomi yang bisa diandalk an masih menjadi perdebatan. Studi terkini tentang crested Gibbon menunjukkan bahwa spesies ini dapat dibedakan secara jelas lewat lagu mereka; bahkan penulis menemukan perbedaan yang nyata pada tingkat populasi. Walaupun ada studi lebih lanjut mengenai perbedaan geografis dan filogenetik dalam suara panggilan keras spesies primata bukan manusia, masih belum jelas sampai sejauh mana suara panggilan keras spesies lain memiliki hubungan yang dekat dengan struktur akustik, hubungan filogenetik dan jarak geografis. Oleh karenanya kami melakukan survey lapang di 19 lokasi di Sumatra, Jawa dan Kepulauan Mentawai untuk merekam suara panggilan keras surili liar (Presbytis), sebuah genus dari monyet daun asia (Colobinae) yang taksonominya masih diperdebatkan, dan membandingkan struktur panggilan keras mereka dengan analisa genetis molekuler.

Hasil: Analisa akustik dari 100 suara panggilan keras surili jantan dari 68 hewan liar mengonfirmasikan perbedaan P. potenziani, P. comata, P. Thomas dan P. melalophos. Dalam analisa akustik yang lebih detail dari subspesies P. melalophos, pemisahan lebih lanjut dari P. m. mitrata di bagian selatan mengonfirmasi usulan parafili dari kelompok ini. Dalam kaitannya dengan penyebaran geografis mereka, kami menemukan korelasi tinggi antara struktur panggilan dan kemiripan genetik, dan korelasi kurang signifikan antara struktur panggilan dan jarak geografis, dan kemiripan genetik dan jarak geografis.

Kesimpulan: Dalam studi ini kami menunjukkan, yaitu pada crested Gibbon, struktur akustik suara panggilan keras surili adalah alat yang dapat diandalkan untuk membedakan spesies dan untuk menverifikasi hubungan filogenetik dan latar belakang migrasi takson bersangkutan. Karena produksi fokal dalam primata bukan manusia lain menunjukkan hambatan serupa, dapat disebut bahwa analisa akustik struktur panggilan dapat membantu klarifikasi taksonomi dan hubungan filogenetik.

4.2 Introduction

Langurs of the Asian colobine genus *Presbytis* (surilis) are exclusively arboreal animals, which inhabit tropical rainforest habitats of Sundaland, i.e., the Malay peninsula and the western Indo-Malay archipelago, comprising of Sumatra, Borneo, Java, the Mentawai islands and some smaller interjacent islands (Oates et al. 1994) (for geographical distribution of *Presbytis* see Figure 3.1 and Figure 4.1). Mainly driven by Sundaland's dramatic geological and climatic changes during the past million years, the genus has undergone an extensive radiation (Meijaard & Groves 2004). With more than 50 described color variants (Brandon-Jones et al. 2004; Groves 2001), *Presbytis* is one of the most diverse primate genera among Old World monkeys.

Like many other primate species, surilis emit loud, conspicuous vocalizations termed loud-calls or long-distance calls. In contrast to *Presbytis*, gibbon loud-calls have a well-adapted acoustic structure (Ryan & Kime 2003; Schneider et al. 2008); with an energy concentration in single frequency bands, a slow modulation of song elements and a transmission range adjusted to the frequency window of rainforest conditions, their songs can be heard over several miles (Padgham 2004; Waser & Waser 1977). Although less well optimized, loud-calls produced by other nonhuman primate species, such as howler monkeys (Da Cunha & Byrne 2006) or surilis (Wich et al. 2003), also exhibit adaptations for long-distance transmission. Loud-calls can have a variety of different functions; they may be used to defend resources, to compete for mates, to mediate intergroup spacing and to promote intragroup cohesion (Da Cunha & Byrne 2006; Waser 1975; Wich & Nunn 2002). In those species in which the structure of loud-calls is well adapted to long-distance transmission, they function predominantly to mark and defend territories.

Although there is general agreement that loud-calls may also serve as phylogenetic traits, systematic studies comparing call structure and genetic relatedness are rare. Amongst gibbons, structural differences are routinely used as a taxonomic tool (Geissmann 2002; Geissmann & Nijman 2006). In a recent study on crested gibbons

carried out in 24 different locations in Vietnam, Laos and Cambodia, Thinh and colleagues (2011) combined a molecular genetic analysis with an acoustic analysis and showed that song structure alone can be used to distinguish the different species. Based on call structure, the authors were also able to distinguish single populations and support not only their phylogentic relatedness, but also their proposed geographic origins. Comparable studies in other nonhuman primates are lacking. However, single studies on loud-calls of orangutans (Ross & Geissmann 2007), Thomas langurs (Wich et al. 2008), chimpanzees (Mitani et al. 1999), black-and-white colobus monkeys (Oates & Trocco 1983) or sportive lemurs (Mendez-Cardenaz et al. 2008) revealed geographic or genetic related differences in the structure of loud-calls of these species. Some previous studies proposed that loud-calls of surilis could be a useful tool to characterize phylogenetic relatedness (Aimi & Bakar 1992; Wilson & Wilson 1975; 1996). According to these studies, the Sumatran surilis were divided into the species *P.melalophos, P.femoralis, P.thomasi* (Aimi & Bakar 1992; Wilson & Wilson 1975; 1996) and *P.potenziani*(Aimi & Bakar 1992; Wilson & Wilson 1975; 1996), and Wilson and Wilson (1975) proposed a successive invasion of Sumatra, Borneo and the Mentawai islands from the Asian mainland. However, all these studies are only based on phonetic descriptions of loud-calls and did not make a systematic analysis of the acoustic structure or a direct comparison between acoustic structure and genetic relatedness.

Here we combine the results of the most comprehensive molecular genetic study on leaf monkeys of the genus *Presbytis* currently available (Meyer et al. 2011) with a systematic field survey in which the loud-calls of *P.potenziani siberu*, *P.comata comata*, *P.thomasi* and the four subspecies of *P.melalophos* (*melalophos, mitrata, bicolor* and *sumatrana*) were recorded (Groves 2001). Previous classifications and phylogenies of *Presbytis* were mainly based on behavioral and anatomical features, in particular coat coloration (Aimi and Bakar 1992, 1996; Brandon-Jones 1996b, 2004; Brandon-Jones et al. 2004; Chasen 1940; Groves 1989, 2001; Hooijer 1962; Napier and Napier 1967; Oates et al. 1994), while genetic studies are extremely limited (Md Zain 2001; Md Zain et al. 2008; Meyer et al. 2011; Vun et al. 2011). In our recent study (Meyer et al. 2011),

mitochondrial DNA was used to propose a revision of Groves' classification (Groves 2001) suggesting species status for the four subspecies of *P.melalophos* and also for both subspecies of *P.comata* and *P.potenziani*. However, for convenience we use here the classification of Groves (2001).

Since surilis intensively responds to stranger call playbacks (Wich et al. 2002a), we used a playback design in order to collect vocalization data under comparable conditions. We hypothesized that, similar to crested gibbons, structural differences in *Presbytis* loud-calls reflect phylogenetic relationships and can support a revision of the current classification.

4.3 Methods

4.3.1 Survey locations and data collection

In 2007 and 2008 we conducted field surveys in 19 locations on Sumatra, Java and the Mentawai islands, and recorded male loud-calls of *P.thomasi, P.potenziani, P.melalophos* and *P.comata* (Figure 4.1). To find and track animals the field sites were explored between 5.30 am and 6 am until noon, and in the evening from 3 pm till sundown. When a group was encountered GPS data of the location (using a handheld GARMIn© GPSMAP 76CSX), information about the group composition and the appearance of the animals (i.e. morphological characters, for instance pelage coloration or scars) were noted on data sheets whenever possible. All visual observations were made by using binoculars (8 x 32 Steiner Sky-Hawk).

Since surilis intensively respond to stranger call playbacks (Wich et al. 2002a), we used a playback design to collect data under comparable conditions. Initially, vocalizations were opportunistically recorded to achieve a high quality call of each population. For the playback the quality of the recorded vocalizations was screened on a notebook using AVISOFT SASLAB Pro software version 5.1 (R. Specht, Berlin, Germany). Undisturbed calls from each population were selected and only one of these was used to stimulate response from respective study populations in the same area. At each site,

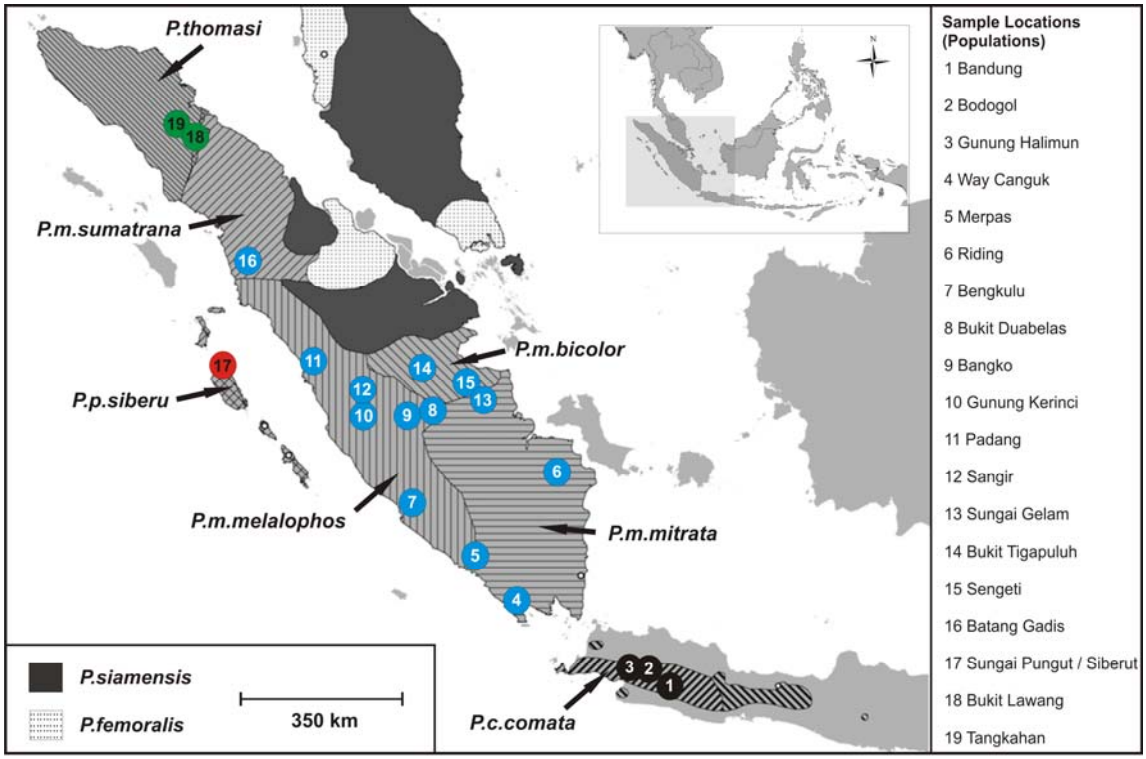

Figure 4.1: Geographical distribution of *Presbytis* taxa on Sumatra, Java and the Malay peninsula. Sampled taxa are labeled in the map. Hatched areas in the map indicate distribution ranges of respective taxa, colors indicate species and numbers indicate the origin of acoustic samples (populations).

we tried to avoid recording the same individuals by direct observations. Each playback comprised of 4 calls, which were played back one by one in 20 second intervals.

For the final data collection, playback treatments were amplified with a Vision David Speaker connected to a MP3-Player (Samsung YP-U3) from about 75 m distance of the focal group at a height of 2 m (Wich et al. 2002a; 2002b). After the performance at least 15 minutes were recorded. If a response was given before the playback was finished the playback was stopped. To record vocalizations a solid state recorder (Mirant PMD 660 (Marantz, Japan); sampling rate: 44.1 kHz, 16 bit amplitude resolution) and a Sennheiser directional microphone (K6 power module, ME66 recording head, MZW66 pro windscreen, Sennheiser, Wedemark, Germany) were utilized. For each playback treatment the GPS position of the location, the group number, the date, time and the identity of a responding male were noted on data sheets.

4.3.2 Acoustic analysis

Male surili loud-calls consist of iterations of single elements. *P.thomasi* and *P.potenziani* produce coughing elements at the beginning of the call. In *P.thomasi*, the successive elements rise in crescendo and increase in volume (see build up phase Figure 4.2), while the coughing elements in *P.potenziani* are equally loud and noisy. Both loud-calls end with howling tonal phrases including inhalation and exhalation elements (Figure 4.2). We considered these calls as completely developed when both parts were produced. *P.comata* loud-calls were considered as completely developed when a boost in loudness and frequency till the end of the call was present. *P.melalophos* loud-calls were considered as completely developed when they included at least 10 elements (the only two calls that were interrupted had less than 10 elements).

AVISOFT SASLAB Pro 5.1 was used to measure acoustic parameters and to generate spectrograms (FFT = 1024 pt, Frequency resolution = app. 27 Hz). To find the point with maximum energy at the beginning, ending and intermediate points of call elements in the spectrogram, the bounded reticule cursor tool of AVISOFT was used. To address different phases within loud-calls, each call was additionally divided into four quarters. Since all taxa produce exhalation elements, the amount of exhalation elements (Ex) was therefore divided by 4 and subsequently multiplied by 1, 2, 3 and 4, respectively. Odd numbers were rounded. If inhalation elements (In) were present; the second, third and fourth quarter always started with an exhalation element (for a detailed description of used parameters see Table 4.1 and Figure 4.3).

Table 4.1: Description of the 23 acoustic parameters that were used in the analysis (numbers of parameters correspond to Additional File 6, for examples see Figure 4.4)

Parameter Number	Parameter description
1	Duration of the entire call [s]: from the starting point of the first element till the ending point of the last element
2	Elements : amount of elements (inhalation and exhalation)
3	Elements per second [e/s]: amount of elements over the duration
4	Maximum frequency start [Hz]: maximum frequency of the starting points of the entire elements
5	Minimum frequency start [Hz]: maximum frequency of the entire starting points of elements
6	Maximum frequency end [Hz]: maximum frequency of the entire ending points of elements
7	Minimum frequency end [Hz]: minimum frequency of the entire ending points of elements
8	Mean frequency start [Hz]: arithmetic mean of the frequency of the entire starting points of elements
9	Mean frequency end [Hz]: arithmetic mean of the frequency of the entire ending points of elements
10	Exhalation elements: amount of exhalation elements
11	Inhalation elements: amount of inhalation elements
12-15	1^{st} -, 2^{nd}-, 3^{rd} - and 4^{th} - quarter elements per second [e/s]: amount of elements over the duration of respective quarters
16	Middle part elements per second [e/s]: amount of elements over the duration of the 2^{nd} and 3^{rd} quarter
17-20	1^{st} -, 2^{nd}-, 3^{rd} - and 4^{th} - quarter mean frequency start [Hz]: arithmetic mean of the frequency of the entire starting points of elements of respective quarters
21	Middle part mean frequency start [e/s]: arithmetic mean of the frequency of the entire starting points of elements of the 2^{nd} and 3^{rd} quarter
22-23	1^{st} and 2^{nd}- quarter mean frequency end [Hz]: arithmetic mean of the frequency of the entire ending points of elements of respective quarters

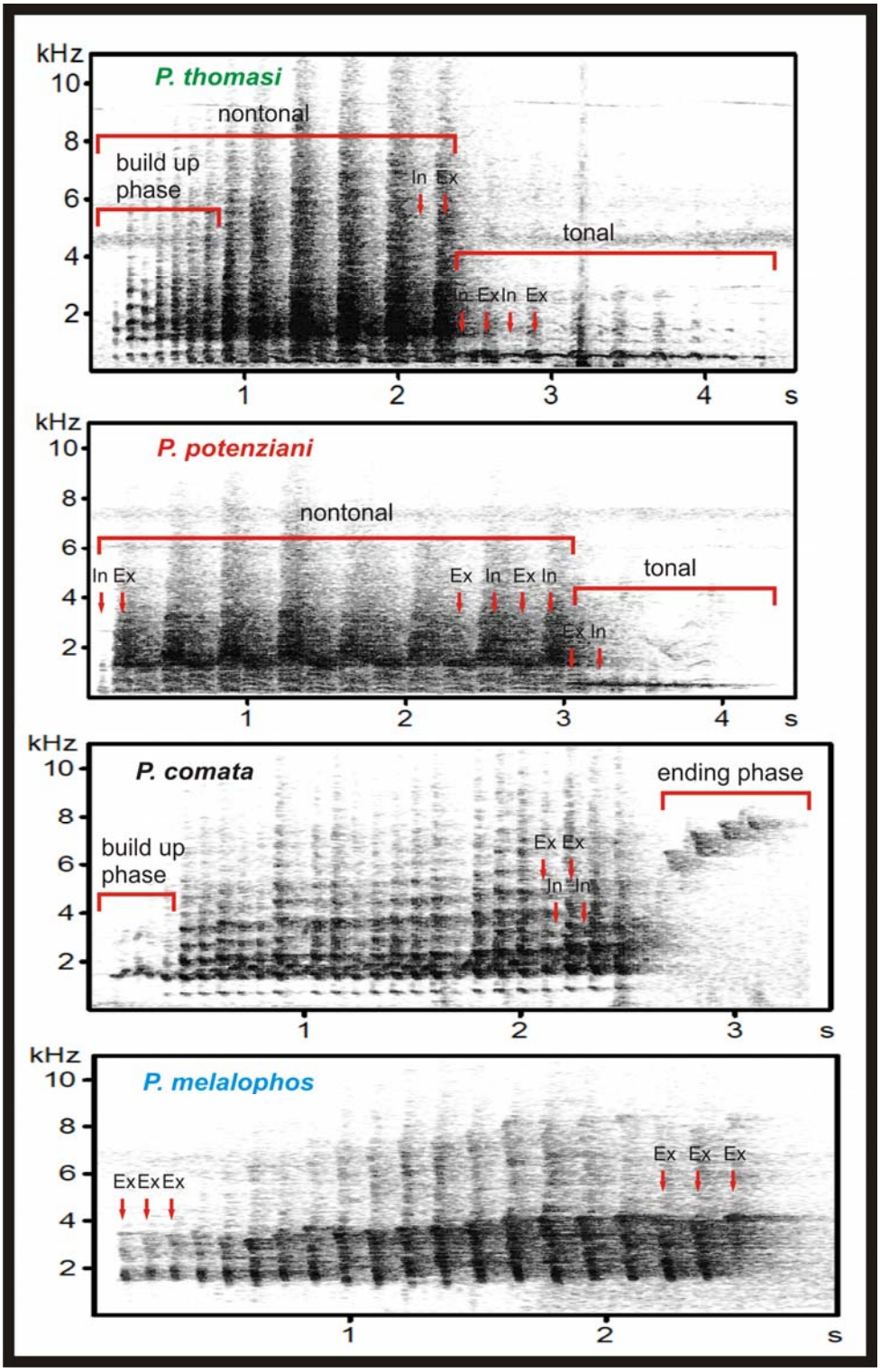

Figure 4.2: Spectrograms of typical loud-calls of *P.thomasi*, *P.potenziani*, *P.comata* and *P.melalophos*.

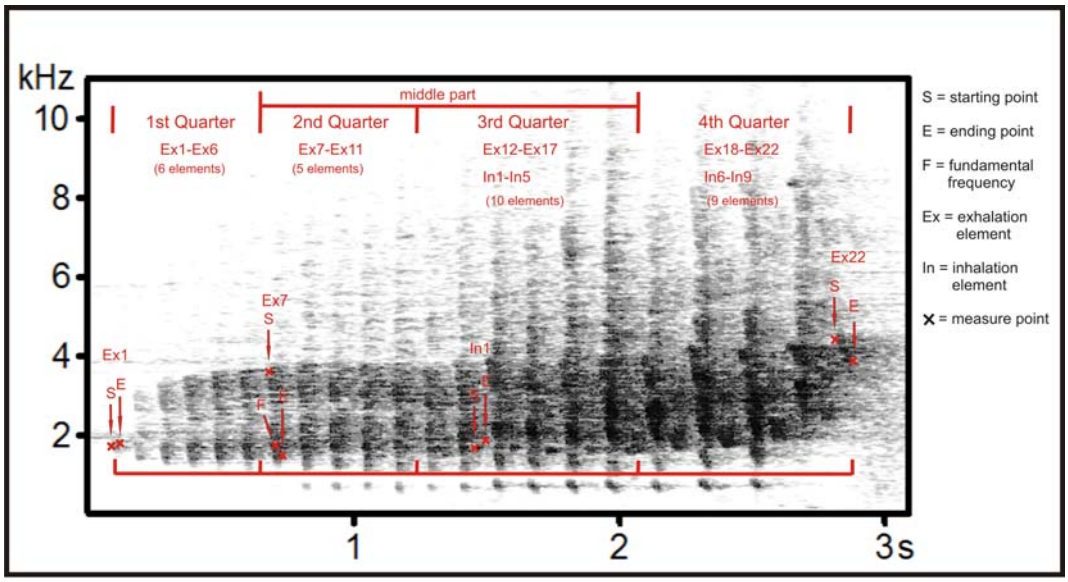

Figure 4.3: Spectrogram of a *Presbytis* loud-call with examples for the measured parameters.

4.3.3 Discriminant Function Analysis

For both Discriminant Function Analyses (DFAs), we excluded acoustic variables that could not be obtained in the majority of loud-calls. In the first DFA we used 23 acoustic parameters for 100 loud-calls from all 19 populations (Table 4.1, Figure 4.1). For the second DFA, including only the four *P.melalophos* subspecies (*melalophos, mitrata, sumatrana, bicolor*), we used the same 23 acoustic parameters for 71 loud-calls (population numbers 4-16, Figure 4.1). All acoustic parameters were conducted to stepwise DFAs in SPSS 19 (Meulman & Heiser 1989). The selection criterion for an acoustic parameter to be entered was p=0.05 and p=0.1 to be removed from the analysis. The assignment of loud-calls to the different populations was cross-validated by the leaving-one-out method (Brockelman & Ali 1987), which involves leaving out each of the cases in turn, calculating the functions based on the remaining n−1 cases and then classifying the left-out case.

4.3.4 Phylogenetic tree reconstruction

For reconstructing phylogenetic relationships of loud-call structure, we used the F values of pairwise distances of the stepwise DFA described above. These F values describe the pairwise similarity of the 19 populations in relation to their overall similarity. Based on these F values, a neighbor-joining tree of acoustic data was reconstructed in the program Neighbor of the PHYLIP package 3.69 (Felsenstein 2005). The molecular-based phylogenetic tree derived from mitochondrial sequence data was redrawn from Figure 3.2 (Meyer et al. 2011) and shows only taxa included in the present study. Respective branch lengths refer to those obtained from the Bayesian reconstruction in (Meyer et al. 2011).

4.3.5 Correlation analysis between vocal structure, genetic and geographical distance

To test the statistical relationship between acoustic structure, and genetic and geographic distance matrices, we used a Mantel Test algorithm programmed in R (R. Mundry, Leipzig, Germany). For the analysis we only used populations where acoustic and genetic data was available (N=17). The acoustic similarity matrices were generated as described above. Geographic coordinates were obtained via GPS and the geographic distance matrices were calculated from the minimum distance of different groups as implemented in GenAlEx 6.4.1 (Peakall & Smouse 2006). GenAlEx was also applied to calculate uncorrected pairwise genetic distances between haplotypes of a 1.8 kb fragment of the mitochondrial genome. Respective haplotypes were recently published by our group (Meyer et al. 2011) (GenBank accession numbers: JF295100-JF295101 [*P.m.mitrata*], JF295104 [*P.m.melalophos*], JF295106-JF295109 [*P.m.bicolor*], JF295117-JF295118 [*P.comata*], JF295124-JF295125 [*P.thomasi*], JF295119-JF295121 [*P.potenziani*]).

4.4 Results

In 2007 and 2008, we conducted field surveys in 19 locations (which resemble 19 populations) on Sumatra, Java and the Mentawai islands, and recorded male loud-calls of seven wild non-habituated *Presbytis* taxa (Figure 4.1). Included are *P.thomasi, P.potenziani siberu, P.comata comata* and all four subspecies of *P.melalophos* (*P.m.melalophos, P.m.mitrata, P.m.bicolor* and *P.m.sumatrana*). In total, we recorded more than 300 loud-calls of 68 male individuals.

In response to the playbacks, males often responded several times, but only one call of this bout was used for the analysis (in total 100 calls). Counter calling males in general decreased the distance to the speaker, while females hid or disappeared. A further common response to playback treatments was alarm calling of group members and in addition juveniles often started to squeal (Gurmaya 1986). Loud-calls were mostly accompanied by a jumping display.

4.4.1 General differences in male loud-calls

P.thomasi, P.potenziani, P.comata and *P.melalophos* can be clearly identified by general acoustic characteristics in their call structure (Figure 4.2). In addition, species' calls are readily distinguished by ear, but P.melalophos subspecific differences are undetectable.

P.thomasi (n=10) and P.potenziani (n=9) calls start with coughing elements at the beginning and end with howling tonal phrases. These two parts include inhalation and exhalation elements. In *P.thomasi*, the initial coughing elements rise in crescendo and increase in volume (build-up phase). In *P. potenziani* (n=9), the build-up phase is missing and the coughing elements are equally loud and noisy (Figure 4.2). Both loud-calls also differ in their mean duration with 3.58 s (SD=0.35) for P.thomasi and 4.17 s (SD=0.42) for *P.potenziani*. On the average, *P.potenziani* produces 28 elements (SD=2) per call with a mean element frequency of 6.42 per second (SD=0.56), while *P.thomasi* produces 30 elements (SD=4) with a mean element frequency of 8.5 elements/s (SD=1)

(Figure 4.2). Detailed differences in the acoustic structure can be found in Additional File 6.

The typical *P.comata* call (n=10) is characterized by a unique staccato-like sequence of 52 (SD=8) alternating exhalation and inhalation elements (mean 18.20, SD=1.91 elements/s). *P.comata* calls, with a mean call duration of 2.86 s (SD=0.26), include a short build-up phase and an end-up phase, both with increasing loudness and frequency (Figure 4.2, Additional File 6).

Loud-calls of *P.m.bicolor* (n=15), *P.m.sumatrana* (n=9), the central Sumatran *P.m.mitrata* (n=7) and *P.m.melalophos* (n=26) from outside of Bengkulu, consist of a sequence of exhalation elements. An exception are the calls of *P.m.melalophos* from Bengkulu (n=3) and the southern Sumatran *P.m.mitrata* (n=12), which differ by producing alternating exhalation and inhalation elements at the end of the call (Figure 4.4). The mean duration of *P.melalophos* calls lies between 2.39 s (SD=0.33) for P.m.mitrata and 2.53 s (SD=0.40) for *P.m.sumatrana*. The mean frequency of produced elements lies between 10.85 elements/s, (SD=2.36) for *P.m.mitrata* and 7.35 elements/s (SD=0.4) for *P.m.bicolor* (Figure 4.4, Additional File 6).

4.4.2 Subtle differences in male loud-calls

Result of discriminant function analysis of all 19 populations (DFA1)

The DFA assigns 72% of the loud-calls (62% of cross-validated, chance level = 5.3%) to the original populations. In relation to taxon identity 83% of the cross-validated cases are correctly classified. Most misclassified cases are found between *P.melalophos* subspecies (Table 4.2).

No misclassification can be found between the four *Presbytis* species, *P.comata* (populations 1-3), *P.melalophos* (populations 4-16), P.potenziani (population 17) and *P.thomasi* (populations 18-19). They form four well separated clusters with a correct assignment of 100% (Table 4.2, Figure 4.5 A). Among the large *P.melalophos* cluster, one further sub-cluster is indicated, which includes *P.m.melalophos* from Bengkulu

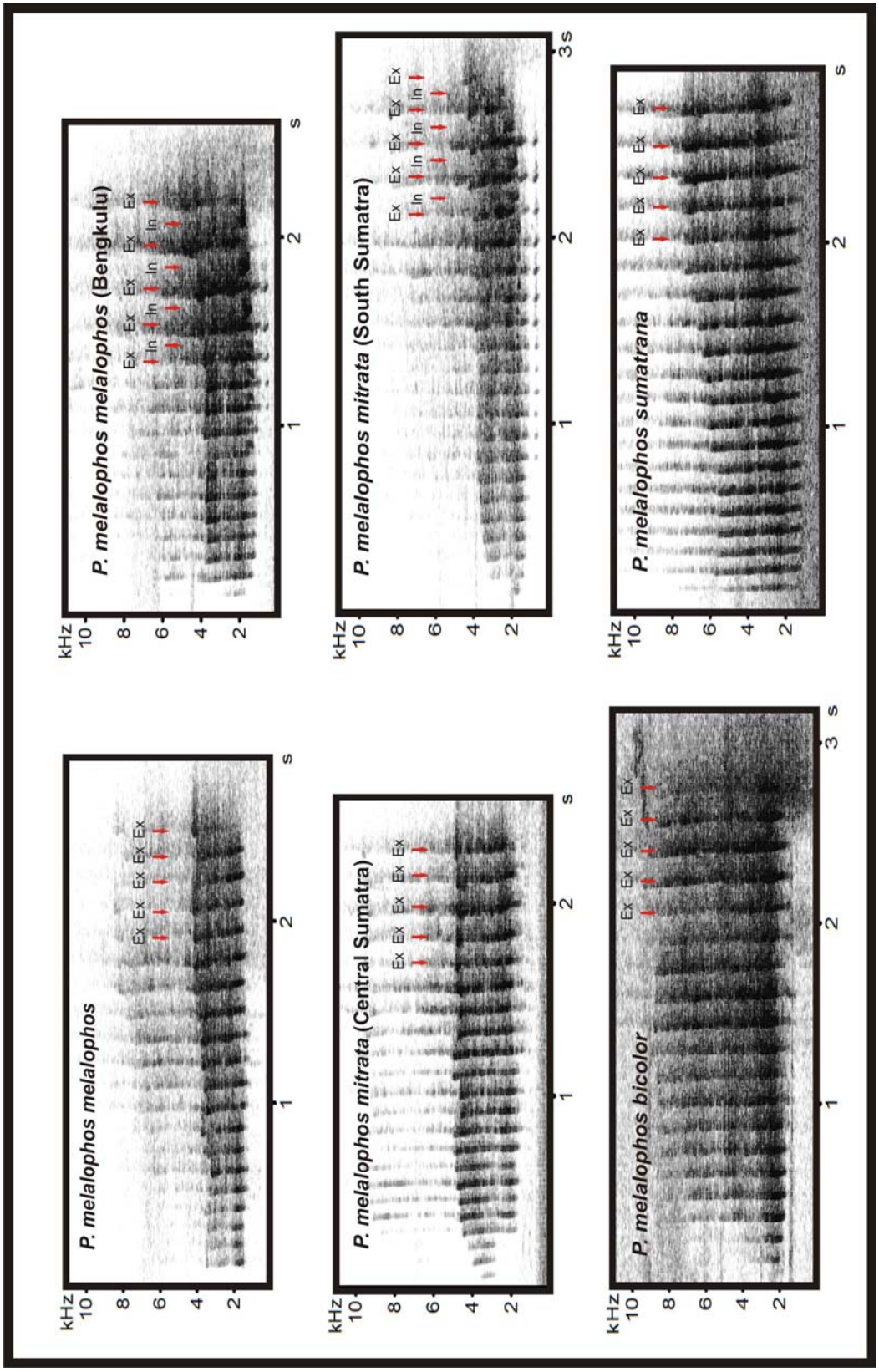

Figure 4.4: Spectrograms of typical loud-calls of *P.melalophos subspecies*.

Table 4.2: Classification results of the first and second DFA in relation to the taxon membership. Relative predicted group membership in % (DFA 1 / DFA 2), n = calls, P = Population(s) number (see also Figure 4.1), I = Individuals.)

	P.comata **n=10** **P 1-3**	***P.m.mitrata*** **n=19** **P 4-6, 13**	***P.m. mellophos*** **n=29** **P 7-12**	***P.m. bicolor*** **n=15** **P 14-15**	***P.m. sumtrana*** **n=8** **P 16**	***P.potenziani*** **n=9** **P 17**	***P.thomasi*** **n=10** **P 18-19**
P.comata I=8	**100**						
P.m.mitrata I=13		**68 / 89**	26 / 11	6 / 0			
P.m.melalophos I=18		7 / 7	**79/ 76**	14 / 10	0 / 7		
P.m.bicolor I=13		7 / 0	20 / 7	**73 / 93**			
P.m.sumatrana I=5			12 / 25	0 / 12	**88 / 63**		
P.potenziani I=4						**100**	
P.thomasi I=7							**100**

(population 7) and the southern Sumatran P*.m.mitrata* (populations 4-6). The scattergram (Figure 4.5 A) shows the separation of the 19 populations according to the first and second discriminant function, explaining 56.3% and 26.7% of the total acoustic variation, respectively. The first discriminant function, which mainly represents the amount of inhalation elements, separates populations 1-3 from all others, while the second discriminant function, which represents rhythmical features, separates population 17 from all others. To focus on the *P.melalophos* cluster (populations 4-16), we conducted a second DFA.

Result of the discriminant function analysis of P.melalophos populations (DFA2)

The second DFA2 assigns 66.2% of the loud-calls (49.3% of cross-validated, chance level = 7.7%) to the original populations and establishes three distinct clusters (Figure 4.5 B), separating the southern Sumatran *P.m.mitrata* (populations 4-6) and the *P.m.melalophos* from Bengkulu (population 7) from the remaining *P.melalophos* populations.

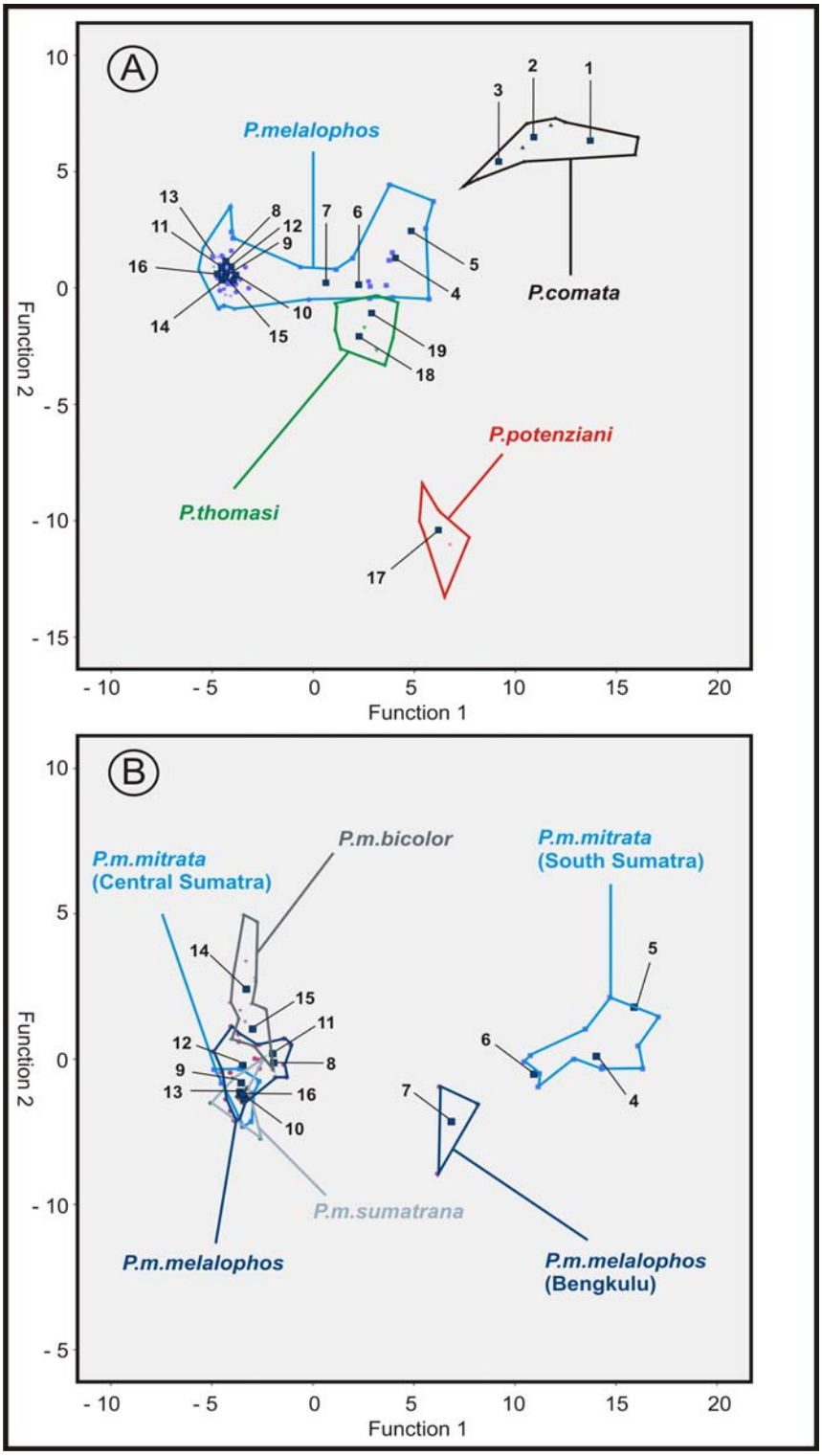

Figure 4.5: Distributions of the different populations belonging to the four species *P.melalophos*, *P.comata*, *P.thomasi* and *P.potenziani* (A), and to the different *P.melalophos* subspecies (B). Rectangles indicate population centroids. Taxa are separated by color.

In relation to the taxon identity 89% *P.m.mitrata*, 76% *P.m.melalophos*, 93% *P.m.bicolor* and 63% *P.m.sumatrana* of the cross-validated population cases are correctly classified (Table 4.2). The scattergram (Figure 4.5 B) shows the separation of the 13 populations according to the first and second discriminant function, explaining 92.9% and 3.8% of the total variation, respectively. The first discriminant function, which explains nearly all structural differences, represents the amount of inhalation elements, separates populations 4-6 from population 7, and the remaining populations. The second discriminant function mainly based on the minimum frequency of the call, indicates the separation of populations 14, 15 and 5 from population 7 and the lasting locations.

4.4.3 Correlation between vocal structure, genetic and geographical distance

A Mantel test was performed to test the concordance between genetic distance, geographical distance and acoustic similarity. All populations where corresponding genetic data was available (N=10) were included in the analysis. We found highest significant correlations between the vocal structure and genetic distance (P=0.001, R_{XY}=0.91), and lower significant correlations between vocal structure and geographical distance (P=0.001, R_{XY}=0.662), and geographical distance and vocal structure (P=0.001, R_{XY}=0.663) between population 3 (*P.comata*), populations 4,6,13 (*P.m.mitrata*), population 9 (*P.m.melalophos*), populations 14, 15 (*P.m.bicolor*), population 17 (*P.potenziani*) and populations 18, 19 (*P.thomasi*) (for details on the locations see Figure 4.1).

4.4.4 Phylogenetic relationships among *Presbytis* taxa based on acoustic data and comparison with molecular data

The vocal- (Figure 4.6 A) and molecular-based phylogenies (Figure 4.6 B) (Meyer et al. 2011) are highly congruent. In both phylogenies, *P.thomasi, P.potenziani, P.melalophos* (excluding *P.m.mitrata* from South Sumatra) and *P.comata* + *P.m.mitrata* from South Sumatra form four distinct clusters/lineages and indicate a similar branching pattern. Contrary to the molecular phylogeny, in the acoustic tree *P.m.mitrata* from South Sumatra (populations 4-6) does not form a monophyletic cluster, and *P.m.sumatrana* (population 16) and *P.m.bicolor* (populations 14-15) are nested within the cluster consisting of *P.m.melalophos* and *P.m.mitrata* from Central Sumatra (populations 7-13). This might be due to the subtle differences found in the vocal structure of respective populations.

4.5 Discussion

Here we report significant differences between loud-call structures of *P.thomasi, P.potenziani, P.comata* and *P.melalophos*. Among the latter species a significant separation between the South Sumatran *P.m.mitrata* and the central Sumatran *P.m.mitrata*, as well as a further separation between *P.m.melalophos* from Bengkulu and the remaining *P.m.melalophos* populations, could be detected. The acoustic discrimination between *Presbytis* taxa was highly positively correlated with their genetic distance. In addition, we found substantial significant correlations between acoustic similarity and geographic distance and between genetic distance and geographic distance.

In our recent molecular genetic study (Meyer et al. 2011) we suggested a paraphyly for *P.m.mitrata*, with the central Sumatran populations being closely related to *P.m.melalophos* and the South Sumatran populations forming a sister lineage to

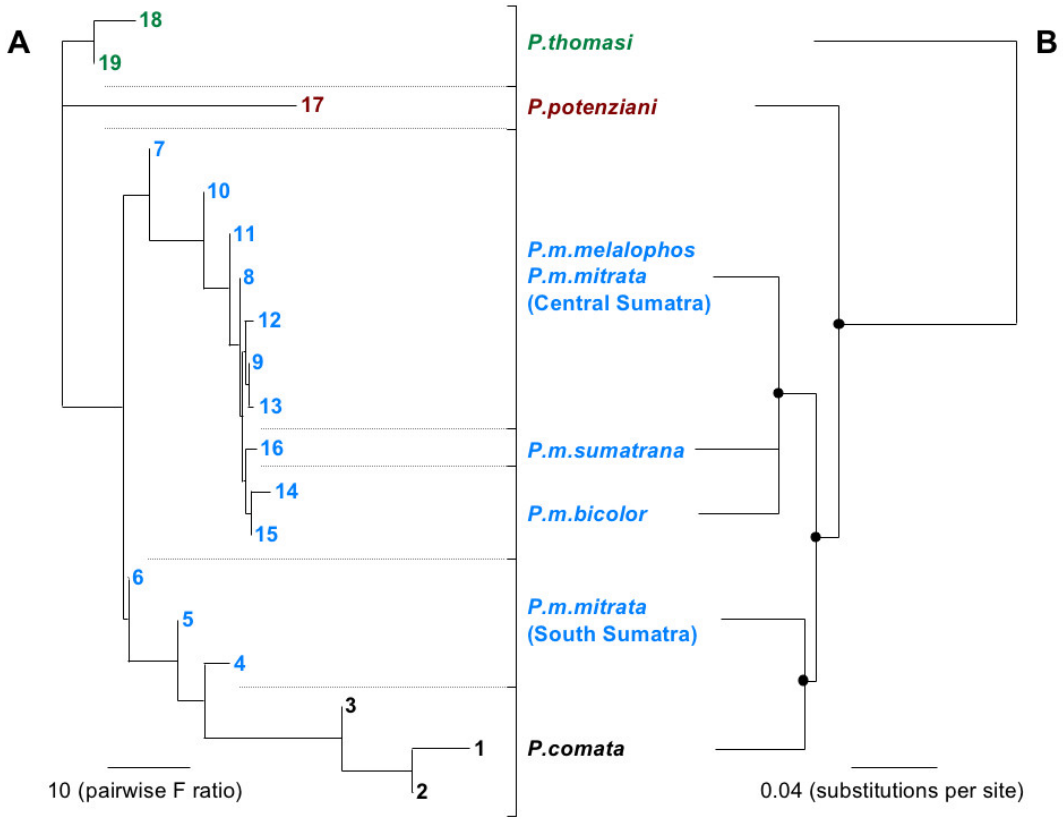

Figure 4.6: (A) Neighbor-joining tree of *Presbytis* taxa based on the acoustic similarity matrix (F values) and (B) their phylogenetic relationships according to mitochondrial sequence data (redrawn from Meyer et al. (2011)). In A, colored letters indicate species and numbers corresponding to sampling locations (see Figure 4.1). In B, branch lengths refer to those obtained from the Bayesian reconstruction in Meyer et al. (2011) and black dots on nodes indicate Bayesian posterior probabilities of >0.96.

P.comata (Figure 4.6 B). Our current findings on the acoustic structure of loud-calls strongly support these results.

P.m.mitrata was reported to inhabit the area southeast of the Batang Hari River, a large river in central Sumatra. In the west, this subspecies does not extend to the Bukit Barisan range, a mountain range on the western side of Sumatra (Aimi & Bakar 1996), where *P.m.melalophos* occurs (Groves 2001). Our samples of the central Sumatran *P.m.mitrata* (population 13) derived from the above described northernmost distribution range of this subspecies, south of the Batang Hari river. Although much paler, the morphological appearance resembles the reddish *P.m.melalophos* more than the gray-

ish white southern Sumatran *P.m.mitrata* (Additional File 7). Whether there might be a transition zone between *P.m.melalophos* and *P.m.mitrata* demands further research. It is highly likely that *P.m.melalophos* gradually intergrades with P.m.mitrata, as may be the case between *P.m.bicolor* and *P.m.melalophos* (Aimi & Bakar 1996). Our results, however, let us conclude that the central Sumatran P.m.mitrata population is the paler color variant of *P.m.melalophos*. Thus, the geographical distribution range of *P.m.melalophos* should be extended from the Bukit Barisan range eastwards towards Jambi. The southern Sumatran *P.m.mitrata* is genetically, morphologically and acoustically distinct from the remaining *P.melalophos* subspecies (see also Additional File 7). Therefore, if the Phylogenetic Species Concept (Cracraft 1983; 1989) is applied, *P.m.mitrata* would be elevated to a monotypic species *P.mitrata* Eschscholtz, (1821).

Among *P.m.melalophos* we found the calls from Bengkulu (population 7) forming a distinct cluster. Unfortunately, genetic data from Bengkulu are lacking, but acoustically, the call types were more closely related to the Southern *P.m.mitrata* mainly due to the presence of inhalation elements. Historically different color morphs of *P.m.melalophos* were described, all of which are currently classified as synonyms of *P.m.melalophos* (Groves 2001). These are a) the much less red and buffer variant from Bengkulu (*Simia melalophos* Raffles, 1821; syn. *flavimanus* Geoffroy, 1830), b) a foxy red northern form (*Presbytis nobilis* Gray, 1842) from Solok [4], c) a less reddish form from Padang (*Semnopithecus ferruginneus* Schlegel, 1876) and d) a golden buff variant (*Semnopithecus sumatranus var. aurata* Müller & Schlegel, 1841) from Gunung Talamau (ca. 150 km northwards from Padang) (Groves 2001). The great diversity of color morphs in *Presbytis*, in particular in *P.melalophos*, has caused much debate over the past decades. Coloration might indicate relatedness, but can often be misleading, in particular, when no broad geographic sampling is available. Our data point out that the taxonomic ranking of some of these historically described taxa possibly should be reconsidered. However, the loud-calls from population 7 are only derived from two individuals and genetic data are missing. Therefore, further molecular genetic and bioacoustic research based on a broader sampling is needed to draw final conclusions. Of

great interest are the acoustic data of the Bornean taxa, in particular data of *P.rubicunda*. Based on molecular genetic results *P.melalophos* is even polyphyletic since *P.rubicunda* is nested within the *P.m.sumatrana*, *P.m.bicolor*, *P.m.melalophos*/central Sumatran *P.m.mitrata* clade (Meyer et al. 2011). Previous studies already proposed a close affiliation of *P.rubicunda* and *P.melalophos* based on the red coat coloration (Brandon-Jones 1996b) or in some aspects of behavior and vocalization (Wilson & Wilson 1975). If species status of *P.rubicunda* is retained, species status of *P.m.sumatrana, P.m.bicolor, P.m.melalophos* will be consequently warranted, otherwise *P.rubicunda* has to be assigned as a subspecies of *P.melalophos*.

The correlation between acoustic structure and genetic differences was higher than the correlation between acoustic structure and geographic distance. This pattern can be explained by the following proposed *Presbytis* migration pattern, which is largely in agreement with Wilson and Wilson (1975). The initial split in *Presbytis* occurred between *P.thomasi* and all other taxa, and *P.thomasi* colonized North Sumatra, which became isolated afterwards. The ancestor of the remaining taxa colonized first Borneo and later Sumatra. An early divergence of Bornean taxa is also supported by previous genetic studies (Md Zain 2001; Md Zain et al. 2008; Meyer et al. 2011; Vun et al. 2011). Of the ancestral Sumatran stock, one lineage invaded the Mentawai islands (*P.potenziani*), the other split into the proto-*P.melalophos* lineage and into the southern *P.m.mitrata/P.comata* lineage (Figure 4.6). Although calls from *P.femoralis/P.siamensis* (eastern Sumatra, Asian mainland) are not analysed in our study, previous publications show similarities in call structures of *P.femoralis* and *P.thomasi* (Kawamura 1984; Megantara 1989). Our genetic study (Meyer et al. 2011) shows that *P.femoralis* diverged relatively early from other lineages and, thus, the similar call structure of *P.femoralis* and *P.thomasi* might be a plesiomorphic feature. Up to this point the genetic, geographic and acoustic differences between populations increased. From this point onwards the geographic distances between populations decreased, because proto-*P.melalophos* subsequently transmuted into various present day subspecies, which were finally distributed across Sumatra as far as to the distribu-

tion range of *P.thomasi* in North Sumatra. Consequently, the geographic distance between *P.thomasi* and the remaining Sumatran populations decreased, while the genetic and the acoustic differences increased. Finally, the southern *P.m.mitrata/P.comata* lineage split into *P.m.mitrata* and *P.comata* that colonized Java. In this case we have a linear migration pattern and thus would expect a similar high correlation between acoustic structure, genetic and geographic distance, as it was currently shown in crested gibbons which are proposed to migrate in a linear fashion from North to South (Thinh et al. 2011).

Surilis and gibbons are limited to rainforest habitats where the selection pressure forces an optimal adaptation of the structure of loud-calls for transmission over longer distances (Schneider et al. 2008; Ey & Fischer 2009). Since the structure of loud-calls is inherited and call adaptation forces a similar structure, gene flow could achieve the major influence on the structural variation of calls (Thinh 2011). By combining the phylogenetic reconstruction of Meyer and colleagues (2011) and the results of our study (Figure 4.2, Figure 4.6 A), we can observe a trend to simplification in call structure over time. However, it is difficult to explain why we found such a simplification in call structure. We cannot answer whether this is a general rule or whether this is a *Presbytis*-specific trait. Crested gibbons show an ambiguous result (Thinh et al. 2011), where after a long period of syllable types with simple frequency modulation, a trend to a slightly more complex modulation appears. More acoustic comparisons with more species and at a higher taxonomic level are necessary to answer this question. Interestingly, *P.potenziani* was regarded as most basal lineage (Brandon-Jones 1993) and due to similarities in call structure, the species was proposed as closely affiliated with *P.thomasi* (Wilson & Wilson 1976). However, neither is the case, since *P.potenziani* derived much later (Meyer et al. 2011). The specific call structure of *P.potenziani* is therefore either the result of an analogous evolution or a pleisiomorphic Presbytis feature. To clarify this issue further research is needed and particularly genetic and acoustic data on the Bornean and Malaysian taxa will help to better understand the evolution and phylogeography of the genus.

For instance, the call structure of *P.rubicunda* seems to be similar to *P.melalophos* calls (Wilson & Wilson 1975) and, as discussed above, molecular genetic data also group *P.rubicunda* with *P.melalophos* (Meyer et al. 2011). This close relationship can partly help to explain the interesting feature of general allopatry of respective *Presbytis* taxa in Sumatra, and sympatry in Borneo (Oates et al. 1994). P.rubicunda originated on Sumatra and subsequently invaded Borneo during the middle Pleistocene via a proposed connection between both islands (Meijaard 2004). At this time Borneo was already colonized by the Bornean species *P.chrysomelas, P.frontata and P.hosei*. As a result of this second colonization, *P.rubicunda* is sympatric today with the three other species wherever their ranges overlap (Nijman 2010).

4.6 Conclusions

In this study we have shown that vocal similarity highly correlates with genetic relatedness; these two measures also correlate significantly with geographic distance, but the strength of the relationship is lower. Accordingly, acoustic analysis of surili loud-calls has been proven to be a promising and powerful tool to support taxon-affiliation and phylogenetic relatedness. In addition, we were able to confirm the proposed paraphyly of *P.melalophos* by differences in loud-call structure. Furthermore, acoustic analysis can be used as a tool to support proposed migration routes. These findings might also help to explain taxonomic relationships and migration backgrounds in other nonhuman primate taxa, as long as they have similar constraints in their vocal communication.

4.7 Acknowledgements

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Chapter 5 General discussion

5.1 Summary of results

Leaf monkeys are a very diverse group of primates which developed a great degree of unique adaptations along their evolutionary history. Eight species of leaf monkeys are listed among the 25 most endangered primates in the world (Mittermeier et al. 2009), but until now they receive less scientific attention. This dissertation was designed to elucidate aspects of phylogeny, taxonomy and phylogeography of leaf monkeys with particular focus on the genus *Presbytis*. Molecular, acoustic and distributional data from various data sources, including museum collections, sequence data from GenBank, and most importantly new data collected from the field were used to answer the following questions:

1. **What can be concluded from the re-examination of the leaf monkey phylogeny regarding *Presbytis*?**

- What is the phylogenetic position of *Presbytis* among the Asian colobines?

Phylogenetic reconstructions based on different molecular genetic markers could not clarify the phylogenetic position of *Presbytis*. Mobile elements indicate a close relationship to the odd-nosed group, nuclear sequence data show a basal position among the Asian colobines and mitochondrial sequence data placed *Presbytis* as sister to *Trachypithecus*.

- Does the colobine monkey phylogeny support a possible hybridization between *Presbytis* and *Trachypithecus* or between *Semnopithecus* and *Trachypithecus* (Ting et al. 2008)?

Our results do not support an ancient hybridization between *Presbytis* and *Trachypithecus*. Nuclear divergence time estimates between *Semnopithecus* and *Trachypithecus* (2.56 mya) postdate the mitochondrial divergence of *Presbytis* and *Trachypithecus* (7.45 mya). They indicate unidirectional gene flow from *Semnopithecus* into

Trachypithecus via male introgression over a long period of time, which finally led to the accumulation of nuclear material of *Semnopithecus* into *Trachypithecus* (nuclear swamping).

2. Are previous classifications of *Presbytis* supported by new data from the field?

- Is *P.comata* a polytypitc species group (Brandon-Jones 1978, 1996c)?

Our genetic results show an early separation of *P.thomasi* and *P.hosei* and a late differentiation of *P.comata*. The structural analysis of loud-calls also clearly separates *P.thomasi* and *P.comata.* Therefore, and in accordance with Groves (2001), *P.hosei*, *P.thomasi*, and *P.comata* should be regarded as distinct species.

- Is *P.melalophos* a polytypic species group (Md Zain 2001)?

According to our genetic results the South Sumatran *P. m. mitrata* forms a sister lineage to *P.comata* and the Central Sumatran *P.m.mitrata* population is placed in the *P.melalophos* clade. Therefore *P.melalophos* is paraphyletic. Furthermore close phylogenetic affiliations between *P.melalophos* and *P.femoralis* could not be detected, since both taxa derive from clearly distinct lineages. Acoustic differences in call structure strongly support the paraphyly of *P.melalophos*. As a consequence, our results indicate a further differentiation of *P.melalophos* rather than a polytypic species proposed by Md Zain (2001). In agreement with the Phylogenetic Species Concept (Cracraft 1983, 1989), a revision of the current classification is appropriate and *P.m.mitrata* should be regarded as distinct species *P.mitrata* (Eschscholtz 1821).

- Is there a conspecific relationship between *P.potenziani* and *P.thomasi* (Wilson and Wilson 1976)?

Our genetic results do not support a conspecific relationship of *P.potenziani* and *P.thomasi*. Instead our results show a basal position of *P.thomasi*, which separated from all other *Presbytis* taxa in the late Miocene around 6.7 mya. *P.potenziani* is nested within *Presbytis* and derived much later in the late Pliocene (2.6 mya) from the *P.melalophos*/*P.comata*/*P.rubicunda* clade. The acoustic structure of *P.thomasi* and

P.potenziani calls is signaficantly distinct, although however, both call types share coughing inhalation and exhalation elements and a tonal howling ending phrase. This particular call structure is more likely pleisiomorphic rather than a product of a convergent evolution.

3. Can existing phylogeographic hypothesis of *Presbytis* be supported by divergence time estimates and a reassessed phylogeny of the genus?

- When did the genus *Presbytis* evolve?

Our divergence time estimates of *Presbytis* suggest that the genus originated during the late Miocene between 5.88 mya (chapter 2) and 14.09 mya (chapter 3), which is in a similar range as previous estimates (Chatterjee et al. 2009; Raaum et al. 2005; Sterner et al. 2006). It is important to note, that results obtained of such an analysis remain a design approach and often differ in various studies. Beside the algorithms used for the estimation and the choice of calibration points (e.g. Drummond et al. 2006; Knoop and Müller 2009; Sanderson 2002, 2003), the total length of the analyzed sequences and the sample size could influence the results. The latter could have biased the results of our divergence time estimation between *Presbytis* and *Trachypithecus* (whole mitochondrial genome and a small sample size: 5.88 mya – 8.86 mya; only cytochrome b and a large sample size: 9.01 mya – 14.09 mya).

- Do our results support an east/west or a west/east dispersal pattern of *Presbytis*?

Since previous models based on a basal position of either *P.potenziani* from the Mentawai Islands (Brandon-Jones 1978; Brandon-Jones 1996a; Meijaard and Groves 2004) or *P.hosei* from North Borneo (Md Zain 2001), our results indicate a different pattern. As mentioned above *P.thomasi* split from an ancestral form first around 6.7 mya. During this period of time the Asian mainland, Borneo and Sumatra were possibly still connected (Meijaard 2004). Shortly afterwards the Bornean and the Malayan *Presbytis* lineages differentiated between 6.1 mya and 5.3 mya. Accordingly *P.thomasi* might have evolved on the Asian mainland and could have subsequently been isolated

to North Sumatra, at the time when Sumatra became disconnected from Malaya due to rising sea-levels (Miller et al. 2005) during the later Miocene or early/middle Pliocene (Meijaard 2004).

Our phylogenetic reconstruction furthermore indicates that some anatomical features, which led to the assumption of a basal position of *P.potenziani* (Brandon-Jones 1978; Brandon-Jones 1996a) are pleisiomorphic. The ancestors of *P.potenziani* were possibly outcompeted and continuously displaced by other *Presbytis* following a route towards Sumatra. Proto-*potenziani* migrated to Sumatra via a landbridge that most likey existed during most of the Pliocene (Meijaard 2004). A further landbridge between the Mentawai Islands and Sumatra, which possibly emerged during a major glacial in the late Pleistocene (Ziegler et al. 2007), enabled proto-potenziani to finaly colonize the Mentawai Islands between 3.4 mya and 1.9 mya. The colonization of the Mentawai Islands by macaques (Roos et al. 2003; Ziegler et al. 2007) and gibbons (Thinh et al. 2010a) during a comparible period of time, also supports the existence of a landbride. The particular climate of the Menatwai islands enabled the archipelago to sustain a requisite habitat, which was almost everywhere else affected by severe glaciations and changed dramatically (Brandon-Jones 1993). Even during the last glacial maximum the Mentawai Islands were most likely one of the only remaining stable rain forest refugias in Sundaland (Brandon-Jones 1998; Gathorne-Hardy et al. 2002).

- Are there any explanations for *Presbytis* allopatry on Sumatra and sympatry on Borneo?

According to our phylogenetic reconstruction *P.rubicunda* is nested in the *P.melalophos* clade. *P.rubicunda* split sometimes during the early/middle Pleistocene and invaded Borneo, which was already colonized by *P. chrysomelas*, *P. frontata* and *P. hosei*. Today, *P. rubicunda* is sympatric with the three other Bornean species wherever their ranges overlap (Nijman, 2010). But with our results we can not explain why *P. chrysomelas*, *P. frontata* and *P. hosei* also occur sympatrically. The reason therefore is proposed to be related to present day ecology (Meijaard and Groves 2004). The soils of Bornean inland areas are generally very poor and low in nutrients (Davies and Baillie

1988) and dipterocarp trees dominate the plant composition (Bennett and Davies 1994). Dipterocarps provide little food for herbivorous mammals, including Colobines (Bennett and Davies 1994). Thus the Bornean species had to specialize to narrow feeding niches. Accordingly, the sympatric representatives on Borneo also stand for ecological distinct groups. On Sumatra, on the other hand, there is ecological scope for only a single folivore-granivore specialist. Accordingly there is considerable competition for the same resources, and therefore species tend to remain allopatric (Meijaard and Groves 2004).

5.2 Consideration of the major results

The present genetic study regarding the genus *Presbytis* is based on mitochondrial DNA (mtDNA) sequence data. MtDNA functions as a single locus (Funk and Omland 2003) and sorting failure of ancestral polymorphisms (incomplete lineage sorting (ILS)) could lead to phylogenetic reconstructions that do not reflect the “true” phylogeny. Because the mitochondrial genome is haploid and only maternally inherited, stochastically lineage sorting is expected to progress more rapidly for mitochondrial alleles than for nuclear allels (Avise 2004; Funk and Omland 2003). Sorting failures due to ILS however could not be ruled out completely (Funk and Omland 2003). Thus the detected paraphyly of *P.melalophos* could be also explained by ILS. Otherwise our acoustic data strongly support our findings and consequently the effect of ILS is unlikely. Another explanation for the detected paraphyly would be hybridization between *P.m.melalophos* and *P.m.mitrata*. This is likely, since both taxa meet on their distribution limit south of the Batang Hari River. To address hybridization the analysis of nuclear markers is mandatory.

In general, we have shown that mtDNA constitutes a useful marker for accurate identification of *Presbytis* taxa. Furthermore, in our study we were also able to confirm Way Kambas as the origin of the *P.m.mitrata* sample from the Ragunan Zoo. Therefore the analysis of mtDNA is being also suitable for conservation issues in terms of locating

hunting spots, indentifying the origin of confiscated animals or selecting individuals for captive breeding programs.

In particular hybridization has been increasingly considered as an important factor in the evolution of species (Arnold and Meyer 2006) and many recent studies in primates uncovered various examples. For instance hybridization was reported in macaques (e.g. Kanthaswamy et al. 2008), baboons (e.g. Alberts and Altmann 2001; Keller et al. 2010; Zinner et al. 2009b), between baboons and geladas (Jolly et al. 1997) and between gray langurs (*Semnopithecus*) and Lutungs (*Trachypithecus*) (Hohmann 1988). The latter study used vocalizations to address the hybridization between both taxa. According to Hohmann (1988) hybrids produce calls which contain species-specific patterns of both, *Semnopithecus entellus* and *Trachypithecus johnii*. Similar results were also obtained in guenons (Gautier and Gautier 1977) and gibbons (Brockelman 1978; Geissmann 1993; Tenaza 1985). On the contrary it is also reported that hybrids could show acoustic traits of either one of their parents. In that case the hybrid exclusively had the morphological characteristics of exactly that parent (Hohmann 1988; Newman and Symmes 1982). Thus, in the case of the central Sumatran *P.m.mitrata*, which has more the morphological appearance of *P.m.melalophos* and its specific call structure, hybridization might still be possible.

However, our study demonstrates that the analysis of acoustic traits is a powerful tool, particularly if acoustic data of respective populations can be directly combined with their genetic information. Various recent acoustic studies used vocal differences to distinguish species, for instance in crickets (Shaw 2000), frogs (Stuart et al. 2011), treeshrews (Esser et al. 2008) and also in other primates i.e. tarsius (Burton and Nietsch 2010). Our study sets itself apart from these studies, because our comparative approach enabled us to address migration backgrounds as well, while we could also distinguish between *Presbytis* species. Such an analysis could therefore also be applied to other animals, where pronounced mating and/or territorial behavior indicate high selective pressure. For instance, it could be a promsing tool to uncover the complex evolution of baboons (Zinner et al. 2009b).

Besides the scientific interest to understand phylogentic patterns or mechanisms of speciation, such studies have also conservational implications. For the evaluation of the conservation status of respective taxa, it is crucial to know the taxonomic status and the phylogenetic relationship between different populations to identify them as targets for conservation (Weisrock et al. 2010). In *Presbytis* the population trend in almost all taxa is decreasing, mostly due to habitat destruction, but also due to hunting and pet trade (IUCN 2010). For instance at least 80% of the natural forest habitat of *P.m.mitrata* has been removed since the colonial time, and at least 50% of this has occurred in the past 30 years (FWI/GFW 2002). Today the remaining forests are severely fragmented (IUCN 2010) and this leads to limited gene flow between populations and could also finally lead to extinction.

5.3 Outlook

1. To understand the phylogeny and phylogeography of the genus all described taxa should be addressed. Therefore complete mitochondrial genome sequences should be generated and used in the analysis, which could increase the resolution of unresolved polytomies (this could refer to the Bornean lineage and the *P.melalophos* group).

2. Assessment of the taxonomic status of the Bornean and Malayan taxa and the Sumatran taxa *P.f.percura*, *P.s.cana* and *P.s.paenulata*.

3. Broader sampling of *P.m.melalophos* and the generation of nuclear sequence data to address possible hybridization between *P.m.mitrata* and *P.m.melalophos*.

4. Assessment of the taxonomic status of *P.rubicunda*. If the species status of *P.rubicunda* would be confirmed, consequently the species status of *P.m.sumatrana*, *P.m.bicolor* and *P.m.melalophos* would be warranted. Otherwise *P.rubicunda* has to be assigned as a subspecies of *P.melalophos*.

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Appendix

Additional File 1: Presence/absence pattern, location, primers and PCR product sizes of mobile elements.

Abbreviations: MM, *Macaca mulatta*; PC, *Papio cynocephalus*; CG, *Colobus guereza*; PB, *Piliocolobus badius*; PV, *Procolobus verus*; SE, *Semnopithecus entellus*; TO, *Trachypithecus obscurus*; PM, *Presbytis melalophos*; RA, *Rhinopithecus avunculus*; PN, *Pygathrix nemaeus*; NL, *Nasalis larvatus*; SC, *Simias concolor*; AT, Annealing temperature; 1, presence of integration; 0, absence of integration; 1/0, sequenced; 1*/0*, not sequenced; Reference 1, Xing et al. 2005; Reference 2, Herke et al. 2007; Reference 3, Osterholz et al. 2008

Name	MM	PC	CG	PB	PV	SE	TO	PM	RA	PN	NL	SC	Forward	Reverse	Filled	Empty	AT	Locus position (Homo, hg18)	Reference
Colobinae (20)																			
DoucL_Yd_14Re	0	0*	1*		1*	1*	1*			1*	1*		TTTTAAAATGTATGCTCCTTGTCA	TCACTGAACTCCTAAATCAGCA	468	158	58	chr11:68568822	1
DoucL_Yd_21	0	0*	1*		1	1*	1*			1*	1*		TGGGAAGTTTGAAGCCTGATA	TGAAGTTCAAAGGCTTAGTTTTATTT	792	482	58	chr14:52024695	1
DoucL_Yd_16	0	0*	1		1	1*	1*			1	1*		CCCTCTGAGCTCCTTCTGAAT	TGGGATCAGTCTTTGCTGACT	566	256	58	chr2:59962224	1
DoucL_Yd_28	0	0*	1*		1	1*	1*			1*	1*		TTGAAAGAACAGGGGAAATCA	TATCTGACAGCCCTTGACCTG	685	375	58	chr20:30348863	1
Kirk_Yd_2Re	0	0*	1*		1	1*	1*			1*	1*		GCCTCACATTCATCTTCCAAAc	TCTATGGTTTCTGGAGGTGCTT	530	220	58	chr4:7697083	1
Kirk_Yd_33Re	0	0*	1*		1	1*	1*	1*		1*	1*		CTCACAATTTCAGTCTTGTGTGA	GGCCTCAGAGAAGAGACTTTCC	464	154	58	chrX:127625240	1
SLL_Yd_1	0	0*	1*		1	1*	1*			1*	1*		TTTGGGGAACTTACGGTCTTT	GAGAAGCCACTCACCATTTGA	554	244	58	chr15:24453270	1
SLL_Yd_8	0	0*	1		1	1	1*			1	1*		CCACCCTTTCCTAAATTTCCA	GTCTTGGCTTTCCCTTCTACG	510	200	50	chr16:46418257	1,2
SLL_PY2_25	0	0*	1*		1	1*	1*			1*	1*		CCTGAGGCTGCTCAGAGAAA	TGCTTATACGAGGCAACCTTTA	684	374	58	chr17:56469575	1,2
Nasalis_PY2_28	0	0*	1		1	1	1*			1*	1		GTGCTAATCCTTGGCCACAT	GAATCTGTCTGCCAAAGTCACA	487	177	60	chr2:116383321	1
N21	0*	0*	1*		1	1*	1*			1*	1*		ACCACTTTTGCTTGGTTCTCAT	TGTCCAGACCAAGAGACATCTG	567	257	58	chr2:242112250	1,2
N16	0*	0*	1*		1	1*	1*			1*	1*		CAGATTCAAAGAGGTGATGTCG	TAGGTGTCTCAGGATGGTGCTA	540	230	58	chr11:56721243	1,2
2499	0		1		1	1	1			1	1		CTTCTTTATCTTCTGTATGCCC	GTGGTCCTAGCCTATGCTG	740	410	58	chr1:43201455	this study
3133	0		1*		1	1	1*			1	1		TGTCTTCTTCCCTCTGGAAATCTA	GTGGTCCCCAATCTCCAAG	450	150	58	chr20:57502278	this study
3141	0		1*		1	1*	1*			1*	1*		CCAGTGTTTTGGTGTCAAAATG	GGGGTTTAAGGTTAATGATGACAG	550	250	58	chr12:4086255	this study
3249	0		1		1	1	1			1	1		ATGTCTTGAGAGTTCAGGGC	CTTAAGATACACAATATAACAAGC	600	300	58	chr2:203941433	this study
3261	0		1		1	1	1			1	1		GTAATATAGGTTGGAAAGATGC	TTATTCTGTGACTTGGAAATAGT	600	300	58	chr2:212208212	this study
3269	0		1		1	1	1			1	1		TGTAGCCAGGGAAGCCTCT	TGGGATTTCTAATACTATGCCTTTG	800	500/350	58	chr11:12743945	this study
3365	0		1		1	1	1			1	1		GTCTTCTTCCCTCTGGAAATCT	AGCTGCCTGGATGAGACCT	500	200	58	chr20:57502300	this study
3369	0		1		1	1	1			1	1		TGGTTTCATGTGTCCACTTAGG	CCAAGAATTTATTGAGCATCCA	900	600	58	chr17:35395755	this study
Piliocolobus + Procolobus (2)																			
6169	0		0	1	1	0	0*	0		0	0		GACAAATCTGATAATCTTATAGG	CAGCAAGAACTCTTTGCATTC	720	400	58	chr15:57476905	this study
6023	0	0	0	1	1	0	0	0		0	0		ACAACCAGAACCAAAGAGTAG	GTTCAGATTCAAACTTTAAGTTG	1960	200	58	chrX:78052011	this study
Piliocolobus/Procolobus + Asian colobines (3)																			
2474	0		0	1	1	1	1	1		1	1		TGGACAAGCTGAAGACATGG	CTGGATCTAGAGCTAGCTAG	500	200	58	chr6:4376475	this study
3371	0		0	1	1	1	1	1		1	1		CAGAGTGCTAAATTCATGCTTC	TGGCTGTTCCAAAGTCAGTTAG	600	300	58	chr9:111700546	this study
3373	0		0	1	1	1	1	1		1	1		TCGTTTGAAGATTTCAGTTGG	CTCTGTCTTGACAGCAGTAAC	600	300	58	chr1:8704528	this study
Asian colobines (30)																			
DoucL_PY2_3	0	0*	0*	0	0	1*	1	1		1	1*		TGCGTATTTCCACATTTCTGAC	GGCACAGCAAATGACTACGTTA	560	250	55	chr3:44778763	1
DoucL_PY2_12RE	0	0*	0*	0	0	1	1*	1		1	1		CCTTGATTTCATCTATGGGCTTA	TGACAAGGGAAATAGAAAGATTGA	743	433	60	chr3:32047343	1
DoucL_PY2_21a	0	0	0	0	0	1	1	1		1	1		CACATTAGAGGGCGCTAGAGTT	TCCTGTTGGTCCTTTGGTAGTT	574	264	55	chr20:41704352	1
Nasalis_PY2_36	0*	0*	0*	0	0*	1*	1*	1*		1*	1*		CAGAAATGTGTTTGTGAAGCAG	ACCCTAATGGCAACATTCAGTT	706	396	60	chr3:194023457	1
DoucL_Yd_2RE	0	0*	0*	0	0	1	1	1		1	1		TCCCACTTCCTCTTTTCTCAG	CTTTTATGGTTTTGCGGCTAAT	578	268	60	chr2:158707448	1
DoucL_Yd_5RE	0	0*	0	0	0	1	1	1		1	1		ATGTGAAGACCTCTGCCCAGTA	TCCTTTTGTTCAAACTGCTTCTT	440	130	60	chr8:123230272	1,2
DoucL_Yd_10	0	0	0	0	0	1*	1*	1		1	1*		TCAAAGAAGCAGCCTTCAAAA	TGCAAAACTCATCTGTGCTGT	610	300	50	chr4:154132124	1
DoucL_Yd_20	0	0*	0*	0*	0*	1	1	1	1	1	1		TCAAAACTTGCATTTCTTCACAA	CACTTTCAAAATTGCATGAACA	680	370	58	chr11:24289704	1
DoucL_Yd_22	0	0*	0	0	0*	1*	1*	1		1*	1*		TTTCATTTAACCCTCCAGCTATTT	TAACCAAACCTGGCCAGAAC	789	479	58	chr3:38767096	1
DoucL_Yd_27	0	0*	0	0	0	1	1	1	1	1*	1*		CAAGGGAGGATTCTAAGTCAGG	CAAATACCTCTTGAGAGCCAGTG	624	314	60	chr8:57979101	1
PFL_PY2_7	0	0*	0*	0	0	1	1	1		1	1		ATCCTGCGGTTCACATTTCTAT	GCCAACTTCTTAGAAACAACAGG	560	250	55	chr2:1048801	1
PFL_PY2_9	0	0*	0*	0	0	1	1	1		1	1		TTCTAAGCAGGACCTAAAAAGCA	TCATTTCAGAGATTTTGCTGATG	561	251	55	chr6:123355226	1,2
PFL_Yd_1	0	0*	0*	0	0	0	1*	1*		1	1*		GTCAGACAAGGTGTGGAACAA	AATGGTTATGTTTGATCTCTTTAACA	466	156	50	chr14:63230793	1
PFL_Yd_11	0	0*	0*	0	0	0	1*	1*		1	1*		CAAATGCTAACCACCTTGCAT	GCCCCATCAAAGAATGTATTTC	555	245	48	chr11:75528642	1

Additonal File 1 continued

PFL_Yd_15	0	0*	0*	0	0	1*	1*	1		1*	1*		TTCAGTTTCATCTGGGAGTGG	CATGTGGAGTGACCTGTGTTG	407	97	50	chr15:63007801	1
SLL_Yd_2	0*	0*	0	0	0	1	1	1		1*	1*		AACACATCAACACATGCCTCA	CCTTTGGGTTACTCTCCAGGT	645	335	57	chr2:149525794	1
SlI_Yd_20	0	0*	0*	0	0	1*	1*	1		1*	1*		TGGTTAAGTAAAGGGGGTGCT	TCATGCTACAACCACAAGCTG	574	264	60	chr1:216592546	1
Nasalis_PY2_27	0	0*	0*	0	0	1	1	1		1	1		CAAATGTTCCGTTGAGTCCA	GAGTCTTGGAAGATGCAGTGA	688	378	60	chr6:43076825	1,2
Nasalis_PY2_32	0	0*	0*	0	0	1*	1*	1		1*	1*		TTTTGTAAACAGCCAAAGCTCA	TTGTTGAAAATATGGCACAAGC	624	314	55	chr10:50351825	1,2
3125	0		0	0	0	1	1	1		1	1		AAGAATCCCAGGGAAGAACACT	TTGCTGGCAAAGTGACTCCT	700	400	58	chr7:107109056	3
3131	0		0	0	0	1	1	1		1	1		CCTGCCACTTCTGTCCATCT	AGAACAAACACCAAGACAACAGC	450	150	58	chr3:193991928	3
3149	0		0	0	0	1	1	1		1	1		GCTTTGCCACATAAAGAGCTG	GGTTAGGTGCAAATGGGAAAC	420	120	58	chr2:109178381	3
3247	0		0	0	0	1	1	1		1	1		TCAATCTTCCAGGGAAAATAAAG	GAATATTAGTTGAAATATTTAGGC	600	300	58	chr15:48692337	3
3253	0		0	0	0	1	1	1		1	1		GACCATGGTAAGACAAATGTG	GACTCAGGCTTAATTTTAAGTC	500	200	58	chr4:39427060	3
3267	0		0	0	0	1*	1	1		1	1		CACCAAGCACAACTGTGAGG	TCTGCCATAGCCATCAGTCA	600	300	58	chr1:217214400	3
3377	0		0*	0	0	1	1	1		1	1		CTCTTGGTTGGGGTGAAGC	GATGGTTGAACAGTGAGACTTGA	500	200	58	chr10:119562900	3
2482	0*		0	0	0*	1	1	1		1	1		ACTGAAACTGGTTCATGTGAC	GTTAGGGATCGTTTCCTCAG	490	160	58	chr3:180793781	this study
2490	0*		0	0	0*	1	1	1		1	1		CATCTGTGGATCTGAAGCAG	TAAGAAACTACATGGAAAAGCC	540	194	58	chr12:25743239	this study
3379	0		0*	0	0	1	1	1		1	1		AGCACCATCAGGCACTCACT	GGGAGATTGGGAAATGGAGT	700	400	58	chr6:104107889	this study
6580	0	0*	0*	0*	0*	1*	1*	1*	1*	1*	1*		GTTTGTTAAATGATGAGAAAAGA	CTCTAGAGAGTGGGCAGG	460	160	56	chr12:21646931	this study
Presbytis + odd-nosed monkeys (2)																			
DoucL_PY2_22	0	0*	0*			0	0	1	1	1	1		TGGTGCTCACTATCAGCAATTT	TGCTTAAAGTCCATCAACATGC	524	214	58	chr12:18654751	2
3277	0		0		0	0	0	1	1*	1	1		ACCTTGATCTCAGGGATCCT	GGTCAAAGTCCTACTTAAGGA	410	110	58	chr7:20757072	this study
odd-nosed monkeys (5)																			
DoucL_PY2_13RE	0	0*	0*			0	0	0	1	1	1		CAATGGGGACAGCTATTTTTCT	CTTTGGGTGTGTAGTTCCCATT	451	141	58	chr13:104854570	1
DoucL_Yd_36	0	0*	0*			0	0	0	1	1	1		CTCAAGCTTCTCCCCTCCTTA	AAGGCACAGGATTCTGCTTTT	434	124	58	chr11:20035364	1
Nasalis_PY2_23	0	0*	0*			0*	0	0	1	1	1		GGGAAAAGGATGAGTGAATCTG	TCTTCTCATAGGATGCCAGTCA	700	351	58	chr4:86118446	1
3143	0		0			0	0	0	1	1	1		AGAAAGTCCCTCCCCAACAC	AAGTTGGCAAAGTGGATTGC	550	250	58	chr1:201638132	3
6578	0	0*	0*			0*	0*	0*	1*	1*	1*		CAATAAACTCTGCTTAATTTATC	ATTCCTCAACCTTGGCCAG	430	130	56	chr15:40251990	this study
Pygathrix + Nasalis/Simias (2)																			
3381	0					0	0	0	0	1	1		GCATGATAAGAGTGGAAATCTGTG	TCAACTGATGCAGAAAATGC	500	200	58	chr18:3757704	this study
DoucL_Yd_33	0	0*	0*			0	0	0	0	1	1		CTGGCACCCAACATTGTACTT	TTGCACTTCCATCTTCATTGC	610	300	58	chr13:56959102	1
Nasalis + Simias (9)																			
Nasalis_PY2_24	0	0*	0*			0*	0*	0	0	0*	1	1*	TTATGGGCCCAATTTAAGTTTT	ATAAAATGGACTTGCCAGATGC	650	340	58	chr7:89850261	1,2
Nasalis_PY2_25	0	0*	0*			0*	0*	0	0	0*	1*	1*	TGCTCCCATTTATGAGGATTTT	ATGGGGTTGTCTGTCTGTCTCT	661	351	58	chr12:97767677	1,2
Nasalis_PY2_33	0	0*	0*			0*	0*		0	0*	1*	1*	CAGTTAGGTGGCTTAGGGAAAA	TCCTATTGGCATTAAAGCATGA	628	318	58	chr17:48297340	1,2
3127	0		0			0	0	0	0*	0	1	1*	TGCATTTATCTTCCCCTTCTTCC	TGGAGCTCCCTCTGTCCTCT	500	200	58	chr11:127217407	this study
3145	0*		0*			0*	0*	0*	0*	0*	1*	1*	CTTAGAGCTCAGATTGTGTTTATGACC	TGACAGCAGCACACTGCATA	623	323	58	chr4:152420536	this study
3153	0*		0*			0*	0*	0*	0*	0*	1*	1*	GGAACCCAGAAGGATATTAGCAG	CCTATATTAGAGCACCATCTTGTGG	590	287	58	chr7:26371431	this study
3367	0		0			0	0	0	0*	0	1	1*	CCAGTGAGACAACATGTGGAA	CCAGAATGTATTTCTATTGATGAG	550	250	58	chr5:18542195	this study
3375	0		0			0	0	0	0*	0	1	1*	CCTGTGCATTCTTGGGATAAC	AGCAGAAGGCCTTGATTCAG	700	400	58	chr2:239017222	this study
6073-6022	0		0			0	0	0	0	0	1	1*	CTTCGCTCTCAATCTTCCCC	CAGGAAACAGCAGAGTTTCC	717	390	58	chrX:78091960	thit study
Trachypithecus + Semnopithecus (10)																			
SlI_Yd_18	0	0*	0*			1	1	0		0	0		GCATTCTGGACAGTGGTGATT	GACGAGATACAATGCTTCTGAAA	897	587	60	chr5:50538553	1
PFL_PY2_14RE	0	0*	0*			1	1	0		0*	0*		TGCCAAAAACTCAGGTTAAGAGA	ATTTTGGGGGAAAACTGCTATC	563	253	60	chrX:14789579	1,2
SLL_PY2_23	0	0*	0*			1	1	0		0	0		ATAGCCTGCACGAAAAGACCTA	CAGGAGTGTTTCTCATTGACCA	513	203	60	chr22:16311986	1
PFL_PY2_2	0	0*	0*			1	1	0		0	0		CATGCTCACCTTGTATTCCTTG	TGAGGTACTGCTCTGGTGAGTT	664	354	55	chr11:111606847	1,2
SLL_PY2_17	0	0*	0*			1	1	0		0	0		TGATCCATCCCTCTTAGGAGTC	AGATCTCGGTGCCACAAATAGT	694	384	50	chr12:118218700	1,2
2457	0		0*			1	1	0		0	0		TGATTAAAGTCAGATGAACACC	GTGTAATGGGATGAAGAACAC	540	240	58	chr15:52962566	3
2652	0		0*			1	1	0		0	0		ATACATAGCATTGACTTAACTCT	GATCCTGAGCCCACTATTCT	520	220	58	chr5:25601752	3
2668	0		0*			1	1	0		0	0		ACATCAGTGACATCAAATAAGG	GAGGAAAAGATACTTTCTCATG	470	170	58	chr8:95826066	3
3257	0		0			1	1	0		0	0		GGATTGAGAGCAATTTTAAAAGGA	GTTCACTCCCAAATCATACTTC	940	640	58	chr15:63693377	3
3269	0		0			1	1	0		0	0		TGTAGCCAGGGAAGCCTCT	TGGGATTTCTAATACTATGCCTTTG	800	500/350	58	chr11:12743945	3

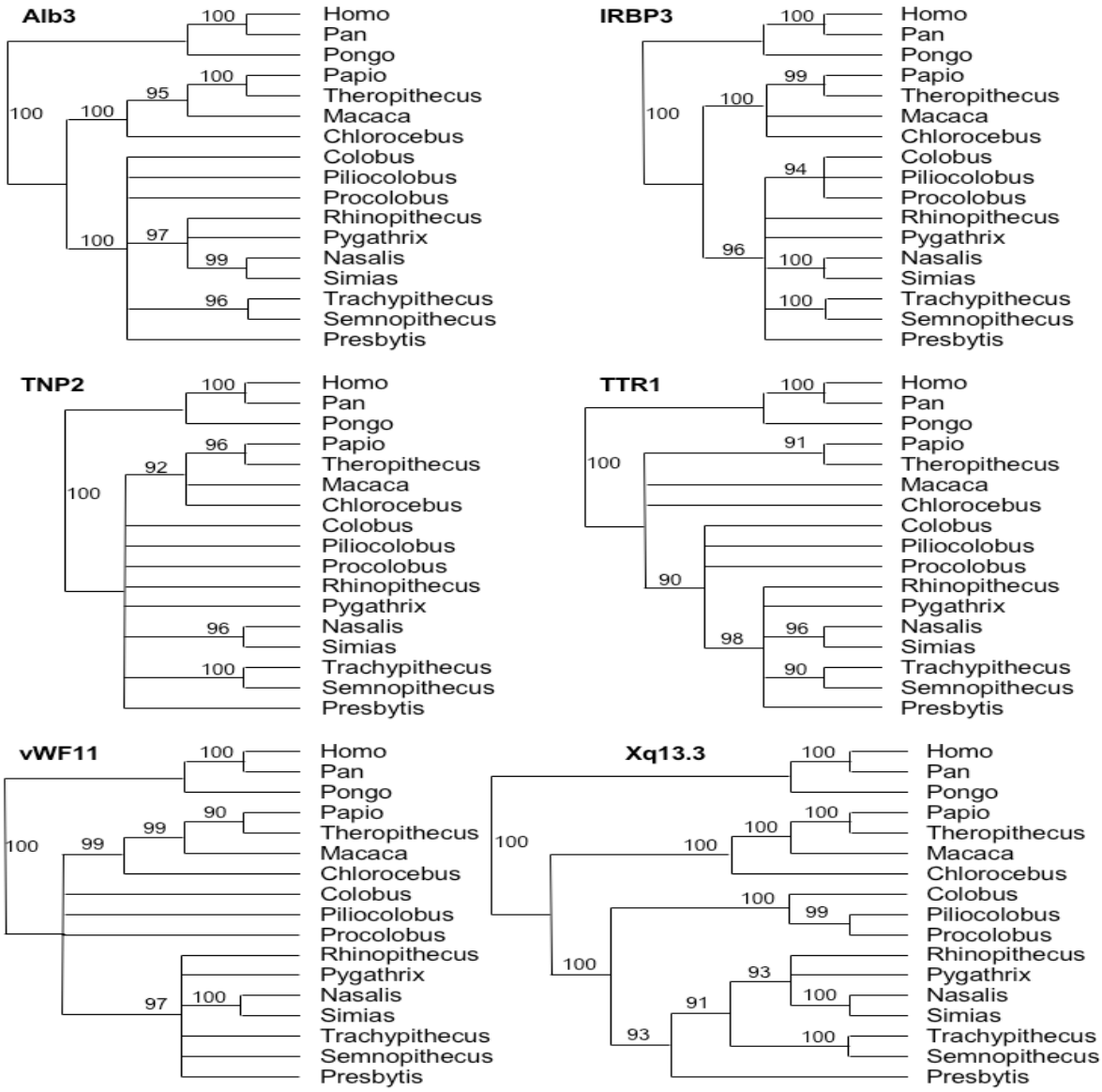
Additional File 2: Primers and PCR conditions for the amplification of nuclear loci.

Locus	Ref.	Forward primer	Reverse Primer	AT
ALB3	-	GCATT-CAAAGTCAACCATG	ACGAA-GAGTTGCAACTGTGC	56°C
IRBP3	-	CTCTGGACA-CACGCCCAG	CACACTGCTGGTCA-GAATGA	58°C
TNP2	-	GCAGGTGTA-CAAAACCAAG	GTCTCATTAGTTGGATTTCC	54°C
TTR1	-	GGCCCTACGGTGAG TGTT	ACTTTGACCATCAGAG-GACA	56°C
vWF11	Chaves et. al 1999			
Xq13.3	Ting et al. 2008; Hellborn et al. 2003			
DBY5	Hellborn et al. 2003			
SMCY7	Hellborn et al. 2003			
SMCY11	Hellborn et al. 2003			
UTY18	Hellborn et al. 2003			
SRY	Whithfield 1993			
ZFYLI	-	CCTGATTCCAGG-CAGTACC	ATCAGGGCCAATAAT-TATTGCT	58°C

Additional File 3: GenBank accession numbers. Sequences in italic are taken from GenBank.

Species	ALB3	IRBP3	TNP2	TTR1	vWF11	DBY5	SMCY7	SMCY11	SRY	UTY18	ZFYLI	Xq13.3	mtDNA
Homo sapiens	*EF649953*	*U05253*	*L03378*	*M11844*	*AC006576*	*AC004474*	*AF273841*	*AF273841*	*X53772*	*AF265575*	*U24118*	*AJ241091*	*X93334*
Pan troglodytes	JF293097	JF293129	JF293193	JF293209	JF293241	JF293113	JF293145	JF293161	JF293177	JF293225	JF293262	*AJ270088*	*D38113*
Pongo abelii	JF293098	JF293130	JF293194	JF293210	JF293242	JF293114	JF293146	JF293162	JF293178	JF293226	JF293263	JF293257	*X97707*
Papio hamadryas	JF293100	JF293132	JF293197	JF293213	JF293243	JF293117	JF293149	JF293164	JF293180	JF293228	JF293265	*AY6899234*	*Y18001*
Theropithecus gelada	JF293101	JF293133	JF293196	JF293212	JF293244	JF293116	JF293148	JF293165	JF293181	JF293229	JF293266	*AY6899236*	*FJ785426*
Macaca sylvanus	JF293099	JF293131	JF293195	JF293211	JF293245	JF293115	JF293150	JF293163	JF293179	JF293227	JF293264	JF293258	*AJ309865*
Chlorocebus aethiops	JF293102	JF293134	JF293198	JF293214	JF293246	JF293118	JF293147	JF293166	JF293182	JF293230	JF293267	*AY6899216*	*AY863426*
Colobus guereza	JF293103	JF293135	JF293199	JF293215	JF293247	JF293119	JF293151	JF293167	JF293183	JF293231	JF293268	*AY6899240*	*AY863427*
Piliocolobus badius	JF293104	JF293136	JF293200	JF293216	JF293248	JF293120	JF293152	JF293168	JF293184	JF293232	JF293269	*EU342361*	*DQ355301*
Procolobus verus	JF293105	JF293137	JF293201	JF293217	JF293249	JF293121	JF293153	JF293169	JF293185	JF293233	JF293270	JF293259	JF293092
Trachypithecus obscurus	JF293111	JF293144	JF293207	JF293223	JF293255	JF293127	JF293159	JF293175	JF293191	JF293239	JF293276	*EU342365*	*AY863425*
Semnopithecus entellus	JF293112	JF293143	JF293208	JF293224	JF293256	JF293128	JF293160	JF293176	JF293192	JF293240	JF293277	*EU342364*	*DQ355297*
Presbytis melalophos	JF293106	JF293138	JF293202	JF293218	JF293250	JF293122	JF293154	JF293170	JF293186	JF293234	JF293271	JF293260	*DQ355299*
Nasalis larvatus	JF293108	JF293140	JF293204	JF293220	JF293252	JF293124	JF293156	JF293172	JF293188	JF293236	JF293273	*EU342359*	JF293094
Simias concolor	JF293109	JF293141	JF293205	JF293221	JF293253	JF293125	JF293157	JF293173	JF293189	JF293237	JF293274	JF293261	JF293095
Pygathrix nemaeus	JF293107	JF293139	JF293203	JF293219	JF293251	JF293123	JF293155	JF293171	JF293187	JF293235	JF293272	*EU342362*	JF293096
Rhinopithecus avunculus	JF293110	JF293142	JF293206	JF293222	JF293254	JF293126	JF293158	JF293174	JF293190	JF293238	JF293275	*EU342363*	JF293093

Additional File 4: Single-locus phylogenetic trees (80% majority rule)



Additional File 5: Divergence ages in mya estimated for each locus separately

	Alb3	IRBP3	TNP2
cercopithecoids-hominoids	23.45 (21.17-25.52)	24.09 (22.08-26.24)	23.57 (21.41-25.77)
Pongo-Homo/Pan	13.81 (12.57-14.91)	13.87 (12.74-14.97)	13.95 (12.79-15.09)
Homo-Pan	6.48 (5.87-7.04)	6.41 (5.83-6.99)	6.48 (5.92-7.07)
cercopithecines-colobines	15.45 (14.31-16.56)	15.15 (14.04-16.24)	15.03 (13.83-16.16)
Chlorocebus-other cercopithecines	8.93 (5.98-12.12)	8.49 (6.17-10.75)	9.42 (6.07-13.38)
Macaca-Theropithecus/Papio	7.12 (4.73-9.86)	6.03 (4.46-7.83)	7.27 (4.60-10.65)
Theropithecus-Papio	3.88 (3.28-4.47)	3.85 (3.28-4.41)	3.93 (3.37-4.51)
Colobus-other colobines	10.87 (8.86-12.95)	10.87 (9.28-12.67)	13.49 (10.97-15.56)
Procolobus/Piliocolobus-Asian colobines	10.26 (8.87-12.14)	10.41 (8.84-12.02)	12.14 (9.79-14.75)
Procolobus-Piliocolobus	7.67 (3.70-11.07)	7.62 (5.00-10.09)	6.19 (2.51-10.11)
Asian colobines	8.73 (7.61-9.81)	8.98 (7.88-9.95)	9.01 (7.93-10.10)
Semnopithecus-Trachypithecus	2.99 (0.61-5.81)	3.24 (1.58-4.94)	1.76 (0.21-3.74)
Presbytis-odd-nosed monkeys	8.04 (6.26-9.46)	8.09 (6.66-9.39)	7.94 (5.90-9.78)
odd-nosed monkeys	5.20 (2.96-7.43)	5.77 (4.10-7.45)	5.69 (3.21-8.16)
Pygathrix-Nasalis/Simias	3.13 (1.22-5.33)	5.41 (3.76-7.09)	4.63 (2.24-7.20)
Nasalis-Simias	0.36 (0.10-1.00)	2.43 (1.07-3.74)	1.10 (0.03-2.58)

	DBY5	SMCY7	SMCY11
cercopithecoids-hominoids	23.63 (21.44-25.80)	22.93 (20.53-25.23)	23.15 (21.04-25.45
Pongo-Homo/Pan	13.94 (12.74-15.02)	14.02 (12.89-15.21)	13.94 (12.77-15.10)
Homo-Pan	6.40 (5.83-6.98)	6.44 (5.83-7.03)	6.50 (5.93-7.07)
cercopithecines-colobines	15.43 (14.32-16.51)	15.44 (14.35-16.63)	15.32 (14.20-16.46)
Chlorocebus-other cercopithecines	9.60 (6.72-12.90)	9.19 (5.25-13.01)	8.62 (5.16-12.41)
Macaca-Theropithecus/Papio	7.23 (4.82-10-04)	6.36 (4.07-9.45)	7.15 (4.22-10.40)
Theropithecus-Papio	3.88 (3.27-4.43)	3.90 (3.35-4.49)	3.91 (3.35-4.53)
Colobus-other colobines	10.15 (8.40-12.16)	11.77 (9.10-14.51)	11.37 (9.11-13.79)
Procolobus/Piliocolobus-Asian colobines	9.20 (7.79-10.66)	10.04 (8.01-12.48)	10.48 (8.54-12.61)
Procolobus-Piliocolobus	2.61 (0.27-3.13)	2.40 (0.02-6.34)	5.33 (1.75-8.90)
Asian colobines	8.67 (7.58-9.76)	8.74 (7.59-9.86)	8.80 (7.67-9.85)
Semnopithecus-Trachypithecus	1.76 (0.26-3.56)	3.21 (0.28-7.04)	3.10 (0.65-6.03)
Presbytis-odd-nosed monkeys	7.74 (5.92-9.49)	7.25 (4.31-9.84)	7.81 (5.74-9.60)
odd-nosed monkeys	6.06 (3.99-8.36)	5.53 (2.53-7.84)	6.43 (3.79-8.71)
Pygathrix-Nasalis/Simias	5.33 (3.07-7.55)	4.60 (1.59-7.90)	5.31 (2.61-7.87)
Nasalis-Simias	1.56 (0.27-3.13)	1.22 (0.02-3.13)	1.53 (0.15-3.31)

Additional File 5 continued

	TTR1	vWF11	Xq13.3
cercopithecoids-hominoids	23.49 (21.33-25.77)	23.44 (21.24-25.73)	24.12 (22.04-26.28)
Pongo-Homo/Pan	13.98 (12.87-15.15)	13.93 (12.72-15.01)	13.82 (12.73-14.97)
Homo-Pan	6.44 (5.87-7.03)	6.41 (5.82-7.01)	6.43 (5.86-7.02)
cercopithecines-colobines	15.37 (14.26-16.49)	15.48 (14.32-16.56)	15.47 (14.40-16.56)
Chlorocebus -other cercopithecines	11.76 (8.09-15.12)	10.33 (7.16-13.40)	9.78 (7.37-12.50)
Macaca-Theropithecus/Papio	9.54 (5.85-13.30)	5.67 (3.87-7.78)	6.65 (4.86-8.84)
Theropithecus-Papio	3.95 (3.36-4.54)	3.96 (3.39-4.53)	3.83 (3.25-4.39)
Colobus -other colobines	12.96 (10.67-15.10)	13.46 (11.35-15.48)	10.80 (9.14-12.34)
Procolobus/Piliocolobus -Asian colobines	12.26 (9.95-14.41)	12.46 (10.35-14.67)	10. 57 (9.01-12.14)
Procolobus-Piliocolobus	7.52 (3.50-11.26)	10.22 (7.56-13.02)	6.94 (4.17-9.63)
Asian colobines	8.56 (7.42-9.70)	8.56 (7.43-9.64)	8.35 (7.25-9.36)
Semnopithecus-Trachypithecus	3.37 (0.67-6.67)	6.64 (3.07-9.22)	2.02 (0.70-3.64)
Presbytis -odd-nosed monkeys	7.22 (4.77-9.18)	7.62 (5.72-9.33)	7.96 (6.71-9.27)
odd-nosed monkeys	6.26 (3.75-8.50)	6.75 (4.66-8.91)	6.28 (4.26-8.08)
Pygathrix-Nasalis/Simias	5.41 (2.88-7.86)	4.12 (1.87-6.37)	5.93 (3.87-7.83)
Nasalis-Simias	0.51 (0.00-1.58)	0.72 (0.01-1.81)	1.13 (0.28-2.23)

	SRY	UTY18	ZFYLI
cercopithecoids-hominoids	23.34 (21.27-25.63)	23.04 (20.85-25.35)	23.23 (21.11-25.43)
Pongo-Homo/Pan	13.93 (12.82-15.06)	14.00 (12.83-15.15)	14.13 (12.99-15.24)
Homo-Pan	6.47 (5.86-7.01)	6.46 (5.90-7.08)	6.39 (5.79-6.97)
cercopithecines-colobines	15.44 (14.35-16.56)	15.47 (14.26-16.54)	15.38 (14.28-16.51)
Chlorocebus -other cercopithecines	9.03 (6.20-12.07)	8.57 (4.96-12.29)	10.20 (6.86-13.25)
Macaca-Theropithecus/Papio	5.98 (4.18-8.23)	5.59 (3.64-8.22)	6.07 (3.96-8.61)
Theropithecus-Papio	3.84 (3.26-4.41)	3.92 (3.34-4.49)	3.92 (3.32-4.46)
Colobus -other colobines	10.97 (8.99-13.24)	11.46 (9.24-13.87)	11.96 (9.73-14.26)
Procolobus/Piliocolobus -Asian colobines	10.32 (8.40-12.28)	10.76 (8.72-13.02)	11.24 (9.19-13.52)
Procolobus-Piliocolobus	4.33 (1.09-8.11)	3.86 (0.35-8.61)	6.46 (2.72-10.35)
Asian colobines	8.66 (7.62-9.78)	8.58 (7.46-9.74)	8.67 (7.55-9.77)
Semnopithecus-Trachypithecus	3.37 (1.14-5.86)	3.03 (0.29-6.57)	2.66 (0.21-5.89)
Presbytis -odd-nosed monkeys	7.69 (5.71-9.38)	7.34 (4.96-9.41)	7.81 (6.02-9.52)
odd-nosed monkeys	6.12 (3.72-8.33)	5.92 (3.22-8.60)	6.53 (4.11-8.65)
Pygathrix-Nasalis/Simias	5.21 (2.57-7.78)	4.84 (2.03-7.68)	4.14 (1.84-6.49)
Nasalis-Simias	1.12 (0.03-2.67)	0.61 (0.00-1.91)	1.00 (0.02-2.37)

Additional File 6: Descriptive statistics to the measured parameters.

		Presbytis comata			*Presbytis m. mitrata*			*Presbytis m.melalophos*		
number	Parameter	arithmetic mean	standard derivation	n	arithmetic mean	standard derivation	n	arithmetic mean	standard derivation	n
1	duration	2,86	,26	10	2,39	,33	19	2,41	,50	29
2	Elements	52	8	10	26	6	19	20	4	29
3	elemente/s	18,20	1,91	10	10,85	2,36	19	8,28	1,05	29
4	f max s	5537	1365	10	4732	623	19	5132	837	29
5	f min s	759	322	10	2103	1089	19	2803	1164	29
6	fmax e	5604	1370	10	3665	1029	19	2429	821	29
7	fmin e	1076	328	10	812	401	19	1160	324	29
8	fmean s	1695	469	10	3429	869	19	4199	832	29
9	fmean e	2289	355	10	1715	303	19	1530	253	29
10	EX	29	4	10	20	3	19	19	4	29
11	IN	24	4	10	6	5	19	1	2	29
12	1/4 t/e	,05	,01	10	,09	,01	19	,09	,01	29
13	2/4 t/e	,04	,01	10	,09	,02	19	,10	,01	29
14	3/4 t/e	,05	,01	10	,09	,03	19	,12	,02	29
15	4/4 t/e	,07	,02	10	,11	,04	19	,14	,02	29
16	middle (2/4-3/4) t/e	,04	,01	10	,09	,02	19	,12	,02	29
17	1/4 mean f start EX	1316	196	10	3227	798	19	3566	794	29
18	2/4 mean f start EX	1394	221	10	3585	742	19	4132	833	29
19	3/4 mean f start EX	1504	186	10	3799	709	19	4434	893	29
20	4/4 mean f start EX	3774	1103	10	4282	569	19	4849	798	29
21	middle (2/4-3/4) mean f start EX	1452	196	10	3692	719	19	4280	859	29
22	1/4 mean f end EX	1539	168	10	1571	294	19	1534	326	29
23	2/4 mean f end EX	1768	232	10	1244	404	19	1399	261	29
24	3/4 mean f end EX	1990	188	10	1071	428	19	1382	309	29
25	4/4 mean f end EX	4284	959	10	1851	391	19	1728	362	29
26	middle (2/4-3/4) mean f end EX	1880	168	10	1155	392	19	1394	272	29
27	first min	1120	166	10	1727	531	19	1736	601	29
28	first fundamental	1547	141	10	2274	680	19	2749	1011	29
29	first max	2401	707	10	2987	732	19	3604	812	29
30	middle min	1262	348	10	1573	259	19	1532	195	29
31	middle fund	1778	235	10	2304	710	19	2402	905	29
32	middle max	2961	1055	10	3371	653	19	4034	1025	29
33	last min	4945	1413	10	2109	569	19	2181	658	29
34	last fund	5273	1417	10	3717	901	19	3366	1250	29
35	last max	5833	1464	10	4636	687	19	4701	1234	29
36	dif min start min end	4057	1291	10	379	763	19	392	843	29
37	dif mid fund mid end	3881	1453	10	1443	1235	19	514	1649	29
38	dif max start max end	3672	1589	10	1648	896	19	881	1264	29
39	mean beginning	1720	269	10	2330	594	19	2814	881	29
40	mean middle	2000	287	10	2416	436	19	2656	592	29
41	mean end	5365	1420	10	3428	487	19	3420	796	29
42	(ex+1) / (in+1) start	2,04	2,47	10	5,87	1,31	19	6,00	1,07	29
43	(ex+1) / (in+1) middle	1,01	,04	10	5,09	4,50	19	9,93	3,12	29
44	(ex+1) / (in+1) end	1,83	,59	10	2,86	2,28	19	5,30	1,55	29

Additional File 5 continued

Presbytis m.bicolor			*Presbytis m.sumatrana*			*Presbytis potenziani*			*Presbytis thomasi*		
arithmetic mean	standard derivation	n	arithmetic mean	standard derivation	n	arithmetic mean	standard derivation	n	arithmetic mean	standard derivation	n
2,52	,53	15	2,53	,40	8	4,17	,42	9	3,58	,35	10
19	4	15	19	3	8	27	2	9	30	4	10
7,35	,40	15	7,45	,18	8	6,42	,56	9	8,50	1,00	10
7587	2665	15	7006	945	8	1757	113	9	1752	226	10
4627	2328	15	4296	1368	8	517	454	9	409	65	10
2609	1132	15	1730	277	8	1779	155	9	1765	122	10
1657	439	15	979	164	8	540	486	9	376	84	10
6484	2492	15	5837	788	8	1119	229	9	972	171	10
2096	792	15	1279	90	8	1115	271	9	993	86	10
19	4	15	19	3	8	13	1	9	19	3	10
0	0	15	0	0	8	13	1	9	12	2	10
,10	,01	15	,09	,00	8	,16	,02	9	,08	,01	10
,12	,01	15	,11	,00	8	,21	,02	9	,11	,02	10
,13	,01	15	,13	,00	8	,14	,02	9	,14	,02	10
,14	,01	15	,16	,01	8	,10	,01	9	,12	,02	10
,14	,01	15	,13	,00	8	,17	,03	9	,12	,02	10
5691	2425	15	5068	878	8	1445	106	9	1199	395	10
6627	2698	15	5560	613	8	1554	93	9	1409	295	10
6880	2695	15	6031	765	8	870	246	9	1076	319	10
6732	2386	15	6719	1038	8	449	14	9	495	54	10
6741	2688	15	5803	678	8	1205	159	9	1250	304	10
2206	1002	15	1400	234	8	1482	156	9	1274	411	10
2143	1035	15	1292	161	8	1504	185	9	1508	198	10
2053	689	15	1167	41	8	887	208	9	1073	223	10
1984	488	15	1252	43	8	448	14	9	470	54	10
2088	844	15	1229	81	8	1190	81	9	1299	178	10
1993	451	15	1507	216	8	1218	339	9	773	360	10
3081	1873	15	2253	598	8	1609	140	9	1561	262	10
4949	2547	15	3623	997	8	2382	426	9	2279	409	10
2100	389	15	1603	269	8	872	386	9	1084	299	10
3299	1802	15	2217	171	8	1688	152	9	1638	221	10
4476	2543	15	3841	673	8	2844	299	9	2382	395	10
2080	340	15	2224	485	8	361	32	9	373	96	10
3503	2307	15	2877	412	8	457	22	9	484	55	10
4948	2331	15	5180	1773	8	564	63	9	633	160	10
-190	797	15	770	595	8	-856	336	9	-324	452	10
-45	1171	15	624	417	8	-1151	132	9	-921	552	10
265	1243	15	1557	2234	8	-1818	448	9	-1418	820	10
3279	1467	15	2309	288	8	1736	150	9	1538	183	10
3292	1500	15	2554	226	8	1801	162	9	1701	156	10
3510	1523	15	3427	694	8	461	25	9	497	52	10
5,80	,94	15	5,88	,83	8	,87	,10	9	5,27	1,84	10
10,27	2,19	15	10,50	1,31	8	1,03	,07	9	1,31	,26	10
5,47	1,19	15	5,50	,93	8	1,13	,20	9	1,09	,11	10

Additional File 7: Photographs of *Presbytis* taxa.

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Publikationen

Meyer, D., Hodges, J., Rinaldi, D., Wijaya, A., Roos, C. & Hammerschmidt, K. **(2012)**: Acoustic structure of male loud-calls support molecular phylogeny of Sumatran and Javanese leaf monkeys (genus Presbytis). BMC Evol Biol 12: 16. doi:10.1186/1471-2148-12-16.

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Eidesstattliche Erklärung

Ich erkläre hiermit, dass ich diese Dissertation selbstständig ohne Hilfe Dritter und ohne Benutzung anderer als der angegebenen Quellen und Hilfsmittel verfasst habe. Alle den benutzten Quellen wörtlich oder sinngemäß entnommenen Stellen sind als solche einzeln kenntlich gemacht. Eigene Beiträge, im Verhältnis zu denen von Koautoren bei bereits publizierten oder zur Publikation eingereichten Teilen dieser Arbeit sind wie folgt:

Kapitel 2: Die Laborarbeit wurde von Dirk Meyer, Christian Roos, Martin Osterholz, Mouya Yang und Cristiane Schwarz ausgeführt. Für die Datenauswertung, sowie die Erstellung des Manuskriptes waren im wesentlichen Christian Roos und Martin Osterholz verantwortlich.

Kapitel 3: Die Studie wurde von Dirk Meyer, Christian Roos und Keith Hodges geplant. Die Proben wurden von Dirk Meyer mit Unterstützung von Ambang Wijaya gesammelt. Die Laborarbeit, sowie die Datenauswertung wurden von Dirk Meyer ausgeführt. Das Manuskript wurde von Dirk Meyer in Rücksprache mit Christian Roos und Keith Hodges erstellt.

Kapitel 4: Die Studie wurde von Dirk Meyer, Keith Hodges und Kurt Hammerschmidt geplant. Die Proben wurden von Dirk Meyer mit Unterstützung von Ambang Wijaya gesammelt. Die Laborarbeit wurde von Dirk Meyer ausgeführt. Die Datenauswertung wurde von Dirk Meyer in Rücksprache mit Kurt Hammerschmidt ausgeführt. Das Manuskript wurde von Dirk Meyer in Rücksprache mit Christian Roos, Keith Hodges und Kurt Hammerschmidt verfaßt.

Die Übersetzungen der Summary und der Abstracts der einzelnen Publikationen in das Indonesische wurden von Dones Rinaldi, bzw. Ambang Wijaya übernommen.

Diese Arbeit ist bislang keiner anderen Prüfungsbehörde vorgelegt worden und auch nicht veröffentlicht worden. Ich bin mir bewusst, dass eine falsche Erklärung rechtliche Folgen haben wird.

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Ort, Datum, Unterschrift