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Phylogeny of Gibbons (Family Hylobatidae) with Focus on Crested Gibbons (Genus *Nomascus*)

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1 General introduction

Gibbons or small apes, family Hylobatidae, inhabit tropical and subtropical rainforests of Southeast Asia and adjacent regions (Figure 2.1) (Groves 1972, 2001; Chivers 1977). Together with great apes and humans, they belong to the primate superfamily Hominoidea (Fleagle 1999; Groves 2001; Geissmann 2002a; Mootnick 2006). Hominoids show some typical characteristics such as no tail, an enlarged brain or a broad thorax, which clearly separates them from other primates (Fleagle 1999). With four genera and up to 16 species, gibbons represent the most diverse group of hominoids. Among hominoids, gibbons are the first to branch off and they differ from great apes and humans in locomotion, morphology, social behaviour and structure, communication and the large number of chromosomal rearrangements (Geissmann 1995; Fleagle 1999; Müller et al. 2003; Mootnick 2006; Cunningham and Mootnick 2009; Giriajan et al. 2009).

Gibbons are diurnal, arboreal and primarily frugivorous (Chivers 1984). They are adapted to a locomotion called brachiation by swinging from tree to tree and they are rarely seen to move quadrupedally or bipedally (Rowe 1996; Geissmann et al. 2000; Groves 2001). As adaptation to their arboreal lifestyle, gibbons have long limbs and they are relatively small compared to great apes and humans (Napier and Napier 1967; Chivers 1984; Geissmann et al. 2000). Their body size reaches a maximum weight of 15kg (Groves 1972; Geissmann 1993; Geissmann et al. 2000). In most gibbon species, adults show a strong sexual dichromatism in fur colouration (Haimoff 1983; Creel and Preuschoft 1984; Haimoff et al. 1984; Hollihn 1984; Geissmann 1993). Typically, the fur colouration ranges from yellow to brown in adult females and grey or black in adult males. Infants are born with a colouration similar to that seen in adult females. After about two years, they change into a dark colour that is similar to that of adult males. When they reach their sexual maturity (about 5-8 years of age), females change again their fur colouration and adopt the light colouration typical of adult females, while males keep their dark colouration (Palombit 1994; Reichard 1995; Brockelman et al. 1998; Geissmann et al. 2000; Lappan 2005).

Gibbons live mainly in small, monogamous and territorial family units consisting of one adult male, one female and their offspring (Groves 1972; Creel and Preuschoft et al. 1984). However, recent research suggests that exceptions with family units comprising more than two adults are common in many gibbon populations (Srikosamatara and Brockelman 1987; Bleisch and Chen 1991; Palombit 1994; Reichard 1995; Brockelman et al. 1998; Jiang et al. 1999; Lappan 2005). Moreover, genetic and behavioural studies confirmed extra-pair paternity (Palombit 1994; Reichard 1995) or immigration of adults or subadults into existing family units (Geissmann et al. 2000; Oka and Takenaka 2001; Lappan 2005).

Gibbon groups often produce the typically duet call in early mornings by mated pairs, which was hypothesized to function as territory defence (Mitani 1985; Cowlishaw 1992). Solo songs appear to be produced only by non-mated individuals, and are heard more frequently from males than from females (Haimoff 1984; Geissmann 1993, 1995; Geissmann et al. 2000). In most species, the song of adult females consists of a loud, stereotyped phrase named great call, which begins with long notes of increasing frequency. Depending on species, great calls typically comprise between 6-100 notes and have a duration of 6-30 minutes (Marshall and Marshall 1976; Haimoff 1984; Geissmann 1993). Adult males produce different phrases, which often become gradually more complex as the song bout proceeds (Haimoff 1984; Geissmann 1993, 2002a; Geissmann et al. 2000).

Although various studies focused on the systematics of gibbons in the last decades, their classification is still highly disputed. Originally, gibbons were divided into two genera, with one (*Symphalangus*) including solely the siamang, and the other (*Hylobates*) all the remaining species (Napier and Napier 1967). However, Groves (1972) divided gibbons into the three subgenera *Symphalangus*, *Nomascus* and *Hylobates,* which he combined in the single genus *Hylobates*. This three-fold division was accepted by Chivers and Gittins (1978), but due to the comparatively large differences between these three groups, Lekagul and McNeely (1977) elevated them all to genera. Based on cytogenetic studies, it became obvious that four, not only three major groups of gibbons exist, which differ from each other in their diploid chromosome number. Accordingly, gibbons were split into four subgenera: *Hoolock* (previously named *Bunopithecus*, 2n = 38), *Hylobates* (2n = 44), *Symphalangus* (2n = 50) and *Nomascus* (2n = 52) (Yunis and

Prakash 1982; Prouty et al. 1983; Müller and Wienberg 2001; Müller et al. 2003). Molecular data supported the division of gibbons into four groups and their elevation from subgenus to full genus rank (Roos and Geissmann 2001). However, the relationship among the four genera can not be regarded as settled. Mitochondrial D-loop sequences depict *Nomascus* as the most basal form, followed by *Symphalangus*, whereas *Hoolock* and *Hylobates* seem to be the last, which diverged from each other (Roos and Geissmann 2001). In contrast, other studies suggest *Symphalangus* (Garza and Woodruff 1992; Hall et al. 1998) or *Hoolock* as the basal genus (Bruce and Ayala 1979; Müller et al. 2003; Takacs et al. 2005).

The genus *Symphalangus* comprises only one species (*S. syndactylus*), which is totally black and endemic to the Malay Peninsula and Sumatra. With 8-15kg, the siamang is the largest living gibbon. Siamangs display a large and inflatable throat sac and males have a long genital tuft (Chivers and Gittins 1978; Ma and Wang 1986; Geissmann 1991; Zhang et al. 1992; Gibbon Research Lab 2010).

The hoolock or white-browed gibbon, genus *Hoolook* is found in eastern Bangladesh, north-eastern India, north-western Myanmar and southern China (Brockelman and Gittins 1984; Marshall and Sugardjito 1986; Geissmann 1991; Groves 2001). The main characteristics of the genus are the white brow band, the absence of light cheeks, a distinct goatee in males and fur on feet in the same colour as on lower leg (Chivers 1977; Chivers and Gittins 1978; Groves 2001; Choudhury 2006). Hoolocks are the only gibbons to produce a guttural growl during their vocalization (Mootnick and Groves 2005). Traditionally, the genus *Hoolock* comprised one species with two subspecies, which, however, were recently elevated to species, the western hoolock (*H. hoolock*) and the eastern hoolock (*H. leuconedys*) (Mootnick and Groves 2005; Moonick 2006; Geissmann 2007).

The genus *Hylobates* is widely distributed in Sundaland, but occurs also on the Southeast Asian mainland. Its northernmost distribution is the westside of the Mekong river in southern China (Brockelman and Gittins 1984; Marshall and Sugardjito 1986; Geissmann 1991). Members of this genus are characterized by a prominent genital swelling in females (Moonick 2006). Concerning their systematics, the genus already comprised at least four species in early

classifications (Napier and Napier 1967; Chivers 1977), but recent studies proposed six or seven species (*H. lar, H. agilis, H. albibarbis, H. moloch, H. muelleri, H. pileatus*, and *H. klossii*) (Groves 2001; Geissmann 2002b, 2007; Takacs et al. 2005; Moonick 2006). The former six species have long been considered to be closely related and, hence, were combined in the lar group (Groves 2001; Brandon-Jones et al. 2004; Mootnick and Groves 2005; Mootnick 2006; Geissmann 2007). The latter, *H. klossii* was sometimes named "dwarf siamang" and recognized as distinct relative of the others (Chivers 1977; Haimoff 1983; Creel and Preuschoft 1984; Haimoff et al. 1984; Groves 1989). Based on genetic data (Takacs et al. 2005; Whittaker et al. 2007), it became obvious that *H. klossii* is not distantly related to other *Hylobates* species, but its closest relative as well as relationships among *Hylobates* species in general are not clarified yet.

The genus *Nomascus* is restricted to the Indochinese bioregion including Vietnam and parts of Laos, Cambodia and southern China. The genus occurs mainly east of the Mekong river and only the west Yunnan crested gibbon (*N. concolor furvogaster*) crossed the Mekong river to the west (Geissmann et al. 2000; Groves 2001). The pelage of adult males is black with small pale yellow or white cheeks in some species. Hairs are dense and shorter compared to other gibbon genera. Males have erected hairs as a crest on the top of their heads, thus the name “crested gibbons" (Groves 1972, 2001; Marshall and Sugardjito 1986; Geissmann 1995; Mootnick 2006). Females are yellow, orange or beige brown (Geissmann et al. 2000). Crested gibbons were originally combined in the single species *N. concolor*, but now they are divided into six species including *N. hainanus, N. nasutus, N. concolor, N. leucogenys, N. siki* and *N. gabriellae* (Roos et al. 2007; IUCN 2009). The subspecies of *N. concolor, N. c. furvogaster* and *N. c. jingdongensis* were suggested as synonyms of the nominate form (Geissmann et al. 2000; Roos et al. 2007). For the southern taxa, the situation is complicated. Originally, three taxa were described, which were recently all classified as species, *N. leucogenys, N. siki* and *N. gabriellae* (Groves 2001, 2007). However, recent acoustic data suggest another, so far undescribed taxon (Geissmann et al. 2000; Konrad and Geissmann 2006), indicating that knowledge about the number of taxa and their distribution areas is still limited for crested gibbons. Based on phylogenetic reconstructions, *N. hainanus* forms a sister lineage to *N. nasutus*. Both are basal among crested gibbons. Among the remaining species, *N. concolor*

branched off first, before finally *N. gabriellae* and *N. leucogenys* diverged (Roos et al. 2007). Captive *N. siki* individuals from unknown location form a sister clade to *N. leucogenys* (Roos et al. 2007).

As mentioned above, the number of gibbon species to be recognized is still a matter of debate. Similarly, phylogenetic relationships among gibbon lineages on various taxonomic levels and respective divergence times are still unresolved. According to molecular studies, gibbons separated from great apes and humans around 12-36 million years ago (mya) (Hayashi et al. 1995; Zehr et al. 1996; Raaum et al. 2005). The initial split among gibbons into genera was proposed to have occurred in the late Miocene (Eudey 1980; Meijaard 2004; Meijaard and Groves 2006), which is in agreement with molecular estimates (Hayashi et al. 1995; Goodman et al. 1998; Chatterjee 2006, 2009). For the lar group, Chivers (1977) and Groves (1972) proposed a radiation in the Pleistocene, and for crested gibbons, Chatterjee (2006, 2009) suggested a radiation in the latest Pleistocene.

The preservation of the natural ecosystem is necessary for the maintenance and existence of wildlife populations. However, a decrease of wildlife populations and deduction of natural habitats has been taking place in recent days, so that immediate actions are required. The lack of information about the biology, status and distribution of gibbons poses a serious problem in terms of how to conduct a long-term conservation program and the establishment of action plans in the region. Recent surveys carried out in areas of the Indochinese bioregion revealed some alarming statistics for some of the most endangered primate species of the world. For example, a recent survey for the Yunnan white-handed gibbon (*H. lar yunnanensis*) was unable to detect any indication that the subspecies survived (Grueter et al. 2009). From *N. hainanus*, only 20 animals remain in Bawangling National Nature Reserve, Hainan island (Zhang and Sheeran 1994; Chan et al. 2005; Mootnick et al. 2007; Cunningham and Mootnick 2009; IUCN 2009), and from *N. nasutus* only approximately 100 individuals occur in north-eastern Vietnam and southern China (IUCN 2009; Long and Nadler 2009). Gibbons in these and all other areas have dramatically decreased and became a critical concern now. Currently, all gibbon species are classified as “Endangered” or “Critically Endangered” (IUCN 2009). Only *Hoolock leuconedys* is classified as “Vulnerable” (IUCN 2009). In fact, most gibbon species are endangered on different levels, mainly due to hunting for food, traditional medicine and their general cultural value.

Most likely, this is the primary cause for the decline of gibbons in all their home countries. However, deforestation through agricultural encroachment into mountainous areas and timber logging from remaining forests as well as infrastructure development of hydroelectric dams and roads is a major threat across their range as well (Geissmann et al. 2000; Geissmann 2007). For example, Vietnam lost approximately 75% of its natural forest cover to deforestation and degradation since the 1990s (Rowcroft 2008). A number of protected forest areas were established in recent years, but often they are poorly managed and wildlife laws are not effectively enforced. Rural poverty and lack of public awareness about threats to gibbons and their forests are additional causes for inadequate gibbon protection (Geissmann et al. 2000; IUCN 2009; Gibbon Conservation Alliance 2010).

Arising from the above outlined state of the art, it is not only crucial to clarify the phylogeny and phylogeography of gibbons, and to establish a reliable classification, but also to provide information and methods, which may improve protection of gibbons and their habitats. Most important in this respect is knowledge about which taxon occurs in a certain area and a clear definition of its exact distributional range as well as its population size. Likewise, tools are required to select gibbons for captive breeding purposes or to trace hunting hotspots. In the wild, the identification of gibbons is problematic, because gibbons live high in the canopy and move fast, so that the few and less prominent characteristics in fur coloration are difficult to be observed. Similarly, also for captive individuals or museum specimens, fur colouration is not always an appropiate distinguishing feature. Accordingly, other, more reliable methods are required. Acoustic analyses have been successfully applied in gibbons (e.g. Creel and Preuschoft 1984; Geissmann et al. 2000; Dallmann and Geissmann 2001a,b; Konrad 2004; Konrad and Geissmann 2006) as well as genetic methods (Roos 2004; Roos at el. 2007). Since all gibbon species can readily be distinguished by their different vocalizations and due to the fact, that sound recordings are relatively easy to be obtained from the field, acoustic analyses might be the most promising tool to identify gibbons in the wild. Based on theses data, population sizes, group compositions, taxon-identity, distribution of taxa and even phylogenetic relationships can be estimated. Genetic studies using samples collected non-invasively in the field or from zoo or museum specimens provide similiar

information. Besides the possibility to assign individuals to taxa, to confirm distribution ranges and to elucidate phylogenetic relationsips, genetic data allow also to estimate divergence ages, which are required to illuminate the phylogeography of gibbons.

To address these issues, this thesis was planned to

1) establish a complete phylogeny of gibbons,
2) elucidate the phylogeography of gibbons,
3) provide a reliable classification of gibbons,
4) establish a marker system to trace hunting hotspots and to select zoo individuals for breeding purposes, and
5) to settle the distribution areas of crested gibbon taxa.

To reach these aims, I analysed DNA sequence of the complete mitochondrial cytochrome b gene, which was shown to be an appropriate marker for phylogenetic analysis on various taxonomic levels in gibbons (Roos 2004; Roos et al. 2007). For crested gibbons, I applied a combined approach including genetic and acoustic data. Genetic materials were collected by myself during field surveys, were provided by colleagues or obtained from zoos and museums. For the study on crested gibbons only clearly provenanced individuals were included. Song bouts of gibbons were recorded during field surveys in Vietnam, Laos and Cambodia.

In the following chapters, these five objectives are discussed in detail. Chapter 2 deals with the taxonomy, phylogeny and phylogeography of the Hylobatidae family in general, while Chapter 3 focuses on the phylogeny and distribution of solely crested gibbons. In Chapter 4, I describe the application of acoustic data as taxonomic and phylogenetic marker in crested gibbons. Finally, in Chapter 5, I discuss a possible correlation between vocal and genetic diversity in crested gibbons.

A general discussion of the findings of this thesis and suggestions for further investigations are finally provided in Chapter 6.

2 Mitochondrial evidence for multiple radiations in the evolutionary history of small apes

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Abstract

Background: Gibbons or small apes inhabit tropical and subtropical rain forests in Southeast Asia and adjacent regions, and are, next to great apes, our closest living relatives. With up to 16 species, gibbons form the most diverse group of living hominoids, but the number of taxa, their phylogenetic relationships and their phylogeography is controversial. To further the discussion of these issues we analyzed the complete mitochondrial cytochrome b gene from 85 individuals representing all gibbon species, including most subspecies.

Results: Based on phylogenetic tree reconstructions, several monophyletic clades were detected, corresponding to genera, species and subspecies. A significantly supported branching pattern was obtained for members of the genus *Nomascus* but not for the genus *Hylobates*. The phylogenetic relationships among the four genera were also not well resolved. Nevertheless, the new data permitted the estimation of divergence ages for all taxa for the first time and showed that most lineages emerged during four short time periods. In the first, between ~6.7 and ~8.3 mya, the four gibbon genera diverged from each other. In the second (~3.0 -~3.9 mya) and in the third period (~1.3 -~1.8 mya), *Hylobates* and *Hoolock* differentiated. Finally, between ~0.5 and ~1.1 mya, *Hylobates lar* diverged into subspecies. In contrast, differentiation of *Nomascus* into species and subspecies was a continuous and prolonged process lasting from ~4.2 until ~0.6 mya.

Conclusions: Although relationships among gibbon taxa on various levels remain unresolved, the present study provides a more complete view of the evolutionary and biogeographic history of the hylobatid family, and a more solid genetic basis for the taxonomic classification of the surviving taxa. We also show that mtDNA constitutes a useful marker for the accurate identification of individual gibbons, a tool which is urgently required to locate hunting hotspots and select individuals for captive breeding programs. Further studies including nuclear sequence data are necessary to completely understand the phylogeny and phylogeography of gibbons.

Key Words: Gibbons, Hylobatidae, *Nomascus, Symphalangus, Hylobates, Hoolock*, mitochondrion, cytochrome b, evolution, biogeography

2.1 Introduction

Gibbons, family Hylobatidae, are small arboreal apes, which inhabit tropical and subtropical rainforests of Southeast Asia and adjacent regions (Figure 2.1). Together with humans and great apes, they belong to the primate superfamily Hominoidea (Fleagle 1999; Groves 2001; Geissmann 2002a; Mootnick 2006). Among hominoids, gibbons were the first to branch off and they display a set of morphological and behavioural characteristics distinctly different from great apes and humans (Fleagle 1999; Geissmann 1995; Cunningham and Mootnick 2009). Most prominent in this respect is the predominantly monogamous life style, their territorial calls, and the typical brachiating locomotion (Geissmann 1995, 2002b; Fleagle 1999; Mootnick 2006; Cunningham and Mootnick 2009). Due to their

extensive karyotypic diversity (Müller et al. 2003; Roberto et al. 2007; Misceo et al. 2008; Giriajan et al. 2009), gibbons provide an excellent model organism to study chromosomal rearrangements and, hence, to better understand human diseases caused by such alterations.

Although in several aspects unique among primates and with up to 16 species the most diverse group of apes, gibbons are still in the shadow of great apes in respect of scientific studies, conservation efforts and public awareness. However, many gibbon species are on the brink of extinction and most of them are classified as “Endangered” or even “Critically Endangered” (IUCN 2009). With approximately 20 individuals left in its native habitat, the Hainan gibbon (*Nomascus hainanus*) is the rarest primate in the world (Chan et al. 2005; Mootnick et al. 2007; Cunningham and Mootnick 2009). Responsible for this critical situation is habitat loss and hunting, which both have seriously reduced gibbon populations throughout their range (Geissmann et al. 2000; Geissmann 2007). Hence, much more attention has to be drawn on the gibbons’ situation and extensive conservation actions are urgently required to save them from extinction (Geissmann 2007).

While gibbons are widely considered to form a monophyletic clade, there is no consensus about the phylogeny and taxonomy within the family. Although various studies based on morphology, behaviour, vocalisation, protein electrophoresis, karyotyping and DNA sequencing were conducted (Napier and Napier 1967; Groves 1972; Haimoff et al 1982; Prouty et al. 1983; Creel and Preuschoft 1984; Shafer 1986; Liu et al. 1987; Garza and Woodruff 1992; Hayashi et al. 1995; Geissmann 1995, 2002a,b; Hall et al. 1998; Roos and Geissmann 2001; Roos 2004; Takacs et al. 2005; Chatterjee 2006; Mootnick 2006; Monda et al. 2007; Roos et al. 2007; Whittaker et al. 2007), neither a congruent phylogeny or a consistent taxonomic classification was obtained. Moreover, incomplete taxon sampling as well as misidentified specimens resulted in only fragmentary or even false conclusions. Accordingly, the classification of gibbon taxa at various taxonomic levels as well as their phylogenetic relationships remain disputed and a consensus is far from being available.

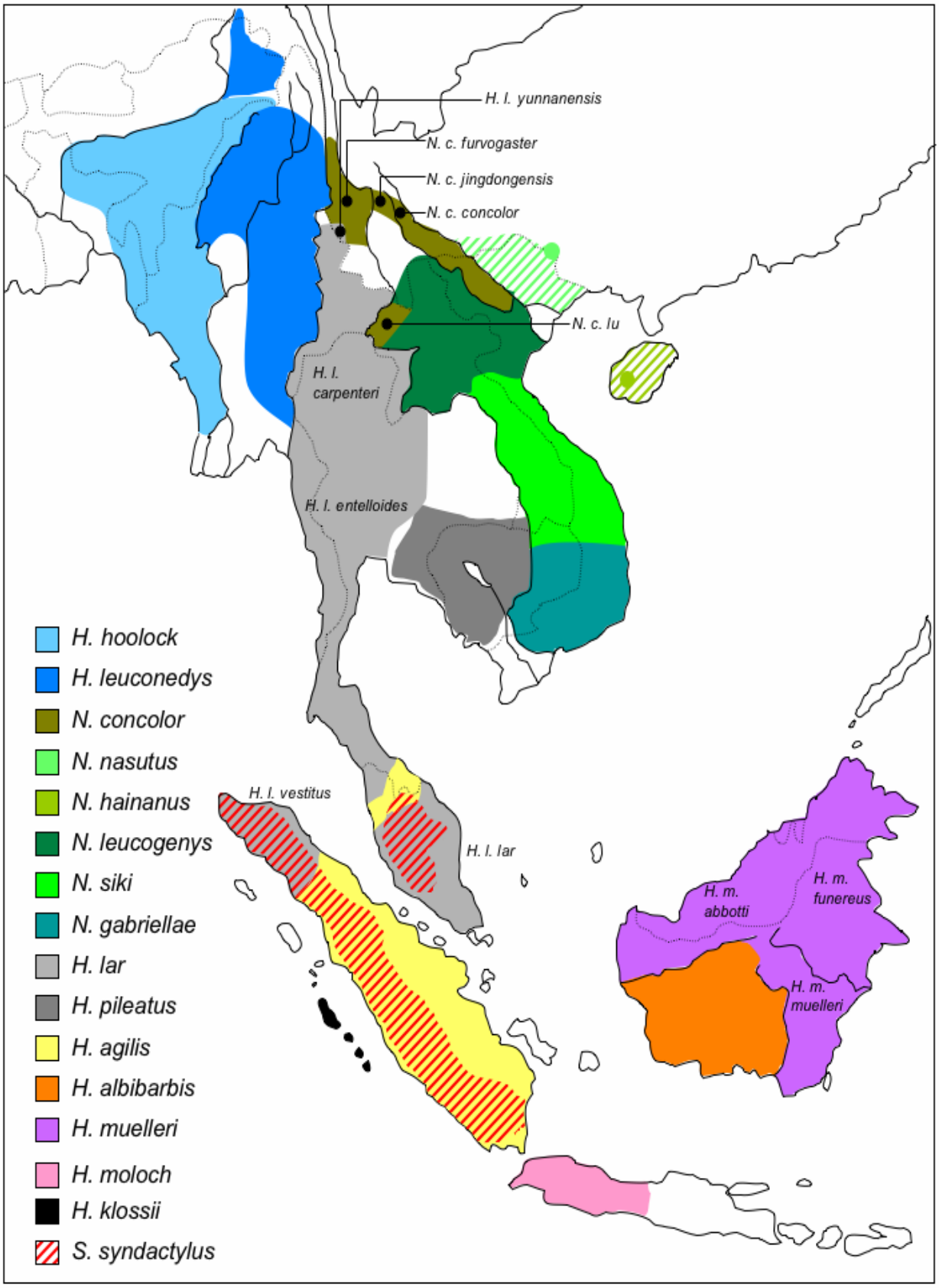

Figure 2.1: Geographical distribution of gibbons based on Marshall and Sugardjito (1986), Geissmann (1995), Groves (2001) and Gibbon Research Lab (2010). Dotted and solid lines indicate country borders and major rivers, respectively. Historical distribution of *N. hainanus* and *N. nasutus* is hatched.

For example, in early studies, small apes were divided into two genera, with one (*Symphalangus*) including the siamang, and the other (*Hylobates*) all the remaining species (Schultz 1933; Napier and Napier 1967). Later on, the family was split into four major clades, which were recognized as subgenera (Prouty et al. 1982; Geissmann 1995; Groves 2001) and eventually as genera (Roos and Geissmann 2001; Brandon-Jones et al. 2004; Mootnick and Groves 2005; Mootnick 2006; Geissmann 2007). This division is now widely accepted and takes into account the fact that species within each of the four major clades share a number of characteristics, most importantly a distinctive diploid chromosome number: *Hoolock* (2n=38), *Hylobates* (2n=44), *Symphalangus* (2n=50) and *Nomascus* (2n=52) (Müller et al. 2003).

Similarly, the number of species and subspecies is a matter of debate as well. While *Symphalangus* is consistently regarded as monotypic, the two *Hoolock* subspecies were recently elevated to species (Mootnick and Groves 2005). In *Nomascus* originally only one species was recognized (Napier and Napier 1967; Groves 1972; Chivers 1977; Haimoff et al. 1982), but in current classifications four to six species were suggested (Groves 2001; Mootnick 2006; Geissmann 2007; Roos et al. 2007). In contrast, the genus *Hylobates* already comprised at least four species in early classifications (Napier and Napier 1967; Chivers 1977), but recent studies proposed six or seven species (Groves 2001; Mootnick 2006; Geissmann 2007). Due to this incongruence we follow the most recent classification of the IUCN Red List (IUCN 2009) with a total of 16 gibbon species (Table 2.1).

In the present study, we analyse the complete mitochondrial cytochrome b (cytb) gene from 85 individuals, which represent all gibbon genera and species, and most subspecies. Based on our data, we are able to 1) provide the most complete phylogeny of gibbons on all taxonomic levels, 2) estimate divergence times between lineages, 3) establish a reliable classification, 4) elucidate gibbon phylogeography, and 5) provide a tool for the species identification of gibbon individuals.

Table 2.1: Common names, IUCN classification and proposed classification of gibbons.

Common name	IUCN classification (IUCN 2009)	Proposed classification
Kloss' s gibbon	*Hylobates klossii*	*Hylobates klossii*
Eastern Müller's Bornean gibbon	*Hylobates muelleri muelleri*	*Hylobates muelleri**
Northern Müller's Bornean gibbon	*Hylobates muelleri funereus*	*Hylobates funereus**
Abbott's Müller's Bornean gibbon	*Hylobates muelleri abbotti*	*Hylobates abbotti**
Agile gibbon	*Hylobates agilis*	*Hylobates agilis**
Bornean white-bearded gibbon	*Hylobates albibarbis*	*Hylobates albibarbis*
Malayan lar gibbon	*Hylobates lar lar*	*Hylobates lar lar**
Sumatran lar gibbon	*Hylobates lar vestitus*	*Hylobates lar vestitus**
Mainland lar gibbon	*Hylobates lar entelloides*	*Hylobates lar entelloides**
Carpenter's lar gibbon	*Hylobates lar carpenteri*	*Hylobates lar carpenteri**
Yunnan lar gibbon	*Hylobates lar yunnanensis*	*Hylobates lar yunnanensis**
Silvery Javan gibbon	*Hylobates moloch*	*Hylobates moloch**
Pileated gibbon	*Hylpobates pileatus*	*Hylpobates pileatus*
Western hoolock gibbon	*Hoolock hoolock*	*Hoolock hoolock*
Eastern hoolock gibbon	*Hoolock leuconedys*	*Hoolock leuconedys*
Siamang	*Symphalangus syndactylus*	*Symphalangus syndactylus**
Hainan gibbon	*Nomascus hainanus*	*Nomascus hainanus*
Cao-vit crested gibbon	*Nomascus nasutus*	*Nomascus nasutus*
Black crested gibbon	*Nomascus concolor concolor*	*Nomascus concolor concolor**
West Yunnan black crested gibbon	*Nomascus concolor furvogaster*	*Nomascus concolor concolor**
Central Yunnan black crested gibbon	*Nomascus concolor jingdongensis*	*Nomascus concolor concolor**
Laotian black crested gibbon	*Nomascus concolor lu*	*Nomascus concolor lu**
Northern white-cheeked gibbon	*Nomascus leucogenys*	*Nomascus leucogenys**
Southern white-cheeked gibbon	*Nomascus siki*	*Nomascus siki**
Red-cheeked gibbon	*Nomascus gabriellae*	*Nomascus gabriellae*
	16 species, 12 subspecies	**18 species, 7 subspecies**

*further research required.

2.2 Materials and methods

2.2.1 Sample collection

A total of 85 specimens representing all species and most subspecies of hylobatids were included in our study. Blood, tissue, faecal or hair samples were collected during field surveys, in zoos or rescue centres, or from museum specimens between 1995 and 2008 (Appendix A.1). Blood and hair samples were taken during routine health checks by veterinarians. Tissue samples were obtained only from deceased animals. Taxon identity of individuals was ascertained by pelage coloration, morphology and if possible by vocalization and geographic

origin. With the exception of some *H. lar* individuals for which subspecies identity could not be traced, only clearly identified specimens were included in our study. Fresh tissue or faecal samples were preserved in 80-90% ethanol and dry samples (tissue, museum skins and hair samples) were placed in plastic bags without any additive. Samples were stored at ambient temperature for up to six months before further processing.

2.2.2 Laboratory methods

Total genomic DNA was extracted with the DNeasy Blood & Tissue and QIAamp DNA Stool Mini kits from Qiagen. When hair follicle cells were used, up to three hairs were directly implemented into the PCR reaction. From high-quality DNA, the complete mitochondrial cytb gene was PCR-amplified in a single fragment with the primers 5'-AATGATATGAAAAACCATCGTTGTA-3' and 5'-TTCATTTCCGGCTTACAAGAC-3'. For low-quality DNA, extracted from faeces or museums material, two to seven overlapping PCR products were amplified with primers constructed on the basis of sequences from conspecifics (respective primers are available from the authors upon request). For all amplifications, wax-mediated hot-start PCRs were performed for 40 cycles, each with a denaturation step at 92°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 0.5-1.5 min, followed by a final extension step at 72°C for 5 min. The results of the PCR amplifications were checked on 1% agarose gels. Subsequently, PCR products were cleaned with the Qiagen Gel Extraction kit and sequenced on an ABI 3130xl sequencer using the BigDye Cycle Sequencing kit. Sequences were assembled with Geneious v4.6.1 (Drummond et al. 2008) and checked for their potential to be correctly transcribed. Gibbon haplotypes were deposited at GenBank and are available under the accession numbers GU321245-GU321329 (see also Appendix A.1).

To prevent cross-species contaminations, laboratory procedures followed described standards (Roos et al. 2008). To exclude contaminations of the dataset with nuclear pseudogenes (numts), we mainly used material in which nuclear DNA is highly degraded (faeces, museum tissue) (Hofreiter et al. 2003; Thalmann et al. 2004). Moreover, the applied primers are known to amplify solely the mitochondrial copy of the gene in hylobatids (Roos et al. 2007), and for cross-validation purposes, for some specimens, sequences were generated using different

material types (blood, faeces).

2.2.3 Statistical methods

For phylogenetic reconstructions, we expanded our dataset with orthologous sequences from various hominids (*Homo, Pan, Gorilla, Pongo*) and *Papio hamadryas*, which was used as outgroup. Phylogenetic trees were constructed with maximum-parsimony (MP) and neighbor-joining (NJ) algorithms as implemented in PAUP v4.0b10 (Swofford 2003) as well as with maximum-likelihood (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 analysis, all characters were treated as unordered and equally weighted throughout. A heuristic search was performed with the maximum number of trees set to 100. For NJ and ML reconstructions, the optimal nucleotide substitution model (GTR + Γ) was chosen using Akaike information criterion (AIC) as implemented in MODELTEST v3.7 (Posada and Crandall 1998). Relative support of internal nodes was performed by bootstrap analyses with 10,000 (MP, NJ) or 500 replications (ML). In GARLI, only the model specification settings were adjusted according to the dataset, while all other settings were left at their default value. ML majority-rule consensus trees were calculated in PAUP. For Bayesian reconstructions, the dataset was partitioned into codon positions, each with its own substitution model. We used four Markov Chain Monte Carlo (MCMC) chains with the default temperature of 0.1. Four repetitions were run for 10,000,000 generations with tree and parameter sampling occurring every 100 generations. The first 25% of samples were discarded as burnin, leaving 75,001 trees per run. Posterior probabilities for each split and a phylogram with mean branch lengths were calculated from the posterior density of trees.

To estimate divergence times, a Bayesian MCMC method, which employs a relaxed molecular clock approach (Drummon et al. 2006), as implemented in BEAST v1.4.8 (Drummond and Rambaut 2007), was used. A relaxed lognormal model of lineage variation and a Yule prior for branching rates was assumed. The alignment 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.

For calibrations we used the fossil-based divergence between *Homo* and *Pan*, which was dated at 6 - 7 million years ago (mya) (Vignaud et al. 2002; Brunet et al. 2005; Lebatard et al. 2008), the separation of *Pongo* from the *Homo*/*Pan* lineage ~14 mya (Kelley 2002), and the divergence of hominoids and cercopithecoids ~23 mya (Benefit and McCorossin 2002; Young and MacLatchy 2004). 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, and for the hominoid - cercopithecoid divergence into a mean of 23 mya and a SD of 2 mya.

Since the estimation of phylogenetic relationships was not the main aim of this analysis, for the calculation an a-priori fixed tree topology as obtained from NJ reconstructions using the GTR + Γ model (Figure 2.2) was implemented. 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 Drummd 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 Results

From all 85 gibbons, we successfully generated sequences of the complete mitochondrial cytb gene (1,140 bp). A contamination of our dataset with numts can be regarded as minimal, because no multiple amplifications of different copies were detected by direct sequencing. All sequences were correctly transcribed, and identical sequences were obtained for the same individual in cases where different material types were available. Moreover, no inconsistent positions were detected in alignments, which were assembled from overlapping sequences. Cross-contamination between individuals can be excluded as well, since all negative

controls revealed no amplifications and randomly repeated PCRs for the same individual produced identical sequences.

Among the 85 individual gibbons studied, no identical haplotypes were detected. The cytb alignment comprising solely gibbons was characterized by 429 variable sites, of which 374 were parsimony-informative. In the complete alignment, which additionally contained great ape, human and hamadryas baboon representatives, we observed 565 variable sites, of which 462 were parsimony-informative.

Phylogenetic tree reconstructions based on MP, NJ, ML and Bayesian algorithms revealed various strongly supported clades, which corresponded to genera, species and subspecies (Figure 2.2). All algorithms led to identical tree topologies, although several branching patterns gained only weak support. According to our reconstructions, hominoids diverged into a clade consisting of gibbons, and another with great apes and human. Among the latter, *Pongo* split off first, followed by *Gorilla*, before finally *Pan* and *Homo* diverged. Within gibbons, a basal position of *Nomascus* and a sister grouping of *Hylobates* and *Hoolock* was indicated, but support for this branching pattern was relatively low (Table 2.2). Similarly, with the exception of a strongly supported *H. agilis* + *H. albibarbis* clade, also the relationships among the species of *Hylobates* were not well resolved. However, at least species monophylies were clearly confirmed, though a common origin of *H. agilis* was only weakly supported.

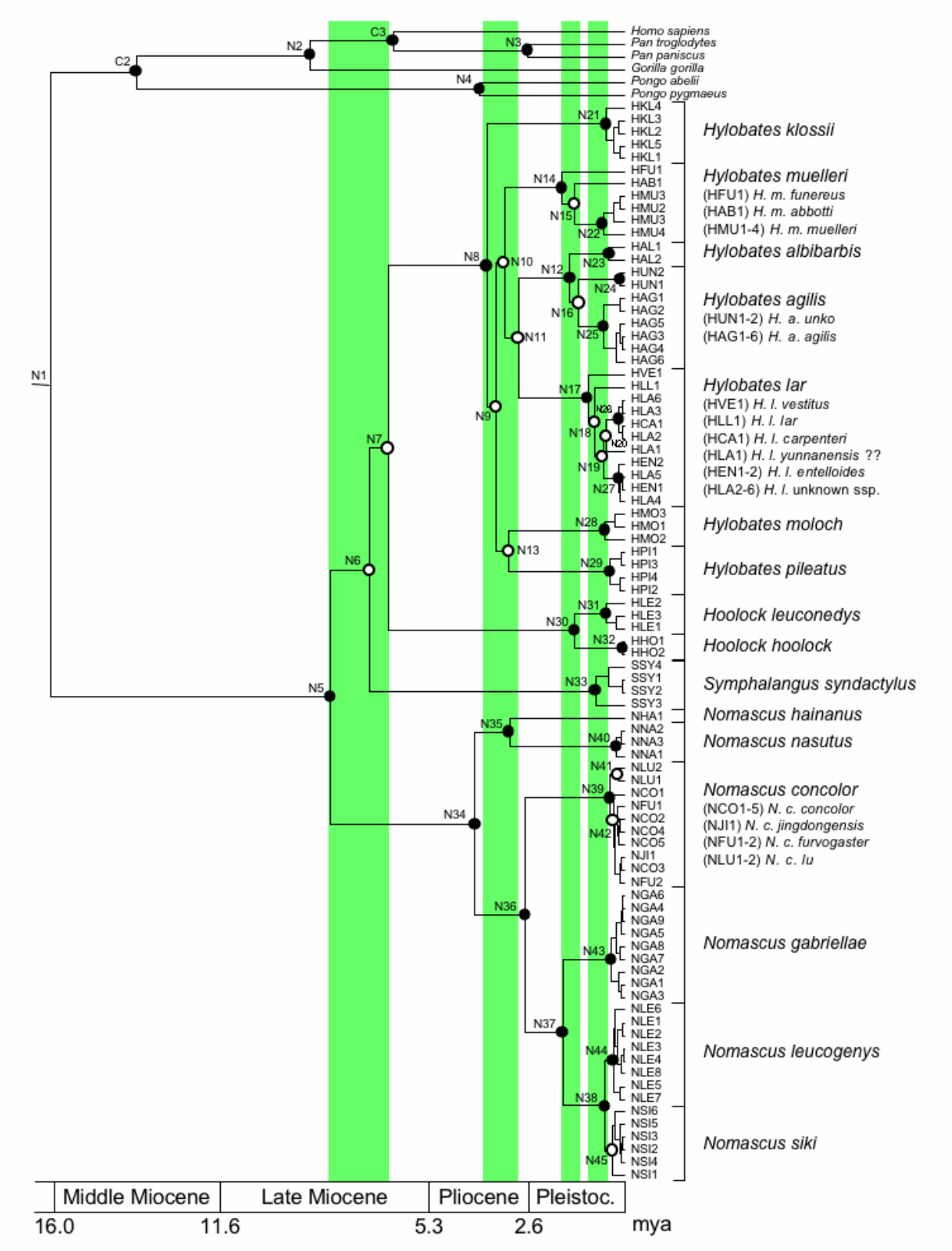

Figure 2.2: Ultrametric tree showing phylogenetic relationships and estimated divergence ages among studied gibbon individuals based on complete mitochondrial cytb sequence data. For individual codes see Appendix A.1. Circles indicate bootstrap or posterior probability values (filled circles: >90%, >0.95, open circles: <70%, <0.80). Nodes of interest are arbitrarily numbered (N1-N45). C2 and C3 refer to two of the three nodes used for calibration (C1 not shown). Light green bars indicate the four radiations. A geological time scale is given below. Full details of age estimates and node supports are presented in Table 2.2.

Table 2.2: Support values and Bayesian divergence date estimates (in mya). Means and 95% credibility intervals (CI) are given for 48 nodes (see also Figure 2.2)*.

Node	Support values**	Divergence	Mean (95% CI)
C1		*Papio* - Hominoidea	24.04 (22.01-26.08)
N1		Hylobatidae - Hominidae	16.26 (14.69-18.16)
C2	96/92/92/0.99	*Pongo - Gorilla/Pan/Homo*	13.83 (13.28-14.41)
N2	91/93/98/1.0	*Gorilla - Pan/Homo*	8.90 (7.58-10.22)
C3	97/96/91/1.0	*Pan - Homo*	6.56 (6.01-7.08)
N3	100/91/97/0.99	*Pan troglodytes - P. paniscus*	2.74 (2.03-3.51)
N4	100/98/96/0.99	*Pongo pygmaeus – P. abelii*	4.12 (3.14-5.13)
N5	100/100/100/1.0	*Nomascus - Symphalangus/Hoolock/Hylobates*	8.34 (7.14-9.68)
N6	56/69/67/0.78	*Symphalangus - Hoolock/Hylobates*	7.22 (5.99-8.44)
N7	65/54/54/0.71	*Hoolock - Hylobates*	6.69 (5.56-7.88)
N8	100/93/94/0.99	*Hylobates klossii - H. pileatus/H. moloch/H. agilis/H. albibarbis/H. lar/H. muelleri*	3.91 (3.25-4.59)
N9	<50/68/<50/<0.50	*H. pileatus/H. moloch - H. agilis/H. albibarbis/H. lar/H. muelleri*	3.65 (3.05-4.25)
N10	<50/<50/<50/0.62	*H. muelleri - H. agilis/H. albibarbis/H. lar*	3.40 (2.81-3.99)
N11	<50/53/<50/0.69	*H. agilis/H. albibarbis - H. lar*	3.02 (2.43-3.60)
N12	100/99/100/1.0	*H. agilis - H. albibarbis*	1.56 (1.19-1.98)
N13	<50/52/<50/<0.50	*H. pileatus - H. moloch*	3.29 (2.64-3.97)
N14	96/96/98/1.0	*H. muelleri funereus - H. m. abbotti/H. m. muelleri*	1.78 (1.33-2.25)
N15	56/57/<50/<0.50	*H. muelleri abbotti - H. m. muelleri*	1.42 (1.02-1.81)
N16	63/<50/67/0.79	*H. agilis agilis - H. a. unko*	1.30 (0.95-1.68)
N17	100/100/99/1.0	*H. lar vestitus - H. l. lar/H. l. entelloides/H. l. carpenteri/H. l. yunnanensis*	1.05 (0.75-1.35)
N18	<50/<50/50/0.76	*H. l. lar - H. entelloides/H. l. carpenteri/H. l. yunnanensis*	0.86 (0.60-1.13)
N19	<50/63/65/0.79	*H. l. entelloides - H. l. carpenteri/H. l. yunnanensis*	0.62 (0.41-0.83)
N20	<50/66/66/0.78	*H. l. carpenteri - H. l. yunnanensis*	0.52 (0.32-0.71)
N21	100/100/99/1.0	MRCA *H. klossii*	0.53 (0.29-0.81)
N22	99/96/97/1.0	MRCA *H. muelleri muelleri*	0.62 (0.38-0.88)
N23	100/100/100/1.0	MRCA *H. albibarbis*	0.44 (0.22-0.68)
N24	100/100/100/1.0	MRCA *H. agilis unko*	0.13 (0.02-0.25)
N25	99/96/94/1.0	MRCA *H. agilis agilis*	0.61 (0.36-0.89)
N26	95/98/92/1.0	MRCA *H. lar carpenteri*	0.17 (0.05-0.28)
N27	96/94/96/1.0	MRCA *H. lar entelloides*	0.18 (0.07-0.31)
N28	100/100/94/1.0	MRCA *H. pileatus*	0.41 (0.21-0.64)
N29	100/100/100/1.0	MRCA *H. moloch*	0.56 (0.30-0.84)
N30	100/100/100/1.0	*Hoolock hoolock - H. leuconedys*	1.42 (0.97-1.90)
N31	99/95/93/0.96	MRCA *H. leuconedys*	0.51 (0.28-0.80)

Node	Support values**	Divergence	Mean (95% CI)
N32	100/100/100/1.0	MRCA *H. hoolock*	0.07 (0.00-0.17)
N33	100/99/99/1.0	MRCA *Symphalangus syndactylus*	0.83 (0.51-1.18)
N34	100/100/99/1.0	*Nomascus hainanus/N. nasutus - N. concolor/N. gabriellae/N. leucogenys/N. siki*	4.24 (3.46-5.06)
N35	91/92/92/0.99	*N. hainanus - N. nasutus*	3.25 (2.49-3.99)
N36	94/91/96/1.0	*N. concolor - N. gabriellae/N. leucogenys/N. siki*	2.83 (2.21-3.50)
N37	96/92/98/1.0	*N. gabriellae - N. leucogenys/N. siki*	1.74 (1.28-2.22)
N38	100/99/93/1.0	*N. leucogenys - N. siki*	0.55 (0.35-0.77)
N39	100/100/100/1.0	*N. concolor lu - N. c. concolor/N. c. furvogaster/N. c. jingdongensis*	0.43 (0.25-0.63)
N40	100/100/99/1.0	MRCA *N. nasutus*	0.23 (0.08-0.39)
N41	<50/<50/67/0.75	MRCA *N. concolor lu*	0.19 (0.05-0.35)
N42	59/<50/<50/<0.50	MRCA *N. concolor concolor/N. c. furvogaster/N. jingdongensis*	0.32 (0.19-0.48)
N43	100/100/98/1.0	MRCA *N. gabriellae*	0.39 (0.21-0.57)
N44	92/91/98/1.0	MRCA *N. leucogenys*	0.33 (0.18-0.47)
N45	<50/<50/<50/0.58	MRCA *N. siki*	0.38 (0.18-0.55)

*Nodes used as calibrations are labelled with a "C", all others with an "N". MRCA denotes the most recent common ancestor. C1 not shown in Figure 2.2. **Support values as obtained from MP, NJ, ML and Bayesian reconstructions, respectively.

The relationships among the subspecies of *H. muelleri* and *H. lar* were less resolved. In *Hoolock,* the two species *H. hoolock* and *H. leuconedys* clearly segregated into two distinct clades. Within *Nomascus*, relationships among species were completely resolved, suggesting a *N. hainanus* + *N. nasutus* clade as sister lineage to the remaining species. Among them, *N. concolor* branched off first, followed by the divergence of *N. gabriellae* and *N. leucogenys/N. siki*. The monophyly of *N. leucogenys* was significantly supported, but evidence for a common origin of *N. siki* individuals was not obtained. Within *N. concolor*, specimens identified as *N. concolor lu* formed a distinct clade, while the remaining subspecies clustered together without further subdivision. However, support for a reciprocal monophyly of both clades was relatively low.

Based on divergence age estimates, gibbons separated from great apes and humans 16.26 mya (for 95% credibility intervals see Table 2.2). Within hominids, *Pongo* branched off first (13.83 mya), followed by *Gorilla* (8.90 mya), before finally *Homo* and *Pan* diverged from each other (6.56 mya). The differentiation of *Pongo* and *Pan* into species occurred 4.12 and 2.74 mya, respectively. In an initial radiation, gibbons diverged within a relative short time

period of only 1.65 million years (6.69-8.34 mya) into four genera. Within *Hylobates*, most species diverged from each other between 3.02 and 3.90 mya. The only exception was the separation of *H. albibarbis* from *H. agilis* 1.56 mya, which was in the time frame of subspecies splits within *H. muelleri* (1.42-1.78 mya). Differentiation of *H. lar* into subspecies occurred even later (0.52-1.05 mya). The two *Hoolock* species diverged 1.42 mya from each other. In *Nomascus*, differentiation into species took place over a longer time period, lasting from 4.24 until 0.55 mya. The most recent species divergence within *Nomascus* occurred between *N. siki* and *N. leucogenys* (0.55 mya), which was in a similar range as the separation of *N. concolor lu* from the other *N. concolor* subspecies (0.43 mya).

2.4 Discussion

By analysing all species and most subspecies, the present study provides the most complete view into the evolutionary history of the gibbon family. However, as in earlier molecular studies on gibbons (Garza and Woodruff 1992; Hayashi et al. 1995; Hall et al. 1998; Roos and Geissmann 2001; Roos 2004; Takacs et al. 2005; Chatterjee 2006; Monda et al. 2007; Roos et al. 2007; Whittaker et al. 2007), relationships on various taxonomic levels are less resolved and partially contradict earlier findings. While the herein depicted branching pattern among genera is identical with that found in earlier studies using also cytb (Chatterjee 2006) or D-loop (Roos and Geissmann 2001) sequences, it differs from another cytb-based study (Hall et al. 1998) in placing *Nomascus* and not *Symphalangus* as most basal genus. Studies based on mitochondrial ND3-ND4 sequences (Takacs et al. 2005) or chromosomal rearrangements (Müller et al. 2003) suggest *Hoolock* as most ancestral lineage, and *Nomascus* together with either *Hylobates* (Takacs et al. 2005) or *Symphalangus* (Müller et al. 2003) as the most recently diverged genera. For *Hylobates*, our data indicate a basal position of *H. klossii*, and a further division into a clade consisting of *H. lar, H. muelleri, H. agilis* and *H. albibarbis*, and another one with *H. moloch* and *H. pileatus*. Various branching patterns among *Hylobates* species are proposed (Hayashi et al. 1995; Takacs et al. 2005; Chatterjee 2006; Whittaker et al. 2007), which all differ from our one, but respective support values are similarly low as in our study. In contrast, the relationships found among species of the genus *Nomascus* are well

resolved and identical with that suggested by Roos (2004), Takacs et al. (2005), Monda et al. (2007) and Roos et al. (2007).

According to our and earlier data, relationships among gibbon genera and *Hylobates* species remain disputed, which most likely can be explained by the separation of respective lineages within relative short time periods. This becomes even more obvious when considering estimated divergence ages, which fall into four temporal windows. In the first, between ~6.7 and ~8.3 mya, the four gibbon genera originated. In a second radiation, between ~3.0 and ~3.9 mya, *Hylobates* split into various species, and in a third burst, between ~1.3 and ~1.8 mya, *H. muelleri*, the *H. agilis* + *H. albibarbis* clade and *Hoolock* further differentiated. Finally, in a fourth radiation, between ~0.5 and ~1.1 mya, *H. lar* diverged into subspecies. In contrast, speciation in *Nomascus* was a continuous process, lasting from 4.24 until 0.55 mya.

2.4.1 Taxonomic implications

Our data show that mitochondrial DNA (mtDNA) provides a powerful tool for the identification and taxonomic classification of gibbons, because taxa form strongly supported monophyletic clades, or at least appear to form distinct lineages in those cases where only one individual per taxon was tested. Moreover, most differentiation events fall into four temporal periods, which allow a hierarchical ranking as proposed by Goodman et al. (1998), though the threshold for the recognition of a certain taxonomic unit whether genus, species, or subspecies remains disputed. Hence, to provide a more reliable classification, we compare divergence ages among gibbon lineages with those among other Asian primates and hominids.

Accordingly and concordant with recent classifications (Roos and Geissmann 2001; Bandon-Jones et al. 2004; Mootnick and Groves 2005; Mootnick 2006; Geissmann 2007; Roos et al. 2007; IUCN 2009; Gibbon Research Lab 2010), the four major gibbon lineages are proposed as distinct genera (Table 2.1), since they split from each other in a similar time range as did colobine genera (Raaum et al. 2005; Sterner et al. 2006; Roos et al. submitted) or African great apes and human (Goodman et al. 1998; Raaum et al. 2005; Gibbon Research Lab 2010). Most species of *Hylobates* and *Nomascus* emerged in or around the second radiation, which is on the same time scale as species splits within *Pongo*

and *Pan*, and the separation of species groups within *Macaca* (Tosi et al. 2003; Ziegler et al. 2007) and *Trachypithecus* (Roos et al. 2008). Thus, taxa originating in this time period should be recognized as distinct species (*H. moloch, H. pileatus, H. klossii, H. lar, H. muelleri, H. agilis/H. albibarbis, H. hoolock/H. leuconedys, N. nasutus, N. hainanus, N. concolor, N. gabriellae/N. leucogenys/N. siki*), and might be even classified as species groups. Further differentiation events among gibbons occurred in the third time period, which is in a similar window as several speciation events within macaques (Tosi et al. 2003; Ziegler et al. 2007). Accordingly, *H. leuconedys* and *H. albibarbis* should be separated from *H. hoolock* and *H. agilis* on species level, respectively, and the three subspecies of *H. muelleri* could be considered for elevation to species level. Moreover, *H. agilis* is divided into two clades, which refer to individuals identified by pelage coloration as *H. agilis agilis* and *H. agilis unko.* However, in a recent work based on a larger number of individuals a reciprocal monophyly of both lineages is doubted (Tanaka et al. 2004), and, hence, we provisionally recognize *H. agilis* as monotypic. For *H. lar*, only a few unambiguously identified specimens were available for our study, but these represent at least four of the five recognized subspecies, while the identity of the putative *H. lar yunnanensis* individual remains uncertain. Based on our data, *H. lar* subspecies form distinct lineages, which diverged relative recently. We provisionally accept all five subspecies, though ongoing studies might reject some or all of them. For *N. concolor*, our data indicate a separation of *N. concolor lu* from the remaining subspecies, which form a clade without further subdivision into taxa. Hence and concordant with Monda et al. (2007) and Roos et al. (2007), we provisionally classify *N. concolor furvogaster* and *N. concolor jingdongensis* as synonyms of *N. concolor concolor*, while we feel *N. concolor lu* is a separate subspecies. We further separate *N. gabriellae* from *N. siki/N. leucogenys* on species level, while it is questionable whether the latter two should be recognized as species or subspecies. Our study reveals a split between both taxa just 0.55 mya, which is in a similar range as the subspecies differentiation within *H. lar* or *N. concolor*. Hence, a separation of both taxa only on subspecies level would be indicated. However, both taxa show slight differences in vocalisation and facial colouration (Mootnick 2006; Geissmann 1995; Geissmann et al. 2000), and Carbone et al. (2009) found a chromosomal inversion unique to *N. leucogenys*. Accordingly, we follow here the current view and recognize *N. leucogenys* and *N.*

siki as distinct species. In summary, we recognize four gibbon genera with 18 species and seven subspecies (Table 2.1).

2.4.2 Biogeographic implications

Multiple radiations in the evolutionary history of gibbons suggest a complicated biogeographic pattern leading to the current distribution of gibbon taxa. Since gibbons are arboreal (Chivers 1977; Geissmann 2002b), radiations most likely were correlated with expanding forest habitats. In fact, the complete range of gibbons experienced complex geographical and environmental changes during the last ten million years. Notably, in the late Miocene as well as in the Plio- and Pleistocene, a series of dramatic climatic changes influenced the geography and vegetation in the region, leading to shifts in the extension and distribution of different habitat types (Eudey 1980; Morley and Flenley 1987; Morley 2000; Meijaard 2004; Bird et al. 2005; Meijaard and Groves 2006). In particular, periods of maximum glaciation might have reduced rainforest cover, resulting in the appearance of more open and deciduous vegetation types in many parts of the region (Heaney 1991; Urushibara-Yoshino and Yoshino 1997; van der Kaars 2001; Meijaard 2004; Bird et al. 2005; Meijaard and Groves 2006; but see Cannon et al. 2009). Moreover, due to the alternately falling and rising sea water levels during the several glacial and interglacial periods (Jablonski and Whitfort 1999; Meijaard 2003; Lisiecki and Raymo 2005; Miller et al. 2005; Naish and Wilson 2009), connections and separations of landmasses were common, and repeated migration between islands and today's mainland was possible (Verstappen 1975; Tougard 2001; Woodruff and Turner 2009).

By combining the available information, we develop the following dispersal scenario for gibbons, which is in general agreement with that proposed by Chatterjee (2006, 2009), Harrison et al. (2006), and Jablonski and Chaplin (2009), but which differs substantially from them in some aspects. Accordingly, gibbons most likely originated on the Asian mainland, because all four gibbon genera occur there. Specifically, the Hengduan mountains in the border region of today's Burma, India and China might have been a possible diversification hotspot (Peng et al. 1993; Jablonski 1998). In the region, all the larger Southeast Asian rivers (Mekong, Salween, Yangtze) rise, which are all well known as barriers for arboreal primates (Meijaard 2004). Although these rivers changed their courses several

times, their upper reaches in the Hengduan mountains exist at least since the early Miocene (Hallet and Molnar 2001). Recently, the Hengduan mountains were also proposed as a region of differentiation for colobine monkeys, and most interestingly, respective splitting events occurred on a similar time scale as in gibbons (Roos et al. submitted). In fact, in the late Miocene, widely distributed rain forest habitats promoted range extension for arboreal primates (Morley and Flenley 1987; Meijaard and Groves 2006). Accordingly, in the late Miocene, *Nomascus* invaded the region east of the Mekong, *Hoolock* entered the region west of the Salween, and *Hylobates* and *Symphalangus* migrated into the area in-between and later on into Sundaland.

Hylobates successfully colonized large parts of Sundaland, but also survived on the Asian mainland. Shortly after its arrival in Sundaland in the Pliocene, populations on the Asian mainland, the Malay peninsula, Sumatra, Borneo, Java and the Mentawai archipelago became isolated. At the same time, various species groups of the genera *Macaca* and *Trachypithecus* diverged (Tosi et al. 2003; Ziegler et al. 2007; Roos et al. 2008), indicating dramatic environmental changes. In fact, this time period was characterized by global warming and sea levels similar to today (Haq et al. 1987; Lisiecki and Raymo 2005; Miller et al. 2005; Meijaard and Groves 2006; Naish and Wilson 2009), which prevented migration between landmasses and, thus, promoted speciation due to vicariance. Whether *Symphalangus* experienced a similar range expansion in Sundaland like *Hylobates*, remains questionable. Today the genus appears only on Sumatra and the Malay peninsula, and fossil data provide only evidence for its historical occurrence on Java and Sumatra (Harrison et al. 2006). In the early Pleistocene, further differentiation in *Hylobates* occurred on Borneo and Sumatra, and in *Hoolock* on the mainland which is on a similar time scale when macaque species diverged (Tosi et al. 2003; Ziegler et al. 2007), and which might has been triggered by the shrinking of forest habitats due to cold phases (Singh and Srinivasan 1993, but see Cannon et al. 2009). Notably, *H. albibarbis* is mitochondrially closer related to Sumatran *H. agilis* than to the other Bornean gibbons and acoustic, morphological and chromosomal data suggest an intermediate position (Geissmann 1995; Groves 2001; Hirai et al. 2005; Hirai et al. 2009). Accordingly, *H. albibarbis* might be the product of an ancient hybridization event, in which proto-*H. agilis* invaded Borneo during sea level lowstands (Haq et

al. 1987; Lisiecki and Raymo 2005; Miller et al. 2005; Naish and Wilson 2009) and successfully reproduced with proto-*H. muelleri*. As we find mtDNA of proto-*H. agilis* in *H. albibarbis*, female introgression is the most likely hybridization scenario, which is in agreement with recent findings, that gibbon females disperse over longer distances than males (Lappan 2007). Finally, in a last range expansion in the early to middle Pleistocene, *H. lar* colonized, starting from its Sumatran refuge, the Malaysian peninsular and mainland Southeast Asia (see also Jablonski and Chaplin 2009).

In contrast to the biogeographic pattern found in *Hylobates* and to the scenario proposed by Chatterjee (2006, 2009), for *Nomascus* not a radiation but a successive migration from North to South over a long time period becomes evident. Based on our data, *Nomascus* originated in the border region of Vietnam and China in the early Pliocene and it took to the early Pleistocene until the genus reached the southern extend of its current distribution in southern Vietnam and Cambodia.

2.4.3 Conservation implications

All gibbon species are on the brink of extinction and, with the exception of *H. leuconedys* (Vulnerable), are classified as “Endangered” or even “Critically Endangered” (Geissmann 2007; IUCN 2009). With approximately 20 individuals left in its native habitat, the Hainan gibbon (*N. hainanus*) is the rarest primate in the world (Cunningham and Mootnick 2009; Chan et al. 2005; Mootnick et al. 2007) and the situation for its closest relative, the Cao-vit crested gibbon (*N. nasutus*) with approximately 100 individuals left (IUCN 2009; Long and Nadler 2009), as well as for other gibbon species, the situation is alarming. Reasons for the decline of gibbons are manifold, but habitat loss due to forest clearance for agricultural use, oil palm or rubber plantations, gold mining, or charcoal and timber production, as well as illegal hunting for food and sport, and the trade for pets or medicine are major threats to wild gibbon populations (Geissmann et al. 2000; Geissmann 2007).

To save gibbons from extinction, urgent actions are required to prevent ongoing habitat destruction and hunting, and to build up a viable gene pool in captivity for later release purposes. Specifically, to prevent or at least reduce hunting, hunting hotspots have to be identified. Therefore, it is crucial to confirm

the taxon identity and if possible the geographical origin of confiscated gibbons or their remains. Similarly, to avoid artificial hybrids, only gibbons with clear taxon identity should be considered for reproduction in zoos or rescue centres. Finally, if captive gibbons are reintroduced into the wild, it has to be ascertained that these gibbons are pure individuals and of the same taxon as those, which naturally occur in the area they are to be released.

An accurate taxonomic identification of gibbons based on vocal data or pelage colouration is sometimes complicated (Geissmann 1995; Mootnick 2006). In this respect, mtDNA analysis might be a promising tool. As shown in our study, gibbon taxa can be diagnosed through mtDNA and, hence, a secure identification can easily be obtained. Yet since mtDNA is only maternally inherited, possible hybrids will not be detected in such analysis, so that additional markers should be studied as well.

2.5 Conclusions

Due to a nearly complete taxon sampling, the present study provides the most comprehensive insights into the evolutionary and biogeographic history of the hylobatid family. Based on estimated divergence ages and unresolved relationships among gibbon taxa on various levels, several radiation-like splitting events are indicated, which suggest a complex biogeographic history of gibbons. Presumably, most of these differentiation events occurred in wave-like range expansions in Sundaland and the Asian mainland followed by vicariance effects, most likely caused by alternately shrinking and expanding rain forest habitats and by repeated separations and connections of landmasses. In contrast, in the region east of the Mekong river gibbons underwent a successive north-to-south migration. Our study also shows that mtDNA provides a solid platform for the taxonomic classification of gibbons and that mtDNA can be successfully applied to accurately identify the species affiliation of gibbon individuals, which is urgently required for conservation purposes. However, to completely understand the phylogeny and phylogeography of gibbons, to identify hybrids in captivity, or to trace possible ancient hybridization events as it might be indicated for *H. albibarbis*, further studies including extended mitochondrial as well as autosomal, X and Y chromosomal sequence data, are necessary.

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3 Phylogeny and distribution of crested gibbons (genus *Nomascus*) based on mitochondrial cytochrome b gene sequence data

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Abstract

Crested gibbons, genus *Nomascus*, are endemic to the Indochinese bioregion and occur only in Vietnam, Laos, Cambodia and southern China. However, knowledge about the number of species to be recognized and their exact distribution zones is still limited. To further elucidate the evolutionary history of crested gibbon species and to settle their distribution ranges, we analyzed the complete mitochondrial cytochrome b gene from 79 crested gibbon individuals from known location. Based on our findings, crested gibbons should be classified into seven species. Within *N. concolor*, we recognize two subspecies*, N. c. concolor* and *N. c. lu*. Phylogenetic reconstructions indicate that the northernmost species, *N. hainanus, N. nasutus* and *N. concolor* branched off first, suggesting that the genus originated in the north and successively migrated to the south. The most recent split within *Nomascus* occurred between *N. leucogenys* and *N. siki*, and between *N.* sp. and *N. gabriellae*. Based on our data, we are also able to revise the currently postulated distribution of the latter four species. Our study also shows that genetic data are concordant with acoustic data and that both in combination or alone can be applied to elucidate phylogenetic relationships among crested gibbons or to elucidate species boundaries.

Key words: *Nomascus*, crested gibbons, phylogeny, taxonomy, distribution, mitochondrial cytochrome b gene

3.1 Introduction

Crested gibbons, genus *Nomascus*, represent one of the four gibbon genera (Roos and Geissmann 2001) and differ from other gibbons in various morphological, anatomical, acoustic and chromosomal features (Groves 1972, 2001; Geissmann et al. 2000; Müller et al. 2003). All taxa of the genus show a strong sexual dichromatism with orange, beige or yellow coloured females, and black males, which in some species have light cheeks (Geissmann et al. 2000; Groves 2001). The crown hair in males is erected, which gave them their common name “crested gibbons”. Crested gibbons are distributed in Vietnam, Laos, Cambodia and parts of southern China. They are mainly restricted to the region east of the Mekong river and only the West Yunnan black crested gibbon (*Nomascus concolor furvogaster*) crossed the upper Mekong to the west (Geissmann et al. 2000; Groves 2001) (Figure 3.1).

Traditionally, crested gibbons were combined in the single species *N. concolor* (Napier and Napier 1967; Groves 1972; Chivers 1977; Haimoff et al. 1982; Marshall and Sugardjito 1986). However, recent investigations based on morphological, genetic and acoustic data divide them into four, five or even six species (Geissmann 1997, 2002, 2007; Geissmann et al. 2000; Groves 2001; Takacs et al. 2005; Mootnick 2006; Monda et al. 2007; Roos et al. 2007; Thinh et

al. 2010), and new acoustic data suggests even an additional, so far undescribed taxon in the range of the Southern white-cheeked gibbon (*N. siki*) (Konrad and Geissmann 2006; Thinh et al. submitted).

Accordingly, the number of taxa to be recognized and their taxonomic classification remains disputed. Due to this taxonomic uncertainty, we follow here the most recent classification with a total of six crested gibbon species, Hainan gibbon (*N. hainanus*), Cao-vit crested gibbon (*N. nasutus*), Black crested gibbon (*N. concolor*), Northern white-cheeked gibbon (*N. leucogenys*), Southern white-cheeked gibbon (*N. siki*) and Red-cheeked gibbon (*N. gabriellae*) (IUCN 2009; Thinh et al. 2010). Additionally, we split *N. siki* into a northern (*N. siki*) and a southern (*N.* sp.) species (Konrad and Geissmann 2006; Thinh et al. submitted).

Besides the discussion about the number of taxa and their classification, also the distributional extend of some taxa is poorly known. However, knowledge about the exact distribution areas is of great importance for conservation purposes since all crested gibbon species are threatened and classified as “Endangered” or even “Critically Endangered” (Geissmann 2007; IUCN 2009). With only about 20 individuals left, *N. hainanus* is the rarest primate in the world (Chan et al. 2005; Mootnick et al. 2007; Cunningham and Mootnick 2009), and for *N. nasutus* with approximately 100 individuals (IUCN 2009; Long and Nadler 2009), as well as for other crested gibbons, the situation is alarming. Reasons for the decline of gibbons are manifold, but illegal hunting for the pet trade, food or the preparation of traditional medicine as well as habitat loss due to forest clearance for agricultural use, rubber, coffee and cashu plantations, gold mining, or charcoal and timber production are major threats to wild gibbon populations (Geissmann et al. 2000; Geissmann 2007).

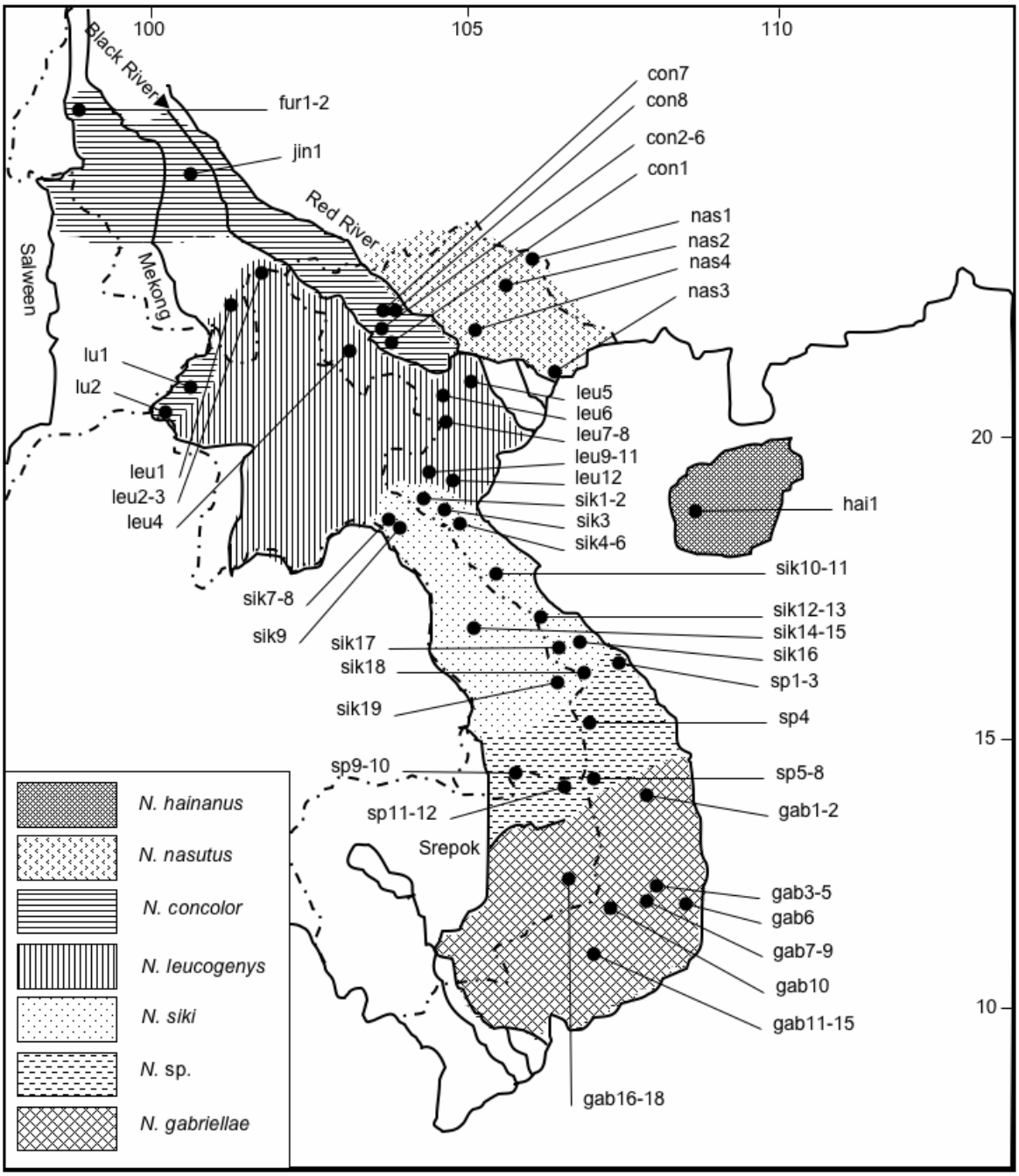

Figure 3.1: Distribution of crested gibbons after Geissmann et al. (2000), and Konrad and Geissmann (2006). Major rivers and country borders are indicated as solid and dashed lines, respectively. Sampling sites are indicated as dots and assigned to individual sample codes (for further details of samples see Appendix B.1).

Although field surveys can confirm the occurrence of gibbons, it is problematic in the field to clarify to which taxon a certain gibbon belongs to, because gibbons live high in the canopy and, hence, characteristic features of fur colouration are normally difficult to be seen. Also the identification of crested gibbon skins in museum collections is hampered by the fact that females of different species show only slight differences and that crested gibbons change

their colour during ontogenesis (Geissmann et al. 2000; Groves 2001). Hence, other methods are required to confirm the taxon-identity of crested gibbon individuals. Most promising in this respect are acoustic analyses, because crested gibbon songs exhibit species-specific characteristics (Geissmann 1993, 2002a; Geissmann et al. 2000; Konrad and Geissmann 2006; Thinh et al. submitted) and sounds can relatively easily be recorded in the field. Similarly, genetic data, which can be extracted from material collected non-invasively in the field or from museum specimens, can be applied as taxon-specific markers (Roos 2004; Takacs et al. 2005; Monda et al. 2007; Roos et al. 2007; Thinh et al. 2010).

In the present study, we analyzed the complete mitochondrial cytchrome b gene (cytb) from 79 gibbon individuals with the aim to 1) further elucidate the phylogenetic relationships among crested gibbon species and 2) to narrow down their distribution zones. Therefore, we collected samples during field surveys in Vietnam, Laos and Cambodia, and from museum specimens. For a more comprehensive analysis, we included further sequences from gibbons with clear provenance available in GenBank.

3.2 Materials and methods

3.2.1 Sample collection

Fecal samples of gibbons were collected during field surveys in 18 protected areas in Vietnam, 4 in Laos and 2 in Cambodia in 2007 and 2008. In total, 48 fecal samples were obtained. 12 tissue samples were gathered from museum specimens stored in the Institute of Ecology and Biological Resources (IEBR), Hanoi, the Zoological Museum of the Vietnam National University (ZMVNU), Hanoi, the Xuan Mai Forestry College (XMFC), Xuan Mai and the National Museum of Natural History (USNM), Washington. Taxon-identity of specimens was ascertained by their presumed distribution using the map presented by Geissmann et al. (2000), which was modified after Konrad and Geissmann (2006) (Figure 3.1). Fecal samples were preserved in 80-90% ethanol and museum samples were placed in plastic bags without any additive. Samples were stored at ambient temperature for up to six months before further processing. Sample collection complied with legal requirements of the countries in which research was conducted. For a more comprehensive overview on crested gibbon

distribution and phylogeny, we included 19 additional *Nomascus* sequences recently published by Thinh et al. (2010). In total, our dataset comprised 79 crested gibbons from 45 locations (Figure 3.1, Appendix B.1).

3.2.2 Laboratory work

Total genomic DNA from tissue and fecal samples was extracted with the DNeasy Blood & Tissue and QIAamp DNA Stool Mini kits from Qiagen, respectively. PCR amplification and sequencing was performed using methods as described in Thinh et al. (2010). Sequences were assembled and aligned with Geneious v4.6.1 (Drummond et al. 2008) and checked for their potential to be correctly transcribed. Haplotypes were deposited in GenBank and are available under the accession numbers GU594996-GU595022 (see also Appendix B.1).

3.2.3 Statistical analysis

For phylogenetic analysis, we expanded our dataset with orthologous sequences available in GenBank from additional crested gibbons from known location and *Hylobates lar*, which was used as outgroup. For further analyses, identical haplotypes were removed. Phylogenetic relationships were constructed with neighbor-joining (NJ), maximum-likelihood (ML) and Bayesian algorithms using the programs PAUP v4.0b10 (Swofford 2003), GARLI v0.951 (Zwickl 2006) and MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003). For all calculations, the optimal nucleotide substitution model (TIM + Γ) was selected using the Akaike information criterion (AIC) as implemented in jModelTest v0.1.1 (Posada 2008). For NJ reconstructions, a heuristic search was performed with the maximum number of trees set to 100. In GARLI, only the model specification settings were adjusted according to the dataset, while all other settings were left at their default value. Support of internal nodes for the NJ and ML tree was assessed by bootstrap analyses with 10,000 and 500 replications, respectively. A ML 50% majority-rule consensus tree was calculated in PAUP. For Bayesian reconstructions, we used four Markov Chain Monte Carlo (MCMC) chains with the default temperature of 0.1. Four repetitions were run for 10,000,000 generations with tree and parameter sampling occurring every 100 generations. The first 25% of samples were discarded as burnin, leaving 75,001 trees per run. Posterior probabilities for each split and a phylogram with mean branch lengths were

calculated from the posterior density of trees.

3.3 Results

We successfully generated sequences of the complete mitochondrial cytb gene (1,140 bp) from 60 crested gibbon individuals from known location. A contamination of our dataset with nuclear pseudogenes (numts) can be excluded because 1) gibbon-specific primers were used (Roos 2004; Roos et al. 2007; Thinh et al. 2010), 2) no multiple amplifications of different copies were detected by direct sequencing, 3) all sequences were correctly transcribed, and 4) no inconsistent positions were detected in alignments, which were assembled from overlapping sequences. Cross-contamination between individuals can be excluded as well, since all negative controls revealed no amplifications and randomly repeated PCRs for the same individual produced identical sequences.

In the complete alignment including 79 crested gibbons, we observed 45 unique haplotypes, which were defined by 200 variable sites, of which 145 were parsimony-informative. Identical haplotypes were mainly found in individuals from the same site, but also from different sites, or were even shared between individuals of different species (*N. leucogenys* and *N. siki*, *N. siki* and *N.* sp.) (Figure 3.2, Appendix B.1).

Phylogenetic reconstructions based on all algorithms revealed identical tree topologies. Most clades and branching patterns were significantly supported (Figure 3.2), although for some only weak support was obtained (see below). According to our reconstruction, *Nomascus* initially diverged into a clade consisting of *N. hainanus* and *N. nasutus*, and another including all the remaining species. However, support for the sister grouping of *N. hainanus* and *N. nasutus* was low. Among the remaining species, *N. concolor* branched off first. Within *N. concolor*, specimens identified as *N. concolor lu* formed a distinct clade, while the remaining subspecies clustered together without further subdivision. The four remaining species further diverged into four clades, which, however, did not support species monophylies. Although two of these clades were composed of only either *N. gabriellae* or *N. siki* individuals, representatives of both species were also nested in at least one other clade. In contrast, *N. leucogenys* and *N.* sp., did not form distinct clades and occurred only in mixed clades. In the first, *N. leucogenys* and *N. siki*, and in the second, *N. gabriellae, N.* sp. and *N. siki* were combined. The

monophyly of these clades was strongly supported, although for the pure *N. siki* clade sufficient support was only obtained from the NJ reconstruction.

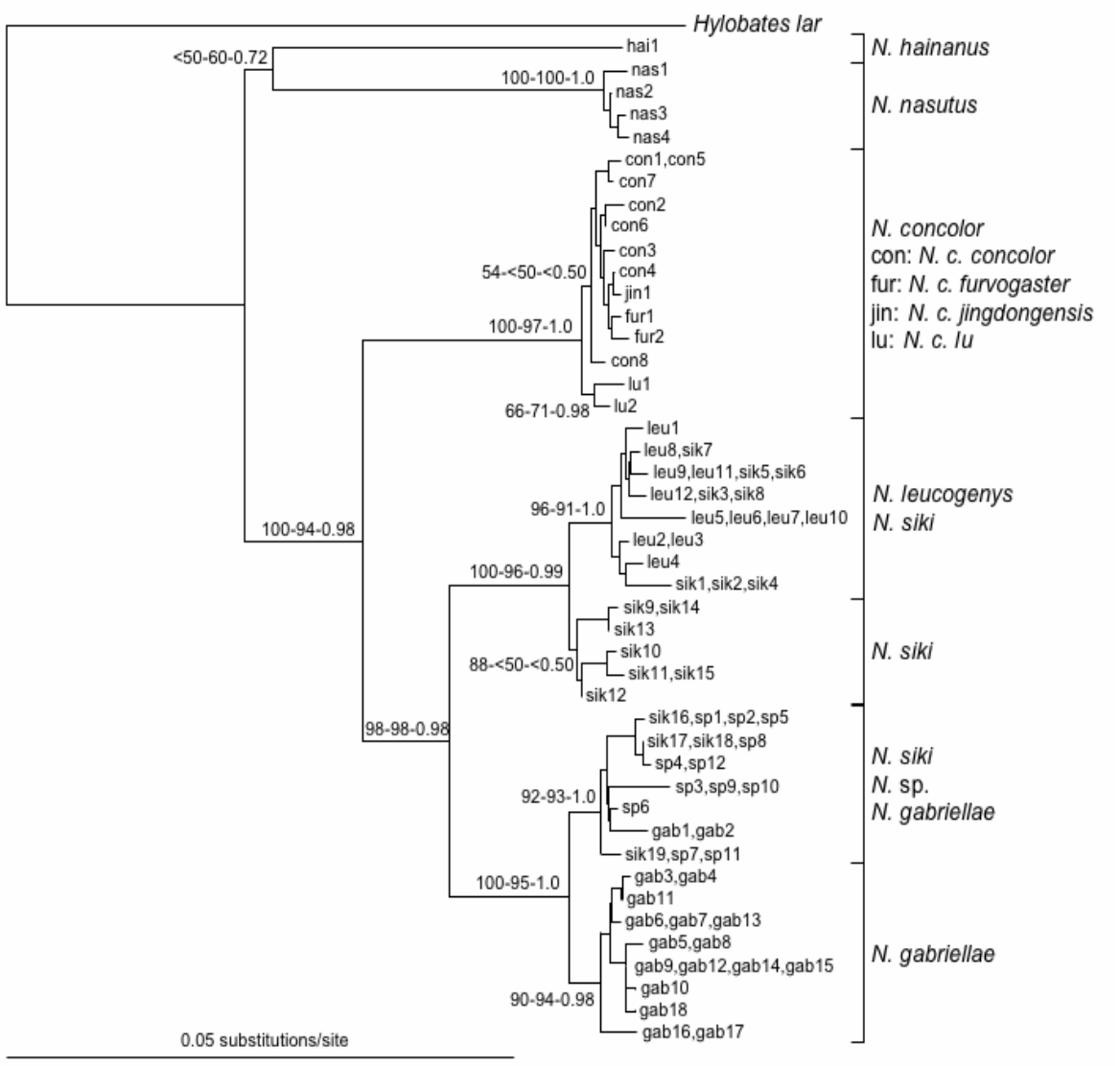

Figure 3.2: Phylogenetic relationships among crested gibbons. Numbers on branches refer to support values as obtained from NJ, ML and Bayesian reconstructions, respectively. Individual codes refer to those mentioned in Figure 3.1 and Appendix B.1.

3.4 Discussion

By analyzing only clearly provenanced individuals and all ten crested gibbon taxa, the present study is the first, which allows reliable and most complete insights into the evolutionary history of the genus *Nomascus* and the distribution of its taxa. Since only individuals from known location are present in our study, a "contamination" of the dataset with misidentified specimens can be excluded. These, however, might have affected earlier studies (e.g. Roos 2004; Chatterjee

2006) and resulted in wrong conclusions.

In general, the herein obtained relationships are concordant with earlier findings based on molecular (Roos 2004; Takacs et al. 2005; Monda et al. 2007; Roos et al. 2007; Thinh et al. 2010) and acoustic data (Geissmann 1993, 2002a; Geissmann et al. 2000; Konrad and Geissmann 2006; Thinh et al. in press, submitted). Accordingly, the three northernmost species with totally black males, *N. hainanus, N. nasutus* and *N. concolor*, represent the deepest splits. Interestingly, the three species are paraphyletic, with *N. concolor* being closer related to the southern species than to *N. nasutus* and *N. hainanus*. Due to this observed branching pattern, the genus most likely originated in the north and successively migrated to the south (Thinh et al. 2010) and, thus, all-black males might represent the ancestral form of the genus. Support for this hypothesis is also gained by the prominent acoustic differences found between these three species and between them and the remaining four species (Geissmann et al. 2000; Geissmann 2002a; Thinh et al. accepted). Within *N. concolor*, *N. c. lu* individuals are separated from the other three subspecies, which cluster together without further subdivision. Hence, we agree with earlier studies (Monda et al. 2007; Roos et al. 2007; Thinh et al. 2010) and recognize *N. c. lu* as distinct subspecies, while *N. c. furvogaster* and *N. c. jingdongensis* are downgraded as synonyms of *N. c. concolor*. In contrast to the clearly confirmed monophyly of *N. nasutus* and *N. concolor*, evidence for a common origin for each of the four species, *N. leucogenys, N. siki, N.* sp. and *N. gabriellae*, is not provided. In fact, all these four species appear to be para- or polyphyletic in our tree, at least when species are identified only on the basis of their presumed distribution.

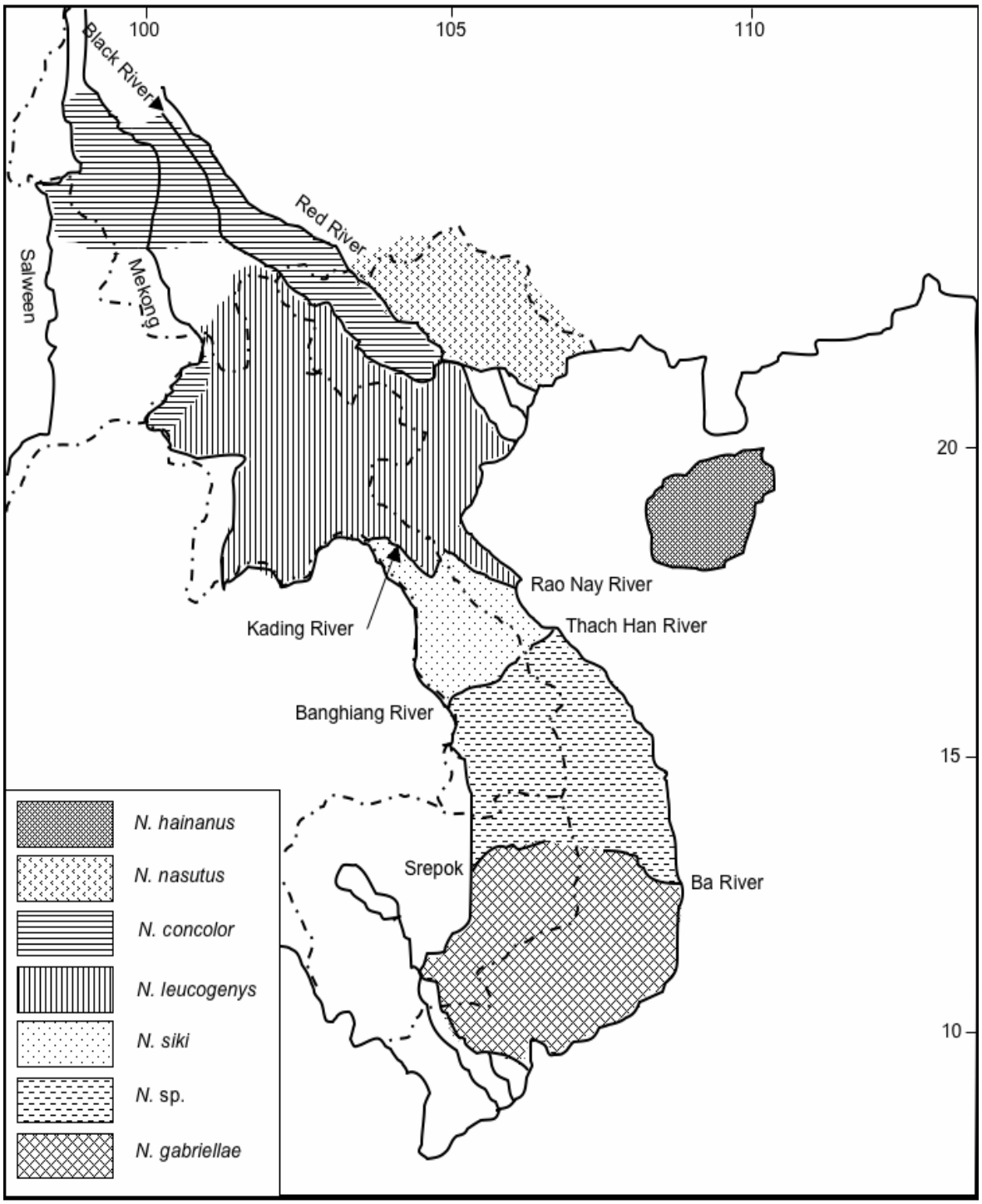

Figure 3.3: Revised distribution of crested gibbons based on genetic (this study) and acoustic (Thinh et al. submitted) data.

Thus, the four species might be indeed paraphyletic or they have distribution areas, which differ from currently postulated ones. Recent acoustic data suggest the latter (Thinh et al. submitted). The herein studied *N. siki* individuals from the proposed northern range of the species (sik1-8) cluster together with *N. leucogenys* and acoustic data clearly show that gibbons from Vu Quang National Park (NP) (origin of samples sik4-6) and northern Nam Kading

National Biodiversity Conservation Area (NBCA) (sik7-8) sing like *N. leucogenys* and not like *N. siki* (Thinh et al. submitted). Similarly, *N. siki* individuals from their presumed southern range (sik16-19) form a clade together with *N.* sp. and *N. gabriellae*, but gibbons from Phong Dien Nature Reserve (NR) (sik16), Da Krong NR (sik17), Sao La NR (sik18) and Xe Sap NBCA (sik19) show acoustic features typical for *N.* sp. (Thinh et al. submitted). Hence, gibbons from these locations might refer to *N.* sp. and not to *N. siki*. Although the majority of *N. gabriellae* individuals form a distinct clade, two of them (gab1-2) appear in a mixed clade together with *N.* sp. and *N. siki*. Both samples are from Kon Ka Kinh NP, a location where gibbons sing like *N.* sp. (Thinh et al. submitted). Accordingly, the Kon Ka Kinh population seems to belong to *N.* sp. and not to *N. gabriellae*.

Based on the herein presented genetic data and information from recent acoustic analyses (Geissmann 2002a; Konrad and Geissmann 2006; Thinh et al. submitted), it becomes obvious that the distributional ranges of the four species, *N. leucogenys, N. siki, N.* sp. and *N. gabriellae* have to be revised (Figure 3.3). According to both genetic and acoustic data (Thinh et al. submitted), Nam Kading NBCA in Laos (origin of samples sik7-9) holds both *N. leucogenys* and *N. siki*, whereas the former occurs north of the Kading river and the latter south of it. The Kading river runs also through Nakai-Nam Theun NBCA (17°36'-18°23'N; 105°02'-105°46'E), suggesting that both species are also present in this area. In Vietnam, *N. siki* is believed to occur as far north as Pu Mat NP (sik1-2) (Geissmann et al. 2000), but genetic data suggest its northernmost distribution in Phong Nha-Ke Bang NP (sik10-11) and acoustic data provide evidence for its northernmost occurrence in Khe Ve NR (17°45'N; 106°00'E). For Ke Go NR (18°00'-18°09'N; 105°50'-106°07'E), between Khe Ve NR and Vu Quang NP, no information is available. Hence, the border between *N. siki* and *N. leucogenys* in Vietnam remains questionable, but the Rao Nay river, north of Khe Ve NR, might be wide enough to prevent dispersal. The southern extend of *N. siki* in Vietnam is Huong Hoa NR (sik12-13), which is well separated from *N.* sp. by the Thach Han river. In Laos, its southernmost range is unknown. Our genetic data confirm the species in Phou Xang He NBCA (sik14-15), but no information is available for Dong Phou Vieng NBCA (16°07'-16°44'N; 105°51'-106°32'E). The Banghiang river running through Dong Phuo Vieng NBCA might be a possible barrier, suggesting that Dong Phou Vieng NBCA holds both *N. siki* and *N.* sp. Whether *N. siki* extends south to

Dong Phou Vieng NBCA or not, the species experiences a dramatic reduction compared to its originally proposed range. Although various protected areas in Laos are still in the range of *N. siki*, in Vietnam the species is found only in Khe Ve NR, Phong Nha-Ke Bang NP and Huong Hoa NR. In contrast, the distribution of *N.* sp. is largely extended. Its northernmost distribution in Vietnam is Phong Dien NR (sik16) and Da Krong NR (sik17), and Xe Sap NBCA in Laos (sik19). Acoustic data further suggest its occurrence in Xe Bang-Nouan NBCA, Laos (15°44'-16°01'N; 105°53'-106°18'E) (Duckworth 2008). Its confirmed southernmost range in Cambodia is Poey (13°57'N; 107°00'E) (Konrad and Geissmann 2006) and Kon Ka Kinh NP (gab1-2) in Vietnam (this study; Thinh et al. submitted). *N. gabriellae* experiences a range reduction with an approved northernmost extend in Phnom Prich Wildlife Sanctury (WS) (gab16-18) in Cambodia and in A Yun Pa NR (13°24'-13°38'N; 108°30'-108°45'E) in Vietnam (Thinh et al. submitted). Most likely, the Srepok river in Cambodia and the Ba river in Vietnam act as barriers for *N.* sp. and *N. gabriellae*.

Our study shows that genetic data from solely provenanced crested gibbon individuals provides detailed and reliable information about phylogeny and distribution of crested gibbon species. Moreover, our data proved to be concordant with acoustic analyses. Hence, both approaches, in combination or alone, can be used to elucidate the species-identity of individuals and to settle species boundaries. This is especially of interest for crested gibbons, for which other characteristics as e.g. fur coloration are difficult to be applied. Although mitochondrial DNA provides a useful tool to study crested gibbon evolution and to identify individuals, also nuclear markers will be required to fully understand the evolutionary history of this enigmatic primate group.

3.5 Acknowledgement

We are grateful to the staff of the protected areas in which field surveys were conducted, to local people in Vietnam, Laos and Cambodia who not only provided support in the administrative procedures but also took part in the field surveys and to respective authorities for the permits. Many thanks go also to the staff of the museums and collections in Hanoi, Xuan Mai and Washington for providing valuable samples. As always, Christiane Schwarz is thanked for her excellent laboratory work. The research was conducted according to respective

national and international laws. This project was financially supported by the German Primate Center and the Biodiversitäts-Pakt of the Wissenschaftsgemeinschaft Gottfried-Wilhelm Leibniz. The authors also wish to thank the United States Fish and Wildlife Service and the Great Apes Conservation Fund (98210-7-G182) for funding parts of this work.

4 Taxon-specific vocal characteristics of crested gibbons (*Nomascus* spp.)

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Abstract

We studied the vocal diversity of various wild crested gibbon populations to assess their taxonomic classification and to elucidate the distribution of taxa as well as their phylogenetic relationships. We recorded gibbon songs within 12 protected areas. 52 recorded groups were selected for analyses including 235 female great call phrases and 254 male multi-modulated phrases. Based on general acoustic features we were able to distinguish *N. nasutus* (Trung Khanh) and *N. concolor* (Muong La and Che Tao) from each other and from all other populations. The southern taxa were difficult to distinguish. Therefore, we performed discriminant function analyses, which could provide additional resolution. The results showed that *N. leucogenys* (Xuan Lien) and *N. siki* (Phong Nha) were difficult to be separated, even with discriminant function analyses. In contrast, *N.* sp. populations (Da Krong, Phong Dien, Bach Ma and Chu Mom Ray) can clearly be discriminated from the Xuan Lien and Phong Nha populations as well as from *N. gabriellae* (Phnom Prich, Ta Dung and Bi Doup-Nui Ba). In general, the study revealed that acoustic analysis could help to distinguish between crested gibbon populations and to confirm and verify phylogenetic relationships.

Key words: *Nomascus*, crested gibbons, taxonomy, distribution, vocalization, discriminant function analysis

4.1 Introduction

Gibbons or lesser apes, family Hylobatidae, are distributed over wide ranges of South- and Southeast-Asia. They are well known for their duet songs and their monogamous social system (Geissmann et al. 2000). In early classifications, the family was divided into two genera, with one, *Symphalangus* including solely the siamang, and the other, *Hylobates* all the remaining species (e.g. Napier and Napier 1967). However, chromosomal studies have shown that gibbons can be split into four major groups (*Nomascus, Symphalangus, Hylobates, Hoolock*), with all of them possessing a different diploid chromosome number (Prouty et al. 1983). Mitochondrial sequence data supported this division and proposed the classification of these four lineages as separate genera (Roos and Geissmann 2001; Takacs et al. 2005). Both, *Symphalangus* and *Hoolock*, include only one species, but *Hylobates* and *Nomascus* are polytypic (Groves 2001).

Especially for crested gibbons, which are endemic to the Indochinese bioregion (Vietnam, Laos, Cambodia, southern China), the number of taxa to be recognized, their phylogenetic relationships and their distribution areas are highly disputed. In early studies, all crested gibbon taxa were listed as subspecies of the single species *N. concolor* (Napier and Napier 1967). Later on, some of them were elevated to species level (e.g. Geissmann et al. 2000; Groves 2001; Roos 2004).

In the most recent classification, *N. nasutus, N. hainanus, N. concolor, N. leucogenys* and *N. gabriellae* are recognized as distinct species, and the subspecies of *N. concolor* were synonymised with the nominate form (Roos et al. 2007). Mitochondrial data have also shown that individuals morphologically classified as *N. siki* cluster either with *N. leucogenys* or *N. gabriellae* (Roos 2004). However, karyotyped *N. siki* specimens, which show the typical chromosomal rearrangements for *N. siki* (Couturier and Lernould 1991), form a sister lineage to *N. leucogenys* and do not cluster with *N. gabriellae* (Roos et al. 2007). Thus, the classification of *N. siki* individuals remains uncertain, but a division of *N. siki* into two species as supported by genetic and acoustic data might be appropriate (Konrad 2004; Konrad and Geissmann 2006). In the following, we will divide *N. siki* provisionally into a southern (*N.*sp.) and a northern (*N. siki*) species.

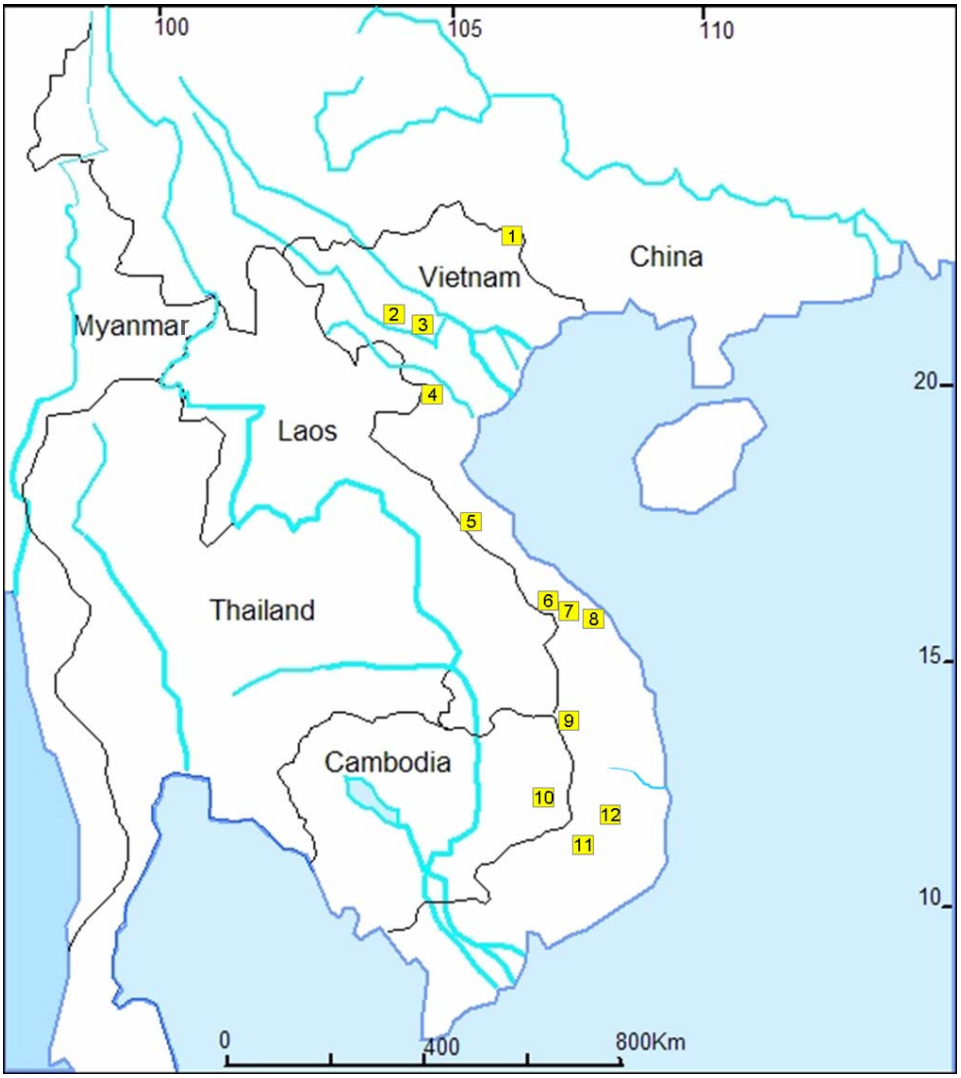

Figure 4.1: Crested gibbon populations for which acoustic data were collected (for location numbers see Table 4.1).

To elucidate the taxon-identity of crested gibbons and to settle their distribution areas, various methods were applied. Due to similar inter-specific fur colouration, this characteristic is inappropriate to distinguish taxa. However, other methods as karyotyping (Couturier and Lernould 1991), mitochondrial DNA sequencing (Roos 2004; Monda et al. 2007; Roos et al. 2007) or acoustic analyses (Haimoff 1983, 1984; Geissmann 1993, 2002a,b; Konrad 2004; Konrad and Geissmann 2006) were successfully applied. In practise, karyotyping is problematic, because fresh blood or tissue samples are required, which are difficult to obtain from free-ranging animals. However, the PCR-based confirmation of chromosomal rearrangements using DNA extracted from faeces or other low-quality material might be a promising alternative (Carbone et al. 2009).

Besides genetic methods, acoustic studies could be a powerful tool to clarify the taxon-identity of gibbons and to describe phylogenetic relationships among taxa. Especially gibbons produce songs with an innate and stereotyped pattern (Groves 1972, 2001; Marshall and Marshall 1976; Haimoff 1984; Schilling 1984; Geissmann 1993, 1995, 2002a, 2003; Geissmann et al. 2000). In addition, the clear, elaborate and loud characteristics of their songs make it easy to record them in the wild.

In order to characterise the vocal diversity of crested gibbon populations and to further elucidate the distribution of taxa and their phylogenetic relationships, we collected songs from 12 populations representing six crested gibbon species. Vocal characteristics were analysed by qualitative and quantitative measurements and tested by discriminant function analyses.

4.2 Materials and methods

4.2.1 Survey locations and data collection

To collect song samples from wild gibbons, field surveys were conducted in 12 protected areas in 2007 and 2008 (Table 4.1, Figure 4.1). Our major aim was to obtain data from all taxa, instead of a dense sampling from only one or a few taxa. Accordingly, we collected acoustic samples from *N. nasutus, N. concolor, N. leucogenys, N. siki, N.* sp. and *N. gabriellae*. The only species missing in our analysis was *N. hainanus*, the crested gibbon species endemic to Hainan Island, China.

Vocalization was recorded in the early morning using a “listening post” approach based on the method described by Brockelman and Ali (1987). When hearing calls, the time, direction and group composition was recorded with compass bearings on angle. With this information, it was possible to distinguish calls from different groups. Group positions were depicted on a map to enable changes in listening posts and to ensure the best coverage to obtain all groups in the observation area. When doubtful, whether the same or a nearby group was recorded, the data were excluded from further analysis.

Vocalizations were recorded with a digital solid state recorder MARANTZ PMD 660; (Marantz, Japan; sampling rate: 44.1 kHz, 16 bit amplitude resolution) and a Sennheiser directional microphone (K6 power module and ME66 recording head with MZW66 pro windscreen; Sennheiser, Wedemark, Germany).

Table 4.1: Taxa, collection sites and number of analysed groups and calls

No.*	Location	Long. (N)	Lat. (E)	Taxon	Time	Analysed groups	Great calls	Male calls
1	Trung Khanh**	22° 51’	106° 42’	*N. nasutus*	09/2007	5	13	26
2	Che Tao**	21° 42’	104° 06’	*N. concolor*	07/2007	2	9	14
3	Muong La**	21° 35’	104° 16’	*N. concolor*	10/2008	4	8	12
4	Xuan Lien**	19° 57’	105° 00’	*N. leucogenys*	06/2007	4	14	17
5	Phong Nha**	17° 29’	106° 21’	*N. siki*	08/2007	5	25	34
6	Da Krong**	16° 24’	107° 05’	*N.* sp.	10/2007	5	24	13
7	Phong Dien**	16° 24’	107° 10’	*N.* sp.	10/2007	4	19	18
8	Bach Ma**	16° 12’	107° 44’	*N.* sp.	11/2007	5	23	24
9	Chu Mom Ray**	14° 25’	107° 42’	*N.* sp.	11/2007	8	53	33
10	Phnom Prich***	12° 44’	107° 02’	*N. gabriellae*	12/2008	3	17	24
11	Ta Dung**	11° 52’	107° 57’	*N. gabriellae*	11/2008	2	11	19
12	Bi Doup-Nui Ba**	12° 11’	108° 41’	*N. gabriellae*	12/2007	5	19	20
	Total					**52**	**235**	**254**

* Location numbers refer to those shown in Figure 4.1; ** Vietnam; *** Cambodia.

4.2.2 Acoustic analysis

For the analysis, 52 group samples consisting of 235 female and 254 male calls from 12 different populations were collected. Crested gibbon songs consist of phrases from both sexes. Males produce multi-modulated and females so-called great call phrases (see Figure 4.2).

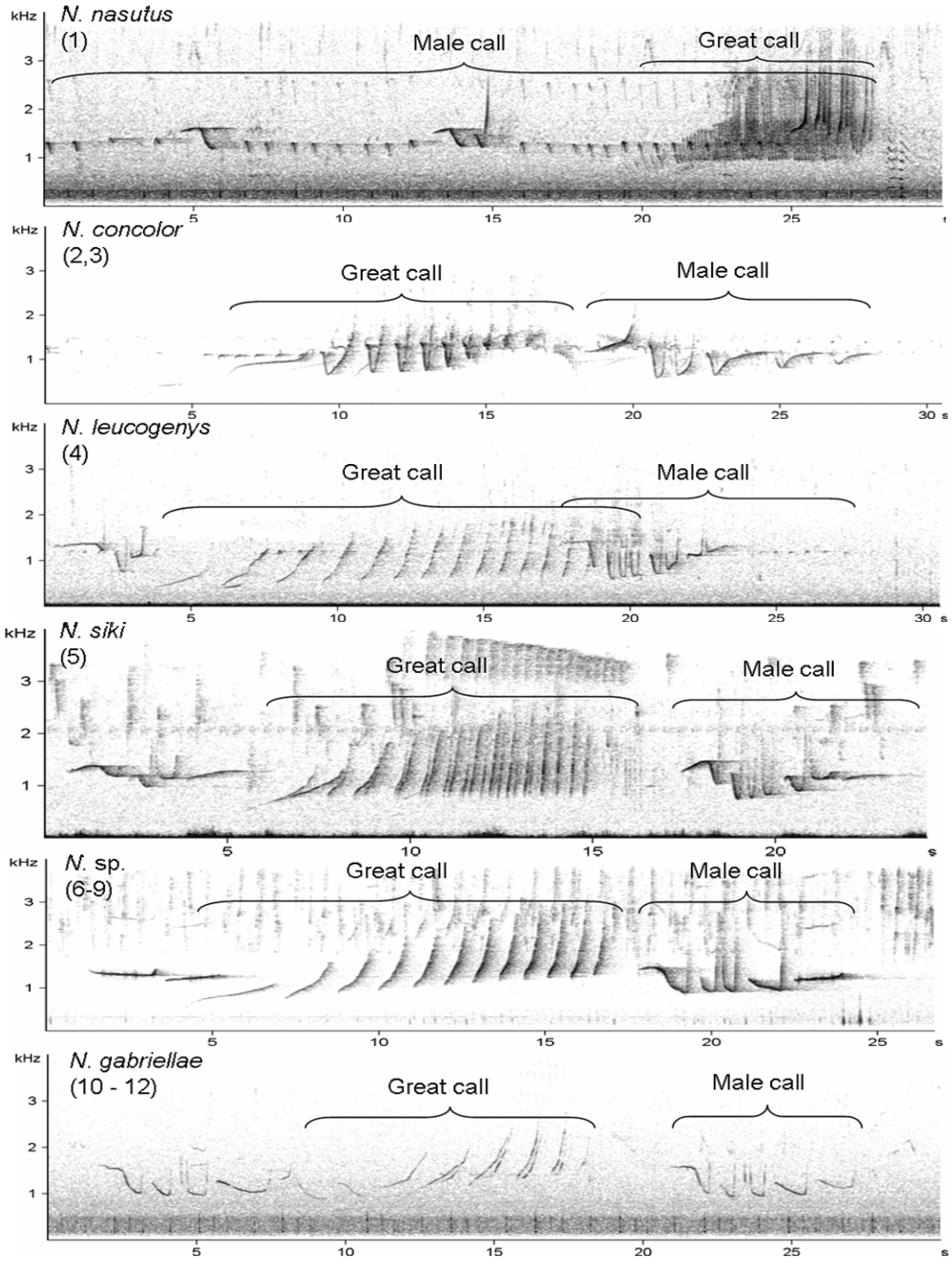

Figure 4.2: Spectrograms of six crested gibbon taxa. Numbers in brackets refer to population numbers shown in Figure 4.1 and Table 4.1.

Table 4.2: Qualitative criteria to describe crested gibbon taxa.

Taxa	Male call	Great call	Assigned populations
N. nasutus	Booms absent. Trough part of first note missing in sweep up frequency. No roll spears and initial part of second note start with short sweep up before sweeping down, then rapid changes of frequency modulation up to the last note. Repeated staccato notes with short and rapid up-down sweeps. Multi-modulated phrase immediately after first few notes of the great call.	8-12 notes and except the first 2-3 very rapid vibrato sounds. All fundamental frequencies < 2.8 kHz. Great call elements sweep up-down as spiral spring.	Trung Khanh
N. concolor	Single booms during inflation of throat sac, staccato phrases and multi-modulated phrases. First note start at high frequency (>1 kHz) and is of ascending, followed by notes with fast up-down modulation.	9-14 notes and except the first, ascending frequency only. From second note fast down-up modulation.	Che Tao Muong La
N. gabriellae - *N.* sp. - *N. siki* - *N. leucogenys*	1a: Booms during inflation of throat sac. 1b: Booms absent during inflation of throat sac. 2a: Stable frequency at the beginning with fast down-up sweep at the end. 2b: Starts at low frequency then increasing with a fast down-up-sweep at the end. 2c: Starts low and holds to the end with stable frequency. 3a: Staccato regular. 3b: Staccato not regular. 3c: Staccato rare. 4a: Modulation of rolls very fast. 4b: Modulation of rolls fast. 4c: Modulation of rolls slow. 5a: Rolls on second and third note. 5b: Rolls only on second note. 5c: Rolls absent on second note.	6a: Series of 9-19 notes and Oo notes <4. 6b: Series of 8-15 notes. 6c: Series of 6-12 notes. 7a: Start frequency of notes low (<=600Hz). 7b: Start frequency of notes high (>600Hz). 8a: Start frequency across all notes constant. 8b: Start frequency across all notes ascending.	Xuan Lien (1a, 2a, 3a, 4c, 5a, 6a, 7a, 8a) Phong Nha (1ab, 2ab, 3b, 4c, 5b, 6b, 7a, 8a) Da Krong (1ab, 2ab, 3b, 4c, 5b, 6b, 7a, 8b) Phong Dien (1ab, 2c, 3c, 4c, 5b, 6b, 7b, 8ab) Bach Ma (1b, 2a, 3c, 4b, 5b, 6b, 7b, 8b) Chu Mom Ray (1b, 2a, 3c, 4b, 5b, 5c, 6c, 7b, 8b) Phnom Prich (1ab, 2b, 3c, 4a, 5bc, 6c, 7b, 8b) Ta Dung (1b, 2b, 3c, 4a, 5c, 6c, 7b, 8b) Bi Doup-Nui Ba (1b, 2a, 3c, 4a, 5c, 6c, 7b, 8b)

We considered male phrases as fully developed if they consisted of two or more notes. Female phrases were considered as fully developed if they consisted of six or more notes. The criteria we used to describe the general differences in song structure are listed in Table 4.2.

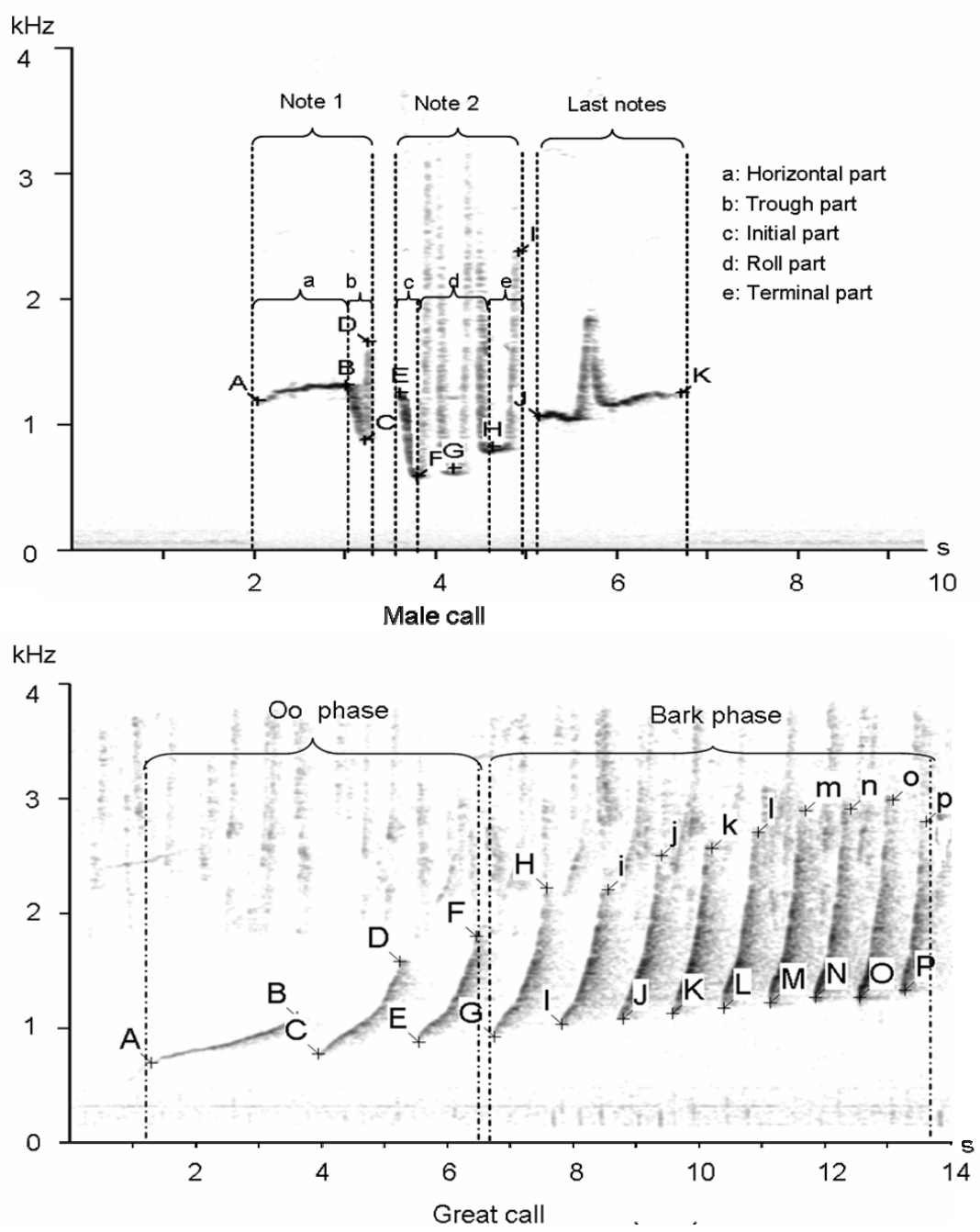

Figure 4.3: Spectrogram describing acoustic parameter estimation. Letters mark points used to calculate acoustic parameter (see also Table 4.3).

For subtle acoustic analysis we used AVISOFT SASLAB Pro (R. Specht, Berlin, Germany) to generate spectrograms and to calculate acoustic parameters. To find the point with maximum energy in the frequency spectrum we used the free reticule cursor tools of AVISOFT (frequency range: up to 500 kHz, frequency resolution: app. 8 Hz, time resolution: 16 ms). In total, we come up with 53 acoustic parameters describing the temporal and frequency structure of male and

female gibbon phrases (Table 4.3). Descriptions how we measured the acoustic parameters are given in Figure 4.3.

Table 4.3: Explanations of acoustic parameters used in the acoustic analysis (DFA). Abbreviations A-P mark the points used to calculate acoustic parameters (see Figure 4.3).

No.	Acoustic parameters	Description
	Male call	
1	Duration of entire male phrase [s]	Time at (J – A)
2	Duration first note [s]	Time at (D – A)
3	Relative duration of first notes [%]	No. 2 in % of No. 1
4	Duration horizontal part [s]	Time at (B – A)
5	Relative duration horizontal part [%]	No. 4 in % of No. 2
6	Duration trough part [s]	Time at (D – B)
7	Relative duration trough part [%]	No. 6 in % of No. 2
8	Start frequency [Hz]	Frequency at A
9	Maximum frequency horizontal part (Hz)	Frequency at B/A
10	Minimum frequency [Hz]	Frequency at C or E or G
11	Frequency range [Hz]	Frequency at (A – E)
12	Duration of second note [s]	Time at (H – F)
13	Relative duration of second notes [%]	No. 12 in % of No. 1
14	Duration initial part [s]	Time at (F – E)
15	Relative duration initial part [%]	No.14 in % of No. 12
16	Duration roll part [s]	Time at (G - F)
17	Relative duration roll part [%]	No. 16 in % of No. 12
18	Duration terminal part [s]	Time at (H – G)
19	Relative duration terminal part [%]	No. 18 in % of No. 12
20	Start frequency of second note [Hz]	Frequency at E
21	Maximum frequency [Hz]	Frequency at E or F or G
22	Minimum frequency [Hz]	Frequency at E or H or G
23	Frequency range [Hz]	No. 21 – No. 22
24	Minimum frequency initial part [Hz]	Frequency at F
25	Frequency range initial part [Hz]	Frequency at (E – F)
26	Frequency range of trough roll part [Hz]	Frequency at (G – F)
27	Frequency range last trough roll part [Hz]	Frequency at (I - G)
28	Minimum frequency terminal part [Hz]	Frequency at G
29	Duration of the last notes [s]	Time at (J - I)
30	Relative duration of last notes [%]	No. 29 in % of No. 1
	Great call	
1	Duration of entire great call [s]	Time at (p – A)
2	Number of notes	Total number of elements
3	Range of start frequencies [Hz]	Frequency at (P – A)
4	Number of Oo notes	Elements with frequency increase of <=1kHz/s
5	Duration of Oo phrase [s]	Time at (F- A)
6	Relative duration of Oo phrase [%]	No. 5 in % of No. 1
7	Number of bark notes	Elements with frequency increase of >1kHz/s
8	Duration of bark phrase [s]	Time at (p – G)
9	Relative duration of bark phrase [%]	No.8 in % of No.1
10	Duration of first note of Oo phrase [s]	Time at (B – A)
11	Duration of second note of Oo phrase [s]	Time at (D – C)
12	Duration of first note of bark phrase [s]	Time at (F – E)
13	Duration of last note of bark phrase [s]	Time at (p – P)
14	Frequency range of first note of Oo phrase [Hz]	Frequency at (B – A)
15	Frequency range of second note of Oo phrase [Hz]	Frequency at (D – C)
16	Frequency range of third note of Oo phrase [Hz]	Frequency at (F – E)
17	Frequency range of first note of bark phrase [Hz]	Frequency at (H – G)

No.	Acoustic parameters	Description
18	First inter-note interval of Oo phrase [s]	Time at (C – B)
19	Second inter-note interval of Oo phrase [s]	Time at (E – D)
20	Last inter-note interval of bark phrase [s]	Time at (P – o)
21	First start freq range between second and first note of Oo phrase [Hz]	Frequency at (C – A)
22	Second start freq range between first note of bark and last note of Oo [Hz]	Frequency at (G – E)
23	First start freq range between last and previous note of bark phrase [Hz]	Frequency at F (G - H)

4.2.3 Statistical analysis

We conducted a discriminant function analysis (DFA) to test whether the songs of the nine populations, which could not be clearly separated by general acoustic description, could be assigned correctly. This was the case for *N. leucogenys, N. siki, N.* sp. and *N. gabriellae* populations (4-12 in Figure 4.1, Table 4.1). Therefore, we used a subject of 205 songs from 41 different groups. We applied a stepwise DFA (SPSS 16) with all 53 acoustic parameters. The selection criterion for an acoustic parameter was p=0.05 to be entered and p=0.1 to be removed from the analysis. The assignment of songs to the different populations was cross-validated by the leaving-one-out method (Jacqueline and Willem 2003), 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 Results

4.3.1 General difference in song structure of *Nomascus*

The population of *N. nasutus* in Trung Khanh and the populations of *N. concolor* in Muong La and Che Tao could be clearly indentified by the general acoustic characteristics of their songs (Figure 4.2, Table 4.2). *N. leucogenys*, *N. siki* and *N.* sp. had very similar song structures. *N. gabriellae* showed only minor differences to them (Figure 4.2).

N. nasutus females produce fast up-down sweeps like a spiral spring, with a vibrato sound on first two notes. Males produce staccato sounds during, before and after their multi-modulated phrases. All male notes start with almost unmodulated frequency, followed by a down sweep and a fast up sweep. Males of *N. concolor* produce their multi-modulated phrase immediately after the climax of the female great call. The first note of male calls has a slightly ascending

characteristic, followed by notes with fast down-up modulation (Table 4.2, Figure 4.2). *N. leucogenys, N. siki, N.* sp. and *N. gabriellae* are difficult to distinguish by pure listening. However, males of *N. leucogenys* give regularly, loud staccato sounds, which appear rarely in *N. siki* and *N.* sp., and which were nearly absent in *N. gabriellae*. *N. leucogenys* can be better distinguished *from N. siki, N.* sp. *and N. gabriellae* by their great calls, which had a longer duration and a faster frequency modulation (Table 4.2, Figure 4.2).

4.3.2 Discriminant function analyses of crested gibbon songs

We conducted a discriminant function analysis (DFA) of the nine populations which could not be satisfyingly distinguished by general acoustic descriptions. The DFA was able to assign correctly 92.7 % of the 205 songs to the nine populations (Figure 4.4). The accuracy of assignment ranged from 66.7% for Phnom Prich to 80.0% for Da Krong and Bach Ma populations to 100% for all other populations. The cross-validation achieved 80.5% of correct assignment. Four populations, Xuan Lien, Chu Mom Ray, Ta Dung and Bi Doup-Nui Ba, remained at 100%. The populations Da Krong and Phnom Prich remained at 80.0% and 66.7%, respectively. Two other populations showed a slight decrease in the assignment accuracy (Phong Nha from 100% to 60.0%, Phong Dien from 100% to 50.0%). Misclassifications occurred only between neighbouring populations.

The DFA selected eight acoustic parameters to assign the songs to the respective populations. The eight acoustic parameters comprised characteristics from males and female calls. The scattergram (Figure 4.4) showed the separation of the nine gibbon population according to the first and second discriminant functions, explaining 63.7 % and 15.7 % of the total variation. The first discriminant function, which mainly represents frequency characteristic of gibbon songs, separates the Xuan Lien and Phong Nha populations from Da Krong, Phong Dien, Bach Ma, Chu Mom Ray, and both from Phnom Prich, Ta Dung and Bi Doup-Nui Ba. The second discriminant function, which represents temporal features of gibbon songs, separates Da Krong, Phong Dien, Phong Dien, Bach Ma and Chu Mom Ray from all other populations.

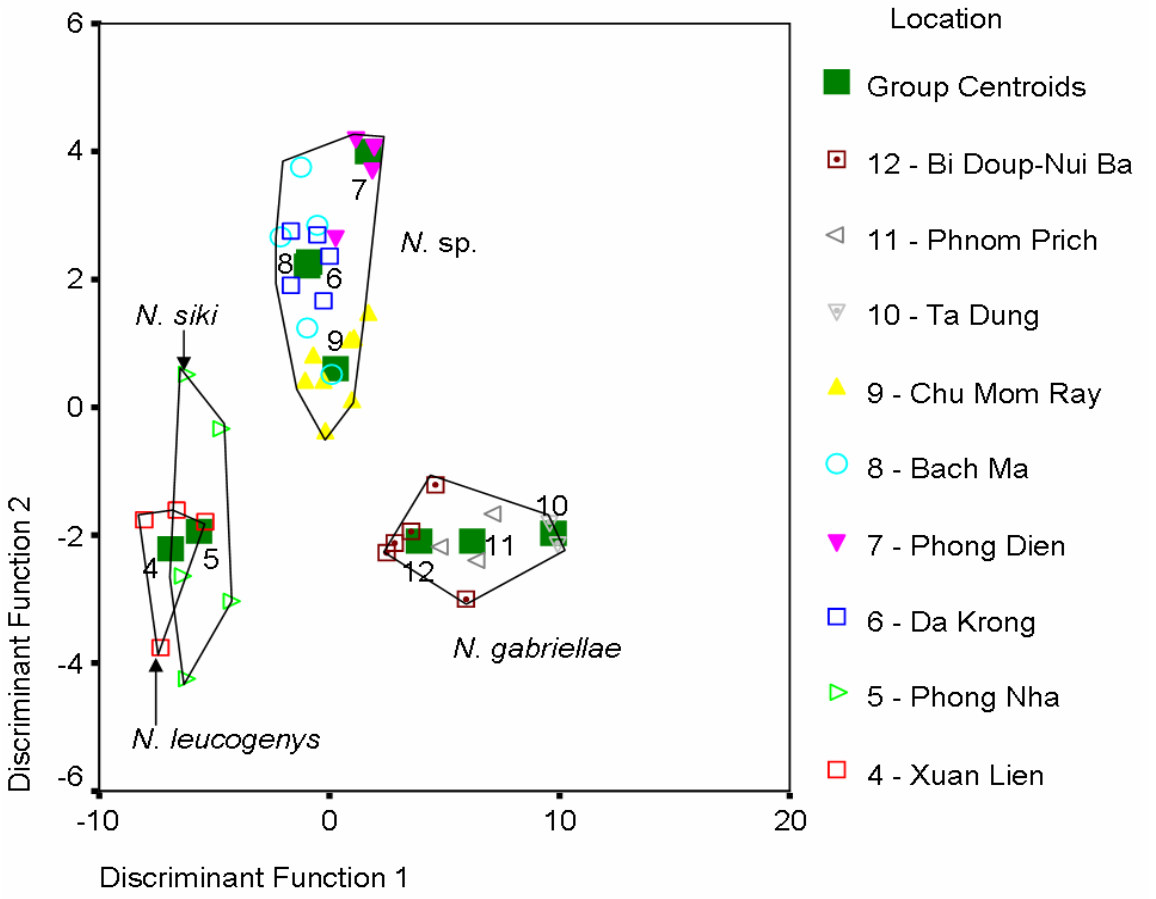

Figure 4.4: Distribution of the different gibbon populations based on the scores of the first and second discriminant function.

4.4 Discussion

Based on morphological and acoustic characteristics, and on genetic data, three (Groves 1997), four (Geissmann 1995, 2002a,b; Geissmann et al. 2000) and recently even five species (Groves 2001) have been identified in the genus *Nomascus*. The system of four species was also the subject of a phylogenetic analysis using vocal, fur colouration, and anatomical data, of which the vocal data produced the most reliable and best resolved tree (Geissmann 2002a). In principal, these results confirm molecular results (Roos 2004; Takacs et al. 2005; Roos et al. 2007). Disputed is the classification of *N. siki.* The taxon was variously classified as subspecies of either *N. leucogenys* or *N. gabriella*e (Geissmann 1995; Geissmann et al. 2000), but Groves (2001) proposed to classify the taxon as distinct species. Roos (2004) showed that *N. siki* representatives are paraphyletic, with some forming a clade together with *N. leucogenys* and others with *N.*

gabriellae. However, karyotyped *N. siki* specimens, which show the typical chromosomal rearrangements for *N. siki* (Counturier and Lernould 1991), form a sister lineage to *N. leucogenys* and do not cluster with *N. gabriellae* (Roos et al. 2007).

In general, the acoustic analysis could confirm the relationships depicted by genetic data. Accordingly, we found a clear distinguishable song structure in *N. nasutus* and *N. concolor*, which separates them from each other and form the remaining populations. The difference in the song structure of the other four species, *N. leucogenys*, *N. siki*, *N.* sp. and *N. gabriellae,* is not well developed. Insofar, it was not possible to separate these taxa on general acoustic descriptions, indicating a close relationship between them, as it was also shown by molecular studies (Garza and Woodruff 1992; Roos 2003, 2004; Monda et al. 2007; Roos et al. 2007). The quantitative acoustic analysis revealed three distinctive clusters, with one including *N. leucogenys* and *N. siki* , one with *N.* sp., and finally one with *N. gabriellae*. Thus, the analysis showed that *N. siki* might be indeed paraphyletic, which supports genetic studies by Roos (2004). To further elucidate the taxonomic status of various crested gibbon populations and specifically to clarify the exact distribution zones of the southern species, further investigations are needed.

4.5 Conclusion

1. Populations of *N. nasutus* (Trung Khanh) and *N. concolor* (Muong La and Che Tao) can clearly be differentiated in their song structure from each one another and from all other populations.

2. *N. leucogenys* in Xuan Lien and *N. siki* in Phong Nha have a similar acoustic structure and, therefore, they form one acoustic cluster together.

3. *N.* sp. populations from Da Krong, Phong Dien, Bach Ma and Chu Mom Ray are highly correlated in stepwise discriminant function analyses and can be clearly separated from *N. gabriellae, N. leucogenys* and *N. siki.*

4. *N. gabriellae* populations (Phnom Prich, Ta Dung and Bi Doup-Nui Ba) are highly correlated and differ in their song pattern from all other taxa.

5. The acoustic results are in agreement with genetic data and hence, show that subtle acoustic analysis could help to confirm and verify phylogenetic relationships.

4.6 Acknowledgement

We are very grateful to the staff of the protected areas, in which field surveys were conducted and to local people in Vietnam, Laos and Cambodia who not only provided support in the administrative procedures but also took part in the field surveys. This study was conducted as PhD project in the frame of the WGL biodiversity network at the German Primate Center.

5 Concordance between vocal and genetic diversity in crested gibbons

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Abstract

The taxonomic classification of crested gibbons (*Nomascus*) has experienced drastic revision in the last decades. Based on recent molecular data, the genus comprises seven species, however, the exact geographical distribution of some taxa remains unclear. In general, song structure is an important trait to confirm phylogenetic relationships. Nevertheless, the four southern species revealed only subtle differences in their songs. Until now it is unclear whether these small differences are immanent enough to use them as an additional taxonomic tool. To further illuminate the phylogenetic relationships among crested gibbons and to settle distribution areas we conducted a survey in the Indochinese bioregion, recording 92 gibbon groups at 24 locations. In total we collected 440 great calls and 447 multi-modulated male calls. The acoustic analysis could distinguish all studied species. Moreover, for the four southern species a highly significant correlation between song structure, geographic distance and genetic similarity was detected. Accordingly, gibbon groups can be assigned to their respective species based on their song structures. The results showed that subtle acoustic analyses, including male and female song features, are helpful to verify taxonomic boundaries and unravel their geographic distribution. Thus, acoustic analyses are also an important tool for conservation purposes.

Key words: *Nomascus*, gibbon songs, vocalization, taxonomy, genetics, geographic distance.

5.1 Introduction

Gibbons are famous for their prominent over long distances audible songs. In most gibbon species both, males and females, sing together (Groves 1972; Chivers 1977; Haimoff at al. 1982; Geissmann et al. 2000). In other species both gender also sing together but in addition males make solo songs. Until now only two species, the Kloss's gibbon (*Hylobates klossii*) and the silvery gibbon (*H. moloch*) are known, where females and males produce only solo songs (Marshall and Marshall 1976; Geissmann 1993, 2002a; Geissmann and Nijman 2006). The structure of gibbon songs, the concentration of energy in single frequency band and slow frequency modulated call elements, showed clear adaptation for improved long-distance transmission (Schneider et al. 2008). In addition, the frequency of song syllable lies in a transmission optimized range. With these features gibbon songs differ from all vocalisations of other nonhuman primates, resembling more songs of typical rainforest birds. It is notable that they are also similar in their proposed functions, like territory advertisement, mate attraction, and strengthening pair bonds (Mitani 1984, 1985, 1987; Raemaekers and Raemaekers 1985; Cowlishaw 1992; Geissmann 1999; Geissmann and Orgeldinger 2000). A

further striking feature of their songs is the fact that they are species-specific and innate. Hence, gibbon songs became a promising tool to identify the taxon affiliation of gibbon individuals and to describe evolutionary relationships among taxa (Haimoff 1983; Haimoff et al. 1984; Marshall et al. 1984; Geissmann 1993, 2002a,b; Geissmann et al. 2000; Konrad and Geissmann 2006; Thinh et al. in press).

Crested gibbons, genus *Nomascus*, are endemic to the region east of the Mekong river and only the West Yunnan black crested gibbon (*Nomascus concolor furvogaster*) crossed the upper Mekong to the west (Figure 5.1). Crested gibbons show a strong sexual dichromatism with orange or yellow coloured females, and black males, which in some taxa have light cheeks. The crown hair in males is erected, which gave them their common name “crested gibbons”. Traditionally, crested gibbons were combined in the single species *N. concolor* (Napier and Napier 1967; Groves 1972; Chivers 1977; Haimoff et al. 1982), but recent investigations based on morphological, genetic and acoustic data split them into four or even six species (Groves 2001; Mootnick 2006; Geissmann 2007; Monda et al. 2007; Roos et al. 2007; Thinh et al. 2010). However, the taxonomic classification of crested gibbon taxa is far from being resolved and even the number of taxa to be recognised is disputed. Recently, Konrad and Geissmann (2006) proposed an additional, so far undescribed taxon, in the range of the Southern white-cheeked gibbon (*N. siki*) based on acoustic data, a finding, which is also supported by genetic data (Thinh et al. accepted). Due to this taxonomic uncertainty, we follow the most recent classification with a total of the six crested gibbon species, Hainan gibbon (*N. hainanus*), Cao-vit crested gibbon (*N. nasutus*), Black crested gibbon (*N. concolor*), Northern white-cheeked gibbon (*N. leucogenys*), Southern white-cheeked gibbon (*N. siki*) and Red-cheeked gibbon (*N. gabriellae*) (Thinh et al. 2010), while we additionally divide *N. siki* into a northern (*N. siki*) and a southern (*N.* sp.) species.

Knowledge about the number of crested gibbon taxa, their distribution areas and their evolutionary relationships is not only of biological interest per se, but also of great conservation importance. All crested gibbons are classified as “Endangered” or even “Critically Endangered” (IUCN 2009). With only 20 individuals left in its native habitat, the Hainan gibbon (*N. hainanus*) is the rarest primate of the world (Mittermeier et al. 2007; Cunningham and Mootnick 2009),

and with approximately 100 remaining individuals, the situation for the Cao-vit crested gibbon (*N. nasutus*) is similar alarming (IUCN 2009; Mittermeier et al. 2009). Reasons for the dramatic decline of gibbons are manifold, but habitat loss due to forest clearance for agricultural use, rubber or oil palm plantations, gold mining, or timber and charcoal production, as well as illegal hunting for sport or food, and the trade for medicine or pets are major threats to wild gibbon populations (Geissmann et al. 2000; Geissmann 2007).

With the aim to further elucidate the phylogenetic relationships among crested gibbon species and to test whether genetic and acoustic data reveal concordant results, we collected more than 400 male and female songs from 92 groups at 24 locations in Vietnam, Laos and Cambodia. In contrast to clear differences in song structure between *N. nasutus, N. concolor* and the four southern species (*N. leucogenys*, *N. siki, N.* sp., *N. gabriellae*), the latter four show a very similar song pattern, which we resolved by subtle acoustic analyses.

5.2 Materials and methods

5.2.1 Survey locations and data collection

In 2007 and 2008, we conducted field surveys in 24 protected areas in Vietnam, Laos and Cambodia (Figure 5.1, Appendix C.1), and recorded songs from *N. nasutus, N. concolor, N. leucogenys, N. siki, N.* sp. and *N. gabriellae*. Recordings from *N. hainanus* were not available for our study, but its song structure differs clearly from all other species (Geissmann et al. 2000; Geissmann 2002a). Vocalizations were recorded in the early morning using a “listening post” approach based on the method described by Brockelman and Ali (1987). When hearing calls, the time, direction and group composition was recorded with compass bearings on angle. With this information, it was possible to distinguish calls from different groups. Group positions were depicted on a map to enable changes in listening posts and to ensure the best coverage in obtaining different groups in the observation area. When doubtful, whether the same or a nearby group was recorded, the data were excluded from further analysis. In total we collected 440 great calls and 447 male calls from 92 different gibbon groups at 24 locations. To record songs, a digital solid state recorder MARANTZ PMD 660; (Marantz, Japan; sampling rate: 44.1 kHz, 16 bit amplitude resolution) and a

Sennheiser directional microphone (K6 power module and ME66 recording head with MZW66 pro windscreen; Sennheiser, Wedemark, Germany) was used.

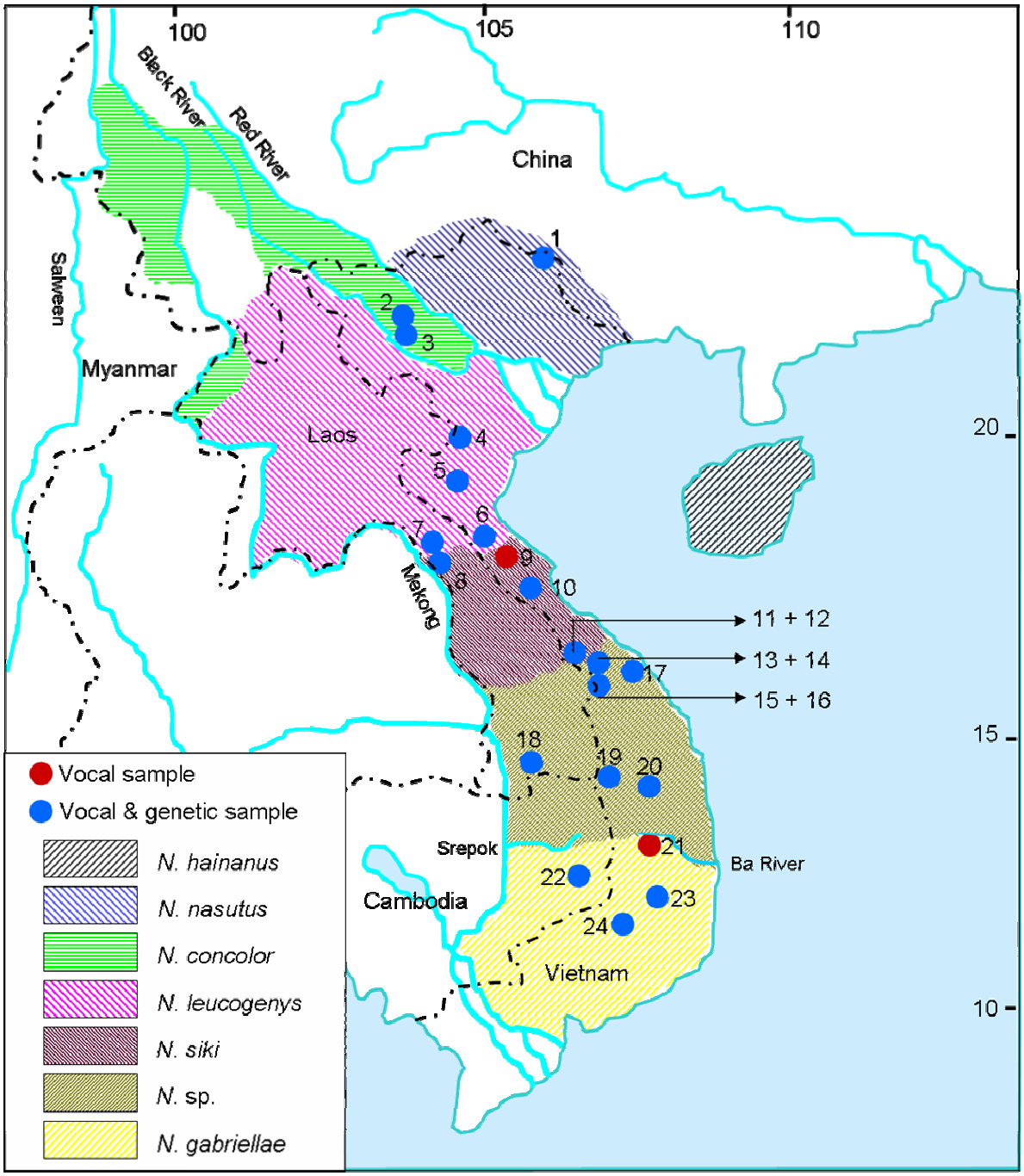


Figure 5.1: Geographic distribution of crested gibbons according to Thinh et al. submitted. Numbers refer to study populations. For detailed description of recording sites see Appendix C.1.

5.2.2 Acoustic analysis

Crested gibbon songs consist of phrases from both sexes. Males produce three different phrases including boom, staccato and multi-modulated phrases and

females so-called great call phrases only (see Figure 5.2). For the analysis we considered male phrases as fully developed if they consisted of two or more notes. Female phrases were considered as fully developed if they consisted of six or more notes. The criteria we used to describe the general differences in song structure are listed in Appendix C.2.

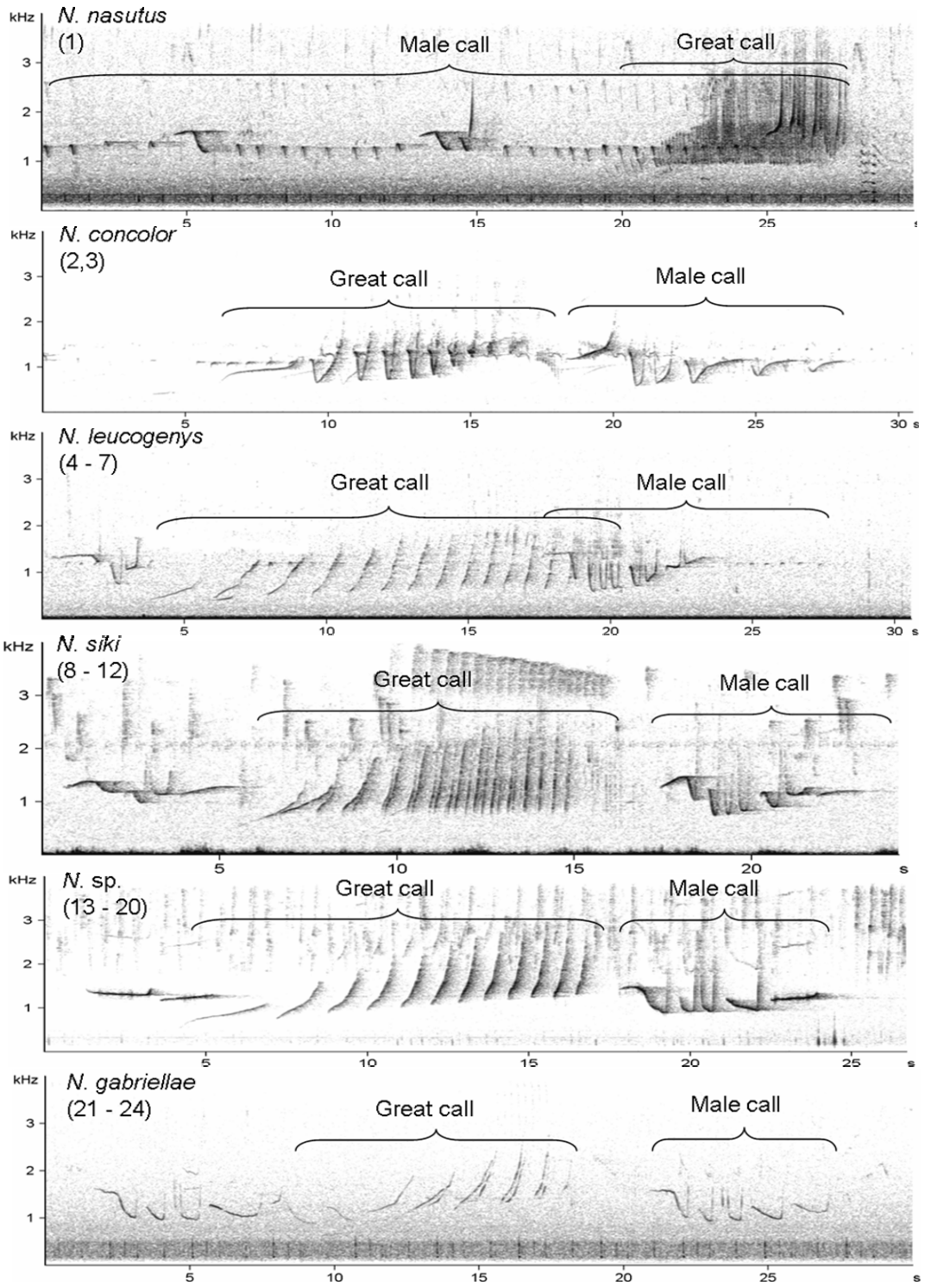


Figure 5.2: Spectrograms of six crested gibbon species. Numbers in brackets refer to population numbers shown in Figure 5.1 and Appendix C.1.

We used AVISOFT SASLAB Pro (R. Specht, Berlin, Germany) to generate spectrograms and to calculate acoustic parameters. To find the point with maximum energy at the beginning, ending and anchor points of notes in the frequency spectrum, we used the free reticule cursor tools of AVISOFT (frequency range: up to 500 kHz, frequency resolution: app. 8 Hz, time resolution: 16 ms). In total, we calculated 53 acoustic parameters describing the temporal and frequency structure of male and female gibbon phrases. A detailed description how we measured the acoustic parameters are given in Figure 4.3. A list with a detailed description of the 53 acoustic parameters is given in Table 4.3.

5.2.3 Statistical analysis

We conducted a discriminant function analysis (DFA) to test whether the four southern species, which are not separable by general acoustic description, can be assigned correctly by the calculated acoustic parameters. We calculated mean values per group using 410 great calls and 395 multi-modulated male calls. In total we analysed 81 different groups from 21 locations (location numbers 4-24, see Figure 5.1, Appendix C.1). We standardized the acoustic parameters and conducted all 53 parameters to a stepwise discriminant function analysis (DFA, SPSS 16). 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 songs to the different populations was cross-validated by the leaving-one-out method (Jacqueline and Willem 2003), 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.

In addition we carried out a hierarchical cluster analysis (CA, SPSS 16) to evaluate the similarity in the acoustic structure of the 81 groups. We calculated the z-score variables of the 13 acoustic parameters selected by the stepwise DFA. As distance measure we used the Euclidean distance and cluster method ‘between groups linkage’.

To test the statistical relationship between acoustic structure, and genetic and geographic distance matrices, we used a Mantel test (GenAlex, Peakall and Smouse 2006). The vocal distance matrices were generated using the F-values of pairwise distances in the stepwise DFA. The geographic distance matrices were calculated from the minimum distance of different groups between the 21 locations

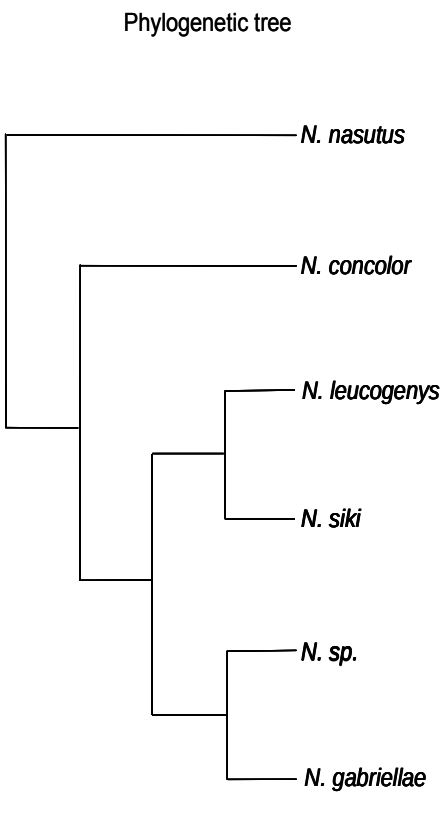
(Appendix C.1). Geographic coordinates were obtained with GPS. Genetic distances were generated using pairwise population F values between haplotypes of mitochondrial cytochrome b gene sequences by the distance function in GenAlex. Respective haplotypes were taken from GenBank (GenBank accession numbers GU321248- GU321319).

5.3 Results

5.3.1 General differences in song structure of crested gibbons

N. nasutus and *N. concolor* could be clearly identified by general acoustic characteristics of their songs (Figure 5.2, Appendix C.2). In contrast, *N. leucogenys*, *N. siki*, *N.* sp. and *N. gabriellae* had very similar song structures and only minor differences could be observed among them (Figure 5.2).

N. nasutus females produced fast up-down sweeps like a spiral spring, with a vibrato sound on first two notes. Males produced staccato sounds during, before and after their multi-modulated phrases. All male notes started with almost unmodulated frequency, followed by a down and a fast up sweep. Males of *N. concolor* produced their multi-modulated phrase immediately after the climax of the female great call. The first note of the male call had slightly ascending structure, followed by notes with fast down-up modulation (Appendix C.2, Figure 5.2). Males of *N. leucogenys* gave regularly, loud staccato sounds, which appeared rarely in *N. siki* and *N.* sp., and were nearly absent in *N. gabriellae*. *N. leucogenys* could be better distinguished from the three southern species by their great calls, which had a longer duration and a faster frequency modulation. Accordingly, only the population from Xuan Lien (location 4, Figure 5.1) could be assigned to *N. leucogenys* in all criteria, while other *N. leucogenys* populations (5-7) showed criteria which occurred also in *N. siki* and *N.* sp. *N. siki* populations (8-12) were more similar in their song structure to *N. leucogenys* than to *N*. sp. populations (13-20). The main criteria to distinguish *N. siki* and *N.* sp. songs were criteria 2 and 4 (see Appendix C.2). In contrast, we found higher concordance between *N.* sp. populations and *N. gabriellae* (21-24). Here the main criteria were 3 and 5 (Appendix C.2). Figure 5.4 gives an overview about the general acoustic differences in relation to a recent phylogenetic reconstruction.



Main qualitative criteria to describe crested gibbon taxa

	1	2	3	4	5	6	7	8
N. nasutus	absent	NA	repeated regular	NA	NA	8-12	low	ascending and descending
N. concolor	present	NA	regular	NA	NA	9-14	low	ascending and descending
N. leucogenys	sometime	stable	regular	slow	present	9-19	low	constant
N. siki	sometime	stable	not regular	slow	absent	8-15	medium	ascending and descending
N. sp.	sometime	increasing	rare	fast	sometime	8-15	medium	ascending and descending
N. gabriellae	absent	increasing	rare	very fast	sometime	6-12	high	ascending

1: Male's booms during inflation of throat sac;
2: Frequency of male call at the beginning with fast down-up sweep;
3: Staccato of male call;
4: Male call modulation of rolls;
5: Rolls on second and third note of great call
6: Great call series of notes;
7: Great call start frequency of notes;
8: Great call start frequency across all notes.

Figure 5.4: Phylogenetic relationships among crested gibbons based on cytochrome b sequences (according to Roos 2004; Roos et al. 2007 and Thinh et al. accepted) and general acoustic features of species (see Appendix C.2).

5.3.2 Subtle vocal differences between *N. leucogenys, N. siki, N.* sp. and *N. gabriellae*

As mentioned above it was not possible to distinguish populations of *N. leucogenys, N. siki, N.* sp. and *N. gabriellae* by their general acoustic song structure (see Figure 5.2). However, a stepwise DFA was able to assign 85.2% of the 81 recorded groups to its correct species. The assignment accuracy ranged from 50% for Sao La (16), 60% for Phong Nha (10) and Bach Ma (17), 75% for Phong Dien (14) and Xe Pian (18), 80% for Da Krong (13), 90% for Vu Quang (6) and 100% for the remaining 14 populations. The cross-validation achieved a classification result of 55.6%. The decline in the cross validation is mainly caused by the fact that at some locations we recorded only one or two groups. Nevertheless, 55.6% is highly significant above the change level of 4.75%.

The stepwise DFA needed 14 out of the 53 acoustic parameters to achieve this classification result. The DFA included acoustic parameters of both sexes, six parameters of the multi-modulated male call (parameters: 1, 11, 14, 19, 23, 28) and eight parameters of the female great call (parameters: 31, 33, 34, 40, 41, 43, 47, 50; for description see Table 4.3. The scattergram (Figure 5.4) showed the separation of the 21 populations according to the first and second discriminant function, explaining 54.3% and 12.8% of variation, respectively. The first discriminant function, which mainly represents frequency characteristics of gibbon songs, separated populations 4-12 from populations 13-24. The second discriminant function, which represents temporal features of gibbon songs, separates populations 21-24 from all other populations. Already the first two functions achieved a good separation of the four species with the exception of one group at Phong Nha-Ke Bang (10), which was assigned to *N. leucogenys* instead of to *N. siki*.

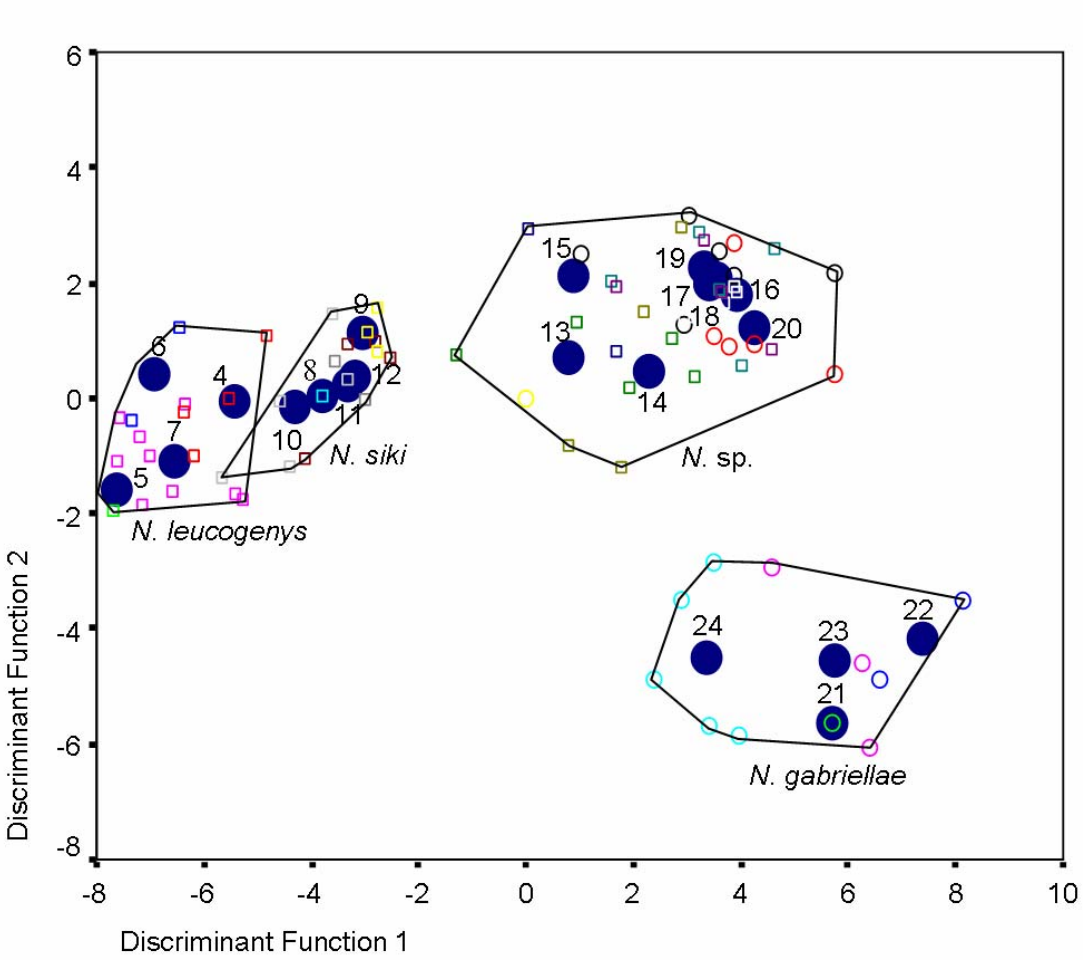


Figure 5.5: Distribution of the different gibbon populations based on the scores of the first and second discriminant function. Classification of species based on Thinh et al. accepted (4 - 7: *N. leucogenys;* 8 - 11: *N siki;* 12 - 20: *N.* sp.; 21 - 24: *N. gabriellae*). Blue circles indicate population centroids.

We conducted hierarchical cluster analysis (CA) to verify the results of the DFA and test whether a CA would come up with the same number of expected categories (i.e., species). Based on the acoustic song structure, the CA algorithm revealed a high concordance between the four species (Appendix C.3). In total, the CA could correctly classify 68 out of 81 groups (84%). In the first cluster, *N. siki* groups clustered together with groups of *N. leucogenys* (6, 9-14) interspersed by two *N.* sp. groups (33, 34). The second and third cluster comprised only of *N. leucogenys* and *N. gabriellae* groups, respectively. The fourth cluster comprised of *N.* sp. groups interspersed by three *N. gabriellae* groups (72, 76, 77).

5.3.3 Correlation between vocal structure, genetic and geographic distance

Among the 21 populations, we found a significant correlation between similarity in vocal structure of gibbon songs and geographic distance (Table 5.1). A similarly significant correlation was also detected between genetic diversity and geographic distance. To test the concordance between genetic diversity and similarity in song structure, we performed comparisons on species and population level. For the comparison among the four species, a significant positive correlation was observed. Also the comparison of the 19 populations, for which both genetic and acoustic data were available, revealed a significant correlation coefficient.

Table 5.1: Correlation between vocal similarity, genetic and geographic distance

Distance matrices compared	Populations of collected samples	Rxy	P(rxy-rand >= rxy-data)	Pairwise comparisons
Vocal vs. Geographic	21 populations (vocal)	0.672	0.01	190
Genetic vs. Geographic	19 populations (genetic)	0.723	0.01	703
Genetic vs. Vocal	19 populations (genetic and vocal)	0.503	0.01	136
Genetic vs. Vocal	4 species (genetic and vocal)	0.868	0.02	6

Note: Rxy = correlation coefficient of Mantel test. P(rxy-rand >= rxy-data) = probability of positive autocorrelation (one tailed).

5.4 Discussion

Acoustic analysis could confirm the general relationships between song structure and phylogeny. We found clear distinguishable song structures between *N. nasutus*, *N. concolor* and the four southern species, *N. leucogenys*, *N. siki*, *N. siki* sp. and *N. gabriellae*, while the latter four revealed only subtle differences in

their songs. However, a detailed acoustic analysis was able to also discriminate between the four southern species. In addition, we found a highly significant correlation between song structure similarity and geographic distance. This relation was significantly positive correlated to their genetic relatedness, indicating a close relationship as it was shown by molecular studies (Roos 2004; Monda et al. 2007; Roos et al. 2007; Thinh et al. 2010, Thinh et al. accepted).

Since the early study of Marshall and Marshall (1976), we know that gibbon songs are an important trait of their taxonomic relationship (Brockelman and Shilling 1984, Dallmann and Geissmann 2001a, Konrad and Geissmann 2006). In many cases species could be easily distinguished by looking at the spectrograms of their songs (see Figure 5.1). However, as can be seen in the same figure, closer related taxa can have very similar song structures. Although there are some studies on individual variation in gibbon songs (Haimoff and Gittins 1985, Dallmann and Geissmann 2001b), there is no systematic study available to confirm whether individual variation or variation at group level is high enough to contradict a possible relation between song structure and genetic relatedness among closely related species. One reason for this lack of information could be the fact that the comparison of single neighbouring groups revealed unambiguous results. The few misclassifications in our study occurred only between neighbouring groups, whereas groups living far away from each other followed the rule, larger distance dissimilar song structure. It remains undecided whether gibbons have a similar system as song birds, in which neighbouring groups show a more distinct vocal repertoire as groups living in adjacent areas (Catchpole and Slater 2008). Song birds seem to use this principle of increased contrast as a tool to clarify territory boundaries. However, song birds must learn their songs (Brainard and Doupe 2002), whereas gibbons have an innate song structure. Therefore, their ability to produce more distinct songs in relation to their direct group neighbours must be limited. This could be the reason why our results could not give a concluding answer in this point. However, the high significant relation between acoustic similarity, geographic distance and genetic relatedness showed that crested gibbon songs are a salient feature of their genetic relatedness.

Due to the concordance between genetic and acoustic data, song structure is a promising tool to identify the taxon-affiliation of gibbon individuals or populations. This is of great importance because samples from free-ranging

gibbons for genetic analyses are difficult to be obtained and fur colouration, especially of female crested gibbons is, due to its extreme intra-taxon variability, unreliable (Geissmann et al. 2000).

According to our study, *N. nasutus* (Trung Khanh) and *N. concolor* (Che Tao and Muong La) clearly differ from each other and from the southern four species, which is in agreement with genetic analysis (Roos 2004; Roos et al. 2007; Thinh et al. 2010, Thinh et al. accepted). Although no acoustic data of *N. hainanus* were included in our study, genetic data support its clear separation from all other crested gibbon species (Roos et al. 2007; Thinh et al. 2010, Thinh et al. accepted). Also concordant with genetic data (Thinh et al. accepted), gibbons from Xuan Lien, Pu Huong, Vu Quang and northern Nam Kading cluster together and represent *N. leucogenys*. They are separated by DFA and CA from *N. siki* populations at Khe Ve, Phong Nha-Ke Bang, Huong Hoa and southern Nam Kading. The central populations of Da Krong, Phong Dien, Xe Sap, Sao La, Bach Ma, Chu Mom Ray, Kon Ka Kinh and Xe Pian, classified as *N.* sp. (Thinh et al. accepted), form their own cluster in DFA and CA analyses as well. The populations from A Yun Pa, Phnom Prich, Bi Dup-Nui Ba and Ta Dung cluster together in both analyses and refer to *N. gabriellae*.

While the distribution of *N. hainanus*, *N. nasutus* and *N. concolor* is well defined and limited by large rivers or islands, the exact distribution zones of the southern species remain unclear. Based on genetic (Thinh et al. accepted) and our acoustic data, the Vu Quang population represents *N. leucogenys* and not *N. siki* as previously believed (Geissmann et al. 2000). On the Laos side, the border between *N. leucogenys* and *N. siki* can be fixed to Nam Kading. The southern extend of *N. siki* in Laos is questionable, but the species could occur as far south as Dong Phou Vieng (Duckworth 2008). In contrast, on the Vietnamese side, the border between *N. siki* and *N.* sp. can be narrowed down to the region between Huong Hoa and Da Krong. Accordingly, the range of *N. siki* is dramatically reduced, at least in Vietnam. The border between *N.* sp. and *N. gabriellae* might be the Srepok and Ba rivers.

In this study, we have shown that in crested gibbons vocal diversity correlates with genetic relatedness. Accordingly, both acoustic and genetic analyses provide a reliable tool to correctly assign the taxon-affiliation of crested gibbon individuals and to settle taxon boundaries. It remains open, whether other

gibbon species have a similar tight relation between subtle acoustic structure and genetics. In addition, it could be possible that loud calls of other nonhuman primate species turn out to be a helpful tool to clarify taxonomic relations as well.

5.5 Acknowledgement

We are very grateful to the staff of the protected areas, in which field surveys were conducted and to local people in Vietnam, Laos and Cambodia who not only provided support in the administrative procedures but also took part in the field surveys. This study was conducted as PhD project in the frame of the WGL biodiversity network at the German Primate Centre. The authors also wish to thank the United States Fish and Wildlife Service, Great Apes Conservation Fund for funding parts of this work.

6 General discussion

The diversity and biology of gibbons, family Hylobatidae, has been studied during the last decades by researches from various scientific fields, which have considerably increased our understanding of these small apes. However, a large number of questions especially concerning their phylogeny, phylogeography and systematics remained unresolved. Although it is now consensus in the scientific community, that gibbons have to be divided into four major groups, it remains disputed, whether these should be classified as subgenera of the genus *Hylobates* (Geissmann 1995; Groves 2001) or as distinct genera (Roos and Geissmann 2001; Müller et al. 2003; Brandon-Jones et al. 2004; Mootnick and Groves 2005; Mootnick 2006; Geissmann 2007). Similarly, also the number of species and subspecies to be recognized is debated, and for some taxa even information about their exact distribution areas is missing. Likewise, the phylogeny and phylogeography of gibbons is less understood and needs further investigations.

Thus, this thesis was set up to contribute to a better understanding of the phylogeny, phylogeography and taxonomy of the gibbon family and especially of crested gibbons, genus *Nomascus*. The main objectives of this dissertation were (i) to reconstruct a phylogeny of gibbons including all taxa, which can be used as basis for a reliable classification and to elucidate the phylogeography of the family, (ii) to narrow down the distribution areas of crested gibbon taxa, which are, at least for some taxa, still inadequately defined, and (iii) to identify a possible correlation between acoustic and genetic differences in crested gibbons with the later aim to use both methods as tool to identify taxa. To obtain these objectives, I conducted genetic analyses by using mitochondrial cytochrome b gene sequence data. For crested gibbons, I applied a multi-dimensional approach, in which genetic and acoustic data were combined. Most importantly, for the genetic study of crested gibbons, only data from animals with clear provenance were implemented, which were obtained from samples collected in the field or from museum specimens. For the acoustic study, I recorded gibbon songs in the field and analysed call

parameters of the female great call and of the multi-modulated phrase of male loud calls.

In Chapter 2, the phylogeny and phylogeography of the gibbon family was examined. Therefore, I sequenced the mitochondrial cytochrome b gene from 85 individuals, which represent all gibbon species and even most subspecies. Accordingly, the most complete view into the evolution of gibbons is provided. The results of this study suggest that the four major gibbon lineages should be classified as distinct genera, *Hylobates, Hoolock, Symphalangus* and *Nomascus*. Within genera, the species status of all currently proposed species (IUCN 2009) is indicated. Since subspecies of *H. muelleri* diverged on a similar time scale as various other gibbon species, we also elevate *H. muelleri abbotti* and *H. muelleri funereus* to species status. With the exception of *N. concolor lu*, we found no further subdivision of *N. concolor* into subspecies-specific clades. Thus, we provisionally classify *N. concolor furvogaster* and *N. concolor jingdongensis* as synonyms of *N. concolor concolor*. In total, we recognize four gibbon genera, 18 species and seven subspecies. The obtained (partially unresolved) phylogeny and estimated divergence ages suggest various radiations in the evolutionary history of gibbons. More or less concordant with recent suggestions (Chatterjee 2006, 2009; Harrison et al. 2006; Jablonski and Chaplin 2009), gibbons most likely originated on the Southeast Asian mainland and diverged into genera in the late Miocene. *Hylobates* split into species and subspecies during radiations in the Pliocene, early Pleistocene and early to middle Pleistocene. In contrast, *Nomascus* successively migrated in the Plio-Pleistocene in the Indochinese bioregion from North to South. This study also shows that mitochondrial DNA constitutes a useful tool to illuminate the taxon-identity of gibbons.

In Chapter 3, I focused on the phylogeny and distribution of crested gibbons. In this study, complete mitochondrial cytochrome b gene sequences of 79 gibbon individuals from known location were analysed. The data were not only able to confirm phylogenetic relationships among crested gibbons as shown in Chapter 2 and earlier studies (Roos 2004; Takacs et al. 2005; Monda et al. 2007; Roos et al. 2007), but also to define and narrow down distribution zones of taxa. Moreover, our study provides the first molecular evidence for an additional, so far undescribed taxon (*N.* sp.) in the range of *N. siki* as proposed by Konrad and Geissmann (2006). However, the most important finding of this study is that the

distribution areas of the southern crested gibbon species, *N. leucogenys, N. siki, N.* sp. and *N. gabriellae* have to be redefined. While *N. leucogenys* and *N.* sp. experienced an expansion of their previously believed range, those of *N. siki* and *N. gabriellae* were largely reduced. This is especially dramatic for *N. siki*, because in Vietnam the species remains only in three protected areas (Huong Hoa NR, Phong Nha-Ke Bang NP, Khe Ve NR). In Laos, the species might be more common (Phu Xang Hae NBCA, Hin Nam No NBCA, Nakai-Nam Theun NBCA), but also here its range is reduced compared to earlier suggestions (e.g. Geissmann et al. 2000, Figure 3.1).

Chapter 4 describes vocal characteristics of crested gibbon taxa and their application as identification marker. Song bouts from 12 locations representing all crested gibbon species, except *N. hainanus,* were collected and analyzed. Qualitative features and discriminant function analyses were applied to distinguish between populations and taxa. We found clear distinguishable song structures in *N. nasutus* and *N. concolor*, which separates them from each other and from the other species. However, differences in the song structure of the four species, *N. leucogenys*, *N. siki*, *N.* sp. and *N. gabriellae* were less prominent. Accordingly, it was not possible to separate these taxa from each other solely on the basis of general acoustic descriptions, which suggests a close phylogenetic relationship among them as also proposed by genetic analyses (Garza and Woodruff 1992; Roos 2004; Roos et al. 2007; Takacs et al. 2005; Monda et al. 2007; Thinh et al. 2010, Thinh et al. accepted). In this case, detailed discriminant function analyses were required to allow a clear separation of species. This study shows that, although sometimes more comprehensive analyses are required, acoustic analyses are in general helpful to discriminate between crested gibbon species and to confirm and verify phylogenetic relationships.

In Chapter 5, I compared differences between crested gibbon taxa on acoustic and genetic level and checked whether they either correlate with each other or each of them at least with the geographic distance between populations. Therefore, we compared 19 crested gibbon populations for which both, acoustic and genetic data, were available. As in Chapter 4, we found clear distinguishable song structures between *N. nasutus, N. concolor* and the southern species. Among the latter, differences were less clear, but as in Chapter 4, these subtle song differences can be distinguished by discriminant function analyses.

Differences in song structure seem to be fixed in populations or at least species, and a significant correlation between vocal similarity and geographic distance, and between vocal and genetic similarity was observed. Accordingly, acoustic and genetic analyses revealed identical results concerning taxon-identification and phylogenetic relationships of crested gibbons, and, hence, both in combination or alone, can be applied for respective purposes.

Taken together, this thesis has successfully contributed new information to better understand the phylogeny, phylogeography and taxonomy of gibbons in general and specifically of crested gibbons. This study also shows, that at least in crested gibbons acoustic and genetic differences among species correlate and, hence, both alone or in combination provide a reliable identification tool to correctly assign the taxon-identity of gibbons, to define distribution areas and to elucidate phylogenetic relationships. This is especially of importance, because other characteristics as e.g. fur coloration are difficult to be applied to verify the species-identity of gibbons.

Gibbon populations throughout their range have dramatically declined in the last decade (Geissmann et al. 2000; IUCN 2009) and all gibbon taxa are now endangered at different levels (IUCN 2009). Hence, prompt and efficient conservation actions are required to save gibbons from extinction. However, to establish a suitable management plan, basic data as e.g. the exact distribution area of a taxon or information about which taxon occurs in a certain protected area is necessary. The herein presented data on phylogeny, taxonomy and most importantly redefined distribution zones provide such basic information to revise management plans or the conservation status of a taxon. For example, *N. siki* in Vietnam experienced a range reduction of about 50% and in Laos of about 30%. Accordingly, in both countries, but especially in Vietnam, *N. siki* should be regarded as a priority species for conservation. Also as a result, *N. siki* should be elevated from “Endangered” to “Critically Endangered” in the IUCN Red List.

Besides elucidating phylogenetic relationships and distribution areas, the applied methods can also be used to identify hunting hotspots, with the later aim to prevent or at least reduce hunting, or to manage captive breeding populations. For captive populations, artificial hybrids should be avoided and only gibbons with clear taxon-identity should be considered for reproduction in zoos or rescue centres. Likewise, if captive gibbons are reintroduced into the wild, it has to be

ascertained that these gibbons are pure individuals and of the same taxon as those, which naturally occur in the area they are to be released.

Although this thesis provided valuable and comprehensive insights into the phylogeny, phylogeography and taxonomy of gibbons and especially of crested gibbons, several topics remain unclear. First, phylogenetic relationships among gibbon genera, and various species and subspecies were not resolved. Although most likely caused by various radiations in the evolutionary history of gibbons, extended mitochondrial sequence data, e.g. from complete mitochondrial genomes, might allow a better resolution of phylogenetic relationships. Moreover, since only the maternally-inherited mitochondrial DNA was studied, possible natural hybridization events among gibbon lineages, as it is indicated in the case of *H. albibarbis,* or artificial hybridization in captive populations, can not be traced. Thus, autosomal, X-chromosomal and Y-chromosomal loci should be implemented in further studies as well. Finally, although the distribution of crested gibbon species was narrowed down, for some protected areas no information about the occuring taxon is available. Since Nakai-Nam Theun NBCA and Dong Phu Vieng NBCA in Laos and Ke Go NR in Vietnam might hold *N. siki* and considering the critical situation of the species, surveys in these protected areas are important to confirm whether *N. siki* is indeed present there.

Summary

Although the phylogeny and phylogeography of gibbons (Hylobatidae), a primate family endemic to Southeast Asia, has been studied in detail, phylogenetic relationships among taxa remain poorly resolved. Likewise, the number of taxa to be recognized is a matter of debate. This dissertation presents the most comprehensive phylogeny in respect to taxon sampling up to date and, hence, allows detailed insights into the phylogeny, phylogeography and taxonomy of gibbons, specifically of crested gibbons, genus *Nomascus*. While for the overall gibbon phylogeny only sequence data of the mitochondrial cytochrome b gene were used, for crested gibbons, acoustic characteristics as obtained from female great calls and male multi-modulated calls were applied as additional independent marker.

According to the obtained phylogeny, in which relationships among various gibbon lineages remain unresolved, and estimated divergence ages, four major radiations in the evolutionary history of gibbons are indicated. Gibbons most likely originated on the Southeast Asian mainland and diverged in an initial split in the late Miocene into genera. *Hylobates* experienced further radiations, which led to various species and subspecies. In contrast, speciation in *Nomascus* was a continuing process associated with a successive North-to-South migration. From a taxonomic view, the data of this thesis suggest that among gibbons four genera with 18 species and seven subspecies should be recognised. Moreover, I provide first molecular evidence for an additional, so far undescribed *Nomascus* species (*N.* sp.). This study also shows, that genetic and acoustic differences between crested gibbon species correlate and, hence, that both in combination or alone can be applied to identify gibbons and to elucidate phylogenetic relationships. Based on acoustic and genetic data, the assumed distribution of *N. leucogenys, N. siki, N.* sp. and *N. gabriellae* has to be redefined. While the ranges of *N. leucogenys* and *N.* sp. are expanded, those of *N. gabriellae* and especially of *N. siki* are largely reduced. Thus, *N. siki* should be regarded as a priority species for conservation in Vietnam and Laos.

Zusammenfassung

Obwohl die Phylogenie und Phylogeographie von Gibbons (Hylobatidae), eine in Südost-Asien vorkommende Primatenfamilie, bereits umfangreich untersucht wurde, bleiben die phylogenetischen Verwandtschaftsbeziehungen zwischen Taxa weiterhin weitestgehend ungeklärt. Auch die Anzahl der anzuerkennenden Taxa ist umstritten. In dieser Doktorarbeit wird die derzeit umfangreichste Phylogenie, in der nahezu alle Gibbon-Taxa vertreten sind, vorgestellt. Dadurch erlaubt diese Arbeit tiefe Einblicke in die Phylogenie, Phylogeographie und Taxonomie von Gibbons, insbesondere von Schopfgibbons. Für die Erstellung der Gesamt-Gibbon-Phylogenie wurden Sequenzdaten des mitochondrialen Cytochrom b-Gens verwendet. Für Schopfgibbons wurden zusätzlich auch akustische Merkmale des weiblichen “great calls” und des männlichen “multi-modulated calls” als unabhängige Marker untersucht.

Entsprechend der ermittelten Phylogenie, die häufig keinen Aufschluss über die Verwandtschaftsbeziehungen zwischen Gibbon-Taxa liefert, und den geschätzten Aufspaltungszeiten, können vier Radiationen in der evolutionären Geschichte von Gibbons angenommen werden. Gibbons entstanden wahrscheinlich auf dem Südostasiatischem Festland und spalteten sich im späten Miozän in Gattungen auf. Innerhalb von *Hylobates* kam es zu weiteren Radiationen, die zur Entstehung von Arten und Unterarten führten. Im Gegensatz hierzu verlief der Artbildungsprocess innerhalb von *Nomascus* kontinuierlich und gekoppelt an eine stufenweise Wanderung von Nord nach Süd ab. Insgesamt werden in dieser Arbeit vier Gibbon-Gattungen mit 18 Arten und sieben Unterarten anerkannt. Zudem kann erstmals ein molekularer Beweis für die Existenz einer weiteren, bisher unbeschriebenen *Nomascus*-Art (*N.* sp.) erbracht werden. Diese Arbeit zeigt auch, dass genetische und akustische Unterschiede miteinander korrelieren und daher beide zusammen oder alleine zur Identifikation von Gibbons oder zur Ermittlung von phylogenetischen Verwandtschaftsbeziehungen herangezogen werden können. Basierend auf den akustischen und genetischen Daten dieser Arbeit müssen die vermeintlichen Verbreitungsgebiete von *N.*

leucogenys, N. siki, N. sp. und *N. gabriellae* korrigiert werden. Während die Daten für *N. leucogenys* und *N.* sp. ein erweitertes Gebiet vermuten lassen, ist dies von *N. gabriellae* und insbesondere von *N. siki* deutlich geschrumpft. Aus diesem Grund sollte *N. siki* ein besonderer Schutzstatus in Vietnam und Laos zugesprochen werden.

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Appendix

Appendix A.1: Origin, material type, sample provider/collector and GenBank accession numbers of studied gibbon specimens.

Species	Code	Origin	Material	Provider/ Collector	GenBank Accession Nr
Nomascus hainanus	NHA1	Bawangling, Hainan, China	feces	L. Ming	GU321248
N. nasutus	NNA1	Kim Hy, Bac Kan, Vietnam	tissue	G. Goldthorpe	GU321245
N. nasutus	NNA2	Trung Khanh, Cao Bang, Vietnam	tissue	T. Nadler	GU321246
N. nasutus	NNA3	"Hinterland von Hon Gai", Vietnam (ZMB)	tissue	T. Geissmann	GU321247
N. concolor concolor	NCO1	Muong La, Son La, Vietnam	tissue	V. N. Thinh	GU321249
N. concolor concolor	NCO2	Che Tao, Yen Bai/Son La, Vietnam	tissue	V. N. Thinh	GU321250
N. concolor concolor	NCO3	Che Tao, Yen Bai/Son La, Vietnam	tissue	V. N. Thinh	GU321251
N. concolor concolor	NCO4	Che Tao, Yen Bai/Son La, Vietnam	tissue	V. N. Thinh	GU321252
N. concolor concolor	NCO5	Liem Phu, Lao Cai, Vietnam	tissue	N. Lormée	GU321253
N. concolor furvogaster	NFU1	Wayao, Yunnan, China (IZCAS)	tissue	T. Geissmann	GU321254
N. concolor furvogaster	NFU2	Wayao, Yunnan, China (IZCAS)	tissue	T. Geissmann	GU321255
N. concolor jingdongensis	NJI1	Wenbu, Yunnan, China (IZCAS)	tissue	T. Geissmann	GU321256
N. concolor lu	NLU1	Nam Kan, Bokeo, Laos	feces	A. Mootnick	GU321257
N. concolor lu	NLU2	Chiang Saen Keo, Laos (USNM)	tissue	T. Geissmann	GU321258
N. leucogenys	NLE1	Twycross Zoo, Great Britain	blood	J. Ray	GU321259
N. leucogenys	NLE2	Mulhouse Zoo, France	feces	P. Moisson	GU321260
N. leucogenys	NLE3	Mulhouse Zoo, France	feces	P. Moisson	GU321261
N. leucogenys	NLE4	Duisburg Zoo, Germany	blood	M. Hartmann	GU321262
N. leucogenys	NLE5	Duisburg Zoo, Germany	blood/feces	M. Hartmann	GU321263
N. leucogenys	NLE6	EPRC, Vietnam	feces	T. Nadler	GU321264
N. leucogenys	NLE7	Phongsaly, Laos (USNM)	tissue	T. Geissmann	GU321265
N. leucogenys	NLE8	Mengla, Yunnan, China (IZCAS)	tissue	T. Geissmann	GU321266
N. siki	NSI1	EPRC, Vietnam	feces	T. Nadler	GU321267
N. siki	NSI2	EPRC, Vietnam	feces	T. Nadler	GU321268

Species	Code	Origin	Material	Provider/ Collector	GenBank Accession Nr
N. siki	NSI3	EPRC, Vietnam	feces	T. Nadler	GU321269
N. siki	NSI4	Phong Nha-Khe Bang National Park, Vietnam	feces	V. N. Thinh	GU321270
N. siki	NSI5	Mulhouse Zoo, France	feces	P. Moisson	GU321271
N. siki	NSI6	Mulhouse Zoo, France	feces	P. Moisson	GU321272
N. gabriellae	NGA1	Mulhouse Zoo, France	feces	P. Moisson	GU321273
N. gabriellae	NGA2	Leipzig Zoo, Germany	blood/feces	K. Eulenberger	GU321274
N. gabriellae	NGA3	EPRC, Vietnam	feces	T. Nadler	GU321275
N. gabriellae	NGA4	EPRC, Vietnam	feces	T. Nadler	GU321276
N. gabriellae	NGA5	EPRC, Vietnam	feces	T. Nadler	GU321277
N. gabriellae	NGA6	EPRC, Vietnam	feces	T. Nadler	GU321278
N. gabriellae	NGA7	Cat Tien National Park, Vietnam	feces	V. N. Thinh	GU321279
N. gabriellae	NGA8	Cat Tien National Park, Vietnam	feces	V. N. Thinh	GU321280
N. gabriellae	NGA9	Cat Tien National Park, Vietnam	feces	V. N. Thinh	GU321281
Symphalangus syndactylus	SSY1	Howletts Wild Animal Park, Great Britain	feces	E. Thetford	GU321282
S. syndactylus	SSY2	Howletts Wild Animal Park, Great Britain	feces	E. Thetford	GU321283
S. syndactylus	SSY3	Berlin Zoo, Germany	blood	R. Göltenboth	GU321284
S. syndactylus	SSY4	Munich Zoo, Germany	blood	J. Hector	GU321285
Hoolock hoolock	HHO1	Dhaka Zoo, Bangladesh	feces	A. Mootnick	GU321286
H. hoolock	HHO2	Dhaka Zoo, Bangladesh	feces	A. Mootnick	GU321287
H. leuconedys	HLE1	Perth Zoo, Australia	hairs	T. Geissmann	GU321288
H. leuconedys	HLE2	Beijing Zoo, China	feces	L. Ming	GU321289
H. leuconedys	HLE3	Beijing Zoo, China	feces	L. Ming	GU321290
Hylobates pileatus	HPI1	Twycross Zoo, Great Britain (NMS)	tissue	A. Kitchener	GU321291
H. pileatus	HPI2	Mulhouse Zoo, France	feces	P. Moisson	GU321292
H. pileatus	HPI3	Zurich Zoo, Switzerland	feces	R. Zingg	GU321293
H. pileatus	HPI4	Khao Yai National Park, Thailand	feces	C. Barelli	GU321294
H. moloch	HMO 1	Munich Zoo, Germany	feces	J. Hector	GU321295
H. moloch	HMO 2	Howletts Wild Animal Park, Great Britain (NMS)	tissue	A. Kitchener	GU321296

Species	Code	Origin	Material	Provider/ Collector	GenBank Accession Nr
H. moloch	HMO 3	Howletts Wild Animal Park, Great Britain (NMS)	tissue	A. Kitchener	GU321297
H. agilis agilis	HAG1	Plock Zoo, Poland	blood	W. Zduniak	GU321298
H. agilis agilis	HAG2	Plock Zoo, Poland	blood	W. Zduniak	GU321299
H. agilis agilis	HAG3	Jakarta Zoo, Indonesia	feces	M. Agil	GU321300
H. agilis agilis	HAG4	Jakarta Zoo, Indonesia	feces	M. Agil	GU321301
H. agilis agilis	HAG5	Bristol Zoo, Great Britain	blood	S. Redrobe	GU321302
H. agilis agilis	HAG6	Paignton Zoo, Great Britain (NMS)	blood	A. Kitchener	GU321303
H. agilis unko	HUN1	Jakarta Zoo, Indonesia	feces	M. Agil	GU321304
H. agilis unko	HUN2	Jakarta Zoo, Indonesia	feces	M. Agil	GU321305
H. albibarbis	HAL1	Louisiana Purchase Gardens and Zoo, USA	feces	A. Mootnick	GU321306
H. albibarbis	HAL2	Jakarta Zoo, Indonesia	feces	M. Agil	GU321307
H. muelleri muelleri	HMU1	Jakarta Zoo, Indonesia	feces	A. Schrod	GU321308
H. muelleri muelleri	HMU2	Jakarta Zoo, Indonesia	feces	A. Schrod	GU321309
H. muelleri muelleri	HMU3	Schwerin Zoo, Germany	hairs	U. Ricker	GU321310
H. muelleri muelleri	HMU4	Schwerin Zoo, Germany	hairs	U. Ricker	GU321311
H. muelleri funereus	HFU1	Banham Zoo, Great Britain (NMS)	tissue	A. Kitchener	GU321312
H. muelleri abbotti	HAB1	Rostock Zoo, Germany	blood/tissue	K. Linke	GU321313
H. klossii	HKL1	Twycross Zoo, Great Britain (NMS)	tissue	A. Kitchener	GU321314
H. klossii	HKL2	Jakarta Zoo, Indonesia	feces	M. Agil	GU321315
H. klossii	HKL3	Siberut, Indonesia	feces	T. Ziegler	GU321316
H. klossii	HKL4	Siberut, Indonesia	feces	T. Ziegler	GU321317
H. klossii	HKL5	Siberut, Indonesia	feces	T. Ziegler	GU321318
H. lar (yunnanensis?)	HLA1	Wuppertal Zoo, Germany	blood	G. Olbricht	GU321319
H. lar	HLA2	Wuppertal Zoo, Germany	blood/feces	G. Olbricht	GU321320
H. lar	HLA3	Wuppertal Zoo, Germany	blood	G. Olbricht	GU321321
H. lar	HLA4	Nuremberg Zoo, Germany	blood	A. Gauckler	GU321322
H. lar	HLA5	Nuremberg Zoo, Germany	blood/feces	A. Gauckler	GU321323
H. lar	HLA6	Besancon Zoo, France	blood	J. Robert	GU321324
H. lar carpenteri	HCA1	Pai, Thailand	feces	C. Roos	GU321325

Species	Code	Origin	Material	Provider/ Collector	GenBank Accession Nr
H. lar entelloides	HEN1	Khao Yai National Park, Thailand	feces	C. Barelli	GU321326
H. lar entelloides	HEN2	Khao Yai National Park, Thailand	feces	C. Barelli	GU321327
H. lar lar	HLL1	Singapore Zoo, Singapore	feces	D. Richardson	GU321328
H. lar vestitus	HVE1	Sumatra, Indonesia (Naturalis)	tissue	C. Smeenk	GU321329
Homo sapiens	-	GenBank	sequence	-	D38112
Pan troglodytes	-	GenBank	sequence	-	D38113
Pan paniscus	-	GenBank	sequence	-	D38116
Gorilla gorilla	-	GenBank	sequence	-	D38114
Pongo pygmaeus	-	GenBank	sequence	-	D38115
Pongo abelii	-	GenBank	sequence	-	NC_002083
Papio hamadryas	-	GenBank	sequence	-	Y18001

EPRC: Endangered Primate Rescue Center, Ninh Binh Province, Vietnam
IZCAS: Institute of Zoology, Chinese Academy of Sciences, Beijing, China
Naturalis: Natural History Museum, Leiden, The Netherlands
NMS: National Museums Scotland, Edinburgh, Great Britain
USNM: National Museum of Natural History, Washington, USA
ZMB: Museum für Naturkunde, Berlin, Germany

Appendix B.1: Information about studied crested gibbon individuals including locality, geographical coordinates, collector or origin and GenBank accession number.

Species	Code	Locality	Longitude/latitude	Origin/ Collector	Genbank Accession no
Nomascus hainanus	hai1	Bawangling NNR, Hainan, China	N19°00'; E109°20'	GenBank	GU321248
Nomascus nasutus	nas1	Trung Khanh NR, Cao Bang, Vietnam	N22°55'; E106°32'	GenBank	GU321246
Nomascus nasutus	nas2	Kim Hy NR, Bac Kan, Vietnam	N22°16'; E106°02'	GenBank	GU321245
Nomascus nasutus	nas3	Hon Gai, Quang Ninh, Vietnam	N20°52'; E106°57'	GenBank	GU321247
Nomascus nasutus	nas4	Tam Dao NP, Vinh Phuc, Vietnam	N21°28'; E105°38'	ZMVNU	GU594996
Nomascus concolor concolor	con1	Muong La District, Son La, Vietnam	N21°35'; E104°16'	GenBank	GU321249
Nomascus concolor concolor	con2	Che Tao, Yen Bai, Vietnam	N21°43'; E104°03'	GenBank	GU321250
Nomascus concolor concolor	con3	Che Tao, Yen Bai, Vietnam	N21°43'; E104°03'	GenBank	GU321251
Nomascus concolor concolor	con4	Che Tao, Yen Bai, Vietnam	N21°43'; E104°03'	GenBank	GU321252
Nomascus concolor concolor	con5	Che Tao, Yen Bai, Vietnam	N21°43'; E104°03'	V. N. Thinh	GU321249
Nomascus concolor concolor	con6	Che Tao, Yen Bai, Vietnam	N21°43'; E104°03'	V. N. Thinh	GU594997
Nomascus concolor concolor	con7	Nam Xay, Lao Cai, Vietnam	N21°57'; E104°10'	V. N. Thinh	GU594998
Nomascus concolor concolor	con8	Liem Phu, Lao Cai, Vietnam	N22°00'; E104°20'	GenBank	GU321253
Nomascus concolor jingdongensis	jin1	Wenbu, Yunnan, China	N24°30'; E100°45'	GenBank	GU321256
Nomascus concolor furvogaster	fur1	Wayao, Yunnan, China	N25°28'; E99°11'	GenBank	GU321254
Nomascus concolor furvogaster	fur2	Wayao, Yunnan, China	N25°28'; E99°11'	GenBank	GU321255
Nomascus concolor lu	lu1	Nam Kan NBCA, Bokeo, Laos	N20°30'; E100°30'	GenBank	GU321257
Nomascus concolor lu	lu2	Chiang Saen Keo, Bokeo, Laos	N20°16'; E100°09'	GenBank	GU321258
Nomascus leucogenys	leu1	Mengla, Yunnan, China	N21°28'; E101°34'	GenBank	GU321266
Nomascus leucogenys	leu2	Phongsaly, Laos	ca. N21°40'; ca. E102°10'	GenBank	GU321265
Nomascus leucogenys	leu3	Phongsaly, Laos	ca. N21°40'; ca. E102°10'	USNM	GU321265
Nomascus leucogenys	leu4	Muong Loi, Lai Chau, Vietnam	N21°02'; E103°14'	IEBR	GU594999
Nomascus leucogenys	leu5	Chi Ne, Hoa Binh, Vietnam	N20°35'; E105°31'	ZMVNU	GU595000
Nomascus leucogenys	leu6	Pu Luong NR, Thanh Hoa, Vietnam	N20°31'; E105°07'	IEBR	GU595000
Nomascus leucogenys	leu7	Xuan Lien NR, Thanh Hoa, Vietnam	N19°57'; E105°00'	V. N. Thinh	GU595000
Nomascus leucogenys	leu8	Xuan Lien NR, Thanh Hoa, Vietnam	N19°55'; E105°10'	ZMVNU	GU595001
Nomascus leucogenys	leu9	Pu Huong NR, Nghe An, Vietnam	N19°42'; E105°05'	IEBR	GU595002

Species	Code	Locality	Longitude/latitude	Origin/ Collector	Genbank Accession no
Nomascus leucogenys	leu10	Pu Huong NR, Nghe An, Vietnam	N19°42'; E105°05'	IEBR	GU595000
Nomascus leucogenys	leu11	Pu Huong NR, Nghe An, Vietnam	N19°17'; E104°53'	V. N. Thinh	GU595002
Nomascus leucogenys	leu12	Nghia Dung, Nghe An, Vietnam	N19°07'; E105°20'	IEBR	GU595003
Nomascus siki	sik1	Pu Mat NP, Nghe An, Vietnam	N18°55'; E104°39'	T. Nadler	GU595004
Nomascus siki	sik2	Pu Mat NP, Nghe An, Vietnam	N18°55'; E104°39'	T. Nadler	GU595004
Nomascus siki	sik3	Thanh Chuong, Nghe An, Vietnam	N18°47'; E105°20'	XMFC	GU595003
Nomascus siki	sik4	Vu Quang NP, Ha Tinh, Vietnam	N18°33'; E105°12'	V. N. Thinh	GU595004
Nomascus siki	sik5	Vu Quang NP, Ha Tinh, Vietnam	N18°13'; E105°25'	V. N. Thinh	GU595002
Nomascus siki	sik6	Vu Quang NP, Ha Tinh, Vietnam	N18°13'; E105°28'	V. N. Thinh	GU595002
Nomascus siki	sik7	Nam Kading NBCA, Bolikhamxay, Laos	N18°39'; E104°11'	C. Hallam	GU595001
Nomascus siki	sik8	Nam Kading NBCA, Bolikhamxay, Laos	N18°38'; E104°21'	C. Hallam	GU595003
Nomascus siki	sik9	Nam Kading NBCA, Bolikhamxay, Laos	N18°25'; E104°06'	C. Hallam	GU595005
Nomascus siki	sik10	Phong Nha-Ke Bang NP, Quang Binh, Vietnam	N17°30'; E106°09'	GenBank	GU321270
Nomascus siki	sik11	Phong Nha-Ke Bang NP, Quang Binh, Vietnam	N17°29'; E106°19'	V. N. Thinh	GU595006
Nomascus siki	sik12	Huong Hoa NR, Quang Tri, Vietnam	N16°56'; E106°35'	V. N. Thinh	GU595007
Nomascus siki	sik13	Huong Hoa NR, Quang Tri, Vietnam	N16°55'; E106°36'	V. N. Thinh	GU595008
Nomascus siki	sik14	Phou Xang He NBCA, Savannakhet, Laos	N16°50'; E105°33'	T. Nadler	GU595005
Nomascus siki	sik15	Phou Xang He NBCA, Savannakhet, Laos	N16°50'; E105°33'	T. Nadler	GU595006
Nomascus siki	sik16	Phong Dien NR, Thua Thien-Hue, Vietnam	N16°32'; E107°10'	V. N. Thinh	GU595009
Nomascus siki	sik17	Da Krong NR, Quang Tri, Vietnam	N16°28'; E107°06'	V. N. Thinh	GU595010
Nomascus siki	sik18	Sao La NR, Thua Thien-Hue, Vietnam	N16°09'; E107°24'	V. N. Thinh	GU595010
Nomascus siki	sik19	Xe Sap NBCA, Sekong, Laos	N16°03'; E107°10'	V. N. Thinh	GU595011
Nomascus siki	sp1	Bach Ma NP, Thua Thien-Hue, Vietnam	N16°13'; E107°55'	V. N. Thinh	GU595009
Nomascus siki	sp2	Bach Ma NP, Thua Thien-Hue, Vietnam	N16°13'; E107°54'	V. N. Thinh	GU595009
Nomascus siki	sp3	Bach Ma NP, Thua Thien-Hue, Vietnam	N16°12'; E107°53'	V. N. Thinh	GU595012
Nomascus sp.	sp4	Song Thanh NR, Quang Nam, Vietnam	N15°30'; E107°37'	V. N. Thinh	GU595013
Nomascus sp.	sp5	Chu Mom Ray NP, Kon Tum, Vietnam	N14°27'; E107°43'	V. N. Thinh	GU595009
Nomascus sp.	sp6	Chu Mom Ray NP, Kon Tum, Vietnam	N14°27'; E107°43'	V. N. Thinh	GU595014

Species	Code	Locality	Longitude/latitude	Origin/ Collector	Genbank Accession no
Nomascus sp.	sp7	Sa Son, Kontum, Vietnam	N14°26'; E107°47'	ZMVNU	GU595011
Nomascus sp.	sp8	Sa Son, Kontum, Vietnam	N14°26'; E107°47'	ZMVNU	GU595010
Nomascus sp.	sp9	Xe Pian NBCA, Champasak, Laos	N14°34'; E106°07'	V. N. Thinh	GU595012
Nomascus sp.	sp10	Xe Pian NBCA, Champasak, Laos	N14°34'; E106°07'	V. N. Thinh	GU595012
Nomascus sp.	sp11	Virachey NP, Ratanakiri, Cambodia	N14°18'; E106°53'	B. Rawson	GU595011
Nomascus sp.	sp12	Virachey NP, Ratanakiri, Cambodia	N14°18'; E106°53'	B. Rawson	GU595013
Nomascus gabriellae	gab1	Kon Ka Kinh NP, Gia Lai, Vietnam	N14°17'; E108°22'	T. Nadler	GU595015
Nomascus gabriellae	gab2	Kon Ka Kinh NP, Gia Lai, Vietnam	N14°20'; E108°23'	V. N. Thinh	GU595015
Nomascus gabriellae	gab3	Chu Yang Sin NP, Dak Lak, Vietnam	N12°30'; E108°25'	V. N. Thinh	GU595016
Nomascus gabriellae	gab4	Chu Yang Sin NP, Dak Lak, Vietnam	N12°25'; E108°29'	V. N. Thinh	GU595016
Nomascus gabriellae	gab5	Chu Yang Sin NP, Dak Lak, Vietnam	N12°25'; E108°30'	V. N. Thinh	GU595017
Nomascus gabriellae	gab6	Hon Ba NR, Khanh Hoa, Vietnam	N12°08'; E108°58'	M. Kenyon	GU595018
Nomascus gabriellae	gab7	Bi Dup-Nui Ba NP, Lam Dong, Vietnam	N12°11'; E108°41'	GenBank	GU595018
Nomascus gabriellae	gab8	Bi Dup-Nui Ba NP, Lam Dong, Vietnam	N12°10'; E108°40'	V. N. Thinh	GU595017
Nomascus gabriellae	gab9	Bi Dup-Nui Ba NP, Lam Dong, Vietnam	N12°08'; E108°23'	V. N. Thinh	GU595019
Nomascus gabriellae	gab10	Ta Dung NR, Dak Lak, Vietnam	N11°53'; E107°57'	V. N. Thinh	GU595020
Nomascus gabriellae	gab11	Cat Tien NP, Dong Nai, Vietnam	N11°27'; E107°14'	GenBank	GU321279
Nomascus gabriellae	gab12	Cat Tien NP, Dong Nai, Vietnam	N11°27'; E107°14'	M. Kenyon	GU595019
Nomascus gabriellae	gab13	Cat Tien NP, Dong Nai, Vietnam	N11°27'; E107°14'	M. Kenyon	GU595018
Nomascus gabriellae	gab14	Cat Tien NP, Dong Nai, Vietnam	N11°27'; E107°14'	M. Kenyon	GU595019
Nomascus gabriellae	gab15	Cat Tien NP, Dong Nai, Vietnam	N11°25'; E107°25'	M. Kenyon	GU595019
Nomascus gabriellae	gab16	Phnom Prich WS, Mondulkiri, Cambodia	N12°44'; E107°02'	V. N. Thinh	GU595021
Nomascus gabriellae	gab17	Phnom Prich WS, Mondulkiri, Cambodia	N12°44'; E107°02'	V. N. Thinh	GU595021
Nomascus gabriellae	gab18	Phnom Prich WS, Mondulkiri, Cambodia	N12°43'; E107°02'	V. N. Thinh	GU595022
Hylobates lar	-	-	-	GenBank	GU321319

NBCA: National Biodiversity Conservation Area
N/NR: National/ Nature Reserve
NP: National Park
WS: Widlife Sanctuary

IEBR: Institute of Ecology and Biological Resources, Hanoi, Vietnam
USNM: National Museum of Natural History, Washington, USA
XMFC: Xuan Mai Forestry College, Xuan Mai, Vietnam
ZMVNU: Zoological Museum, Vietnam National University, Hanoi, Vietnam

Appendix C.1: Information about sample locations, molecular identification and number of analysed calls.

No.*	Samples**	Location	Province, Country***	Longitude (N)	Latitude (E)	Molecular Identification	Recording time	Analysed groups	Great calls	Male calls
1	v + g	Trung Khanh	Cao Bang, VN	22° 51’ 10’’	106° 42’ 58’’	*N. nasutus*	09/2007	5	13	26
2	v + g	Che Tao	Yen Bai, VN	21° 42’ 30’’	104° 06’ 26’	*N. concolor*	07/2007	2	9	14
3	v + g	Muong La	Son La, VN	21° 35’ 14’’	104° 16’ 18’’	*N. concolor*	10/2008	4	8	12
Populations (1-3) can be distinguished by quantitative analyses								**11**	**30**	**52**
4	v + g	Xuan Lien	Thanh Hoa, VN	19° 57’ 01’’	105° 00’ 18’’	*N. leucogenys*	06/2007	4	14	17
5	v + g	Pu Huong	Nghe An, VN	19° 21’ 42’’	104° 56’ 02’’	*N. leucogenys*	12/2007	1	2	2
6	v + g	Vu Quang	Ha Tinh, VN	18° 16’ 29’’	105° 26’ 35’’	*N. leucogenys*	06/2008	2	11	12
7	v + g	Nam Kading (N)	Bolikhamxai, Laos	18°39’ 00’’	104° 26’ 07’’	*N. leucogenys*	2007	7	36	40
8	v + g	Nam Kading (S)	Bolikhamxai, Laos	18°18’ 45’’	104° 26’ 57’’	*N. siki*	2007	3	9	16
9	v	Khe Ve	Quang Binh, VN	17° 54’ 08’’	105° 46’ 40’’	No data	06/2008	3	12	14
10	v + g	Phong Nha-Ke Bang	Quang Binh, VN	17° 29’ 09’’	106° 21’ 10’’	*N. siki*	08/2007	5	25	34
11	v + g	Huong Hoa	Quang Binh, VN	16° 59’ 28’’	106° 35’ 59’’	*N. siki*	07/2008	2	17	17
12	v + g	Huong Hoa	Quang Tri, VN	16° 55’ 49’’	106° 35’ 45’’	*N. siki*	07/2008	4	17	24
13	v + g	Da Krong	Quang Tri, VN	16° 24’ 40’’	107° 05’ 26’’	*N.* sp.	10/2007	5	24	13
14	v + g	Phong Dien	Thua Thien-Hue, VN	16° 24’ 22’’	107° 10’ 01’’	*N.* sp.	10/2007	4	19	18
15	v + g	Xe Sap	Sekong, Laos	16° 04’ 04’’	107° 15’ 04’’	*N.* sp.	08/2008	2	15	11
16	v + g	Sao La	Thua Thien-Hue, VN	16° 06’ 46’’	107° 26’ 34’’	*N.* sp.	08/2008	4	21	15
17	v + g	Bach Ma	Thua Thien-Hue, VN	16° 12’ 03’’	107° 44’ 45’’	*N.* sp.	11/2007	5	23	24
18	v + g	Xe Pian	Champasak, Laos	14° 34’ 46’’	106° 08’ 04’’	*N.* sp.	10/2008	5	27	18
19	v + g	Chu Mom Ray	Kon Tum, VN	14° 25’ 56’’	107° 42’ 47’’	*N.* sp.	11/2007	8	53	33
20	v + g	Kon Ka Kinh	Gia Lai, VN	14° 20’ 20’’	108° 24’ 50’’	*N.* sp.	09/2008	6	32	20
21	v	A Yun Pa	Gia Lai, VN	13° 18’ 59’’	108° 22’ 05’’	No data	08/2009	1	6	4

No.*	Samples**	Location	Province, Country***	Longitude (N)	Latitude (E)	Molecular Identification	Recording time	Analysed groups	Great calls	Male calls
22	v + g	Phnom Prich	Mondulkiri, Cambodia	12° 44’ 37’’	107° 01’ 54’’	*N. gabriellae*	12/2008	3	17	24
23	v + g	Bi Dup-Nui Ba	Lam Dong, VN	12° 11’ 37’’	108° 41’ 06’’	*N. gabriellae*	12/2007	5	19	20
24	v + g	Ta Dung	Dak Lak, VN	11° 52’ 51’’	107° 57’ 27’’	*N. gabriellae*	11/2008	2	11	19
Populations (4-24) can not be distinguished by quantitative analyses								**81**	**410**	**395**
Total								**92**	**440**	**447**

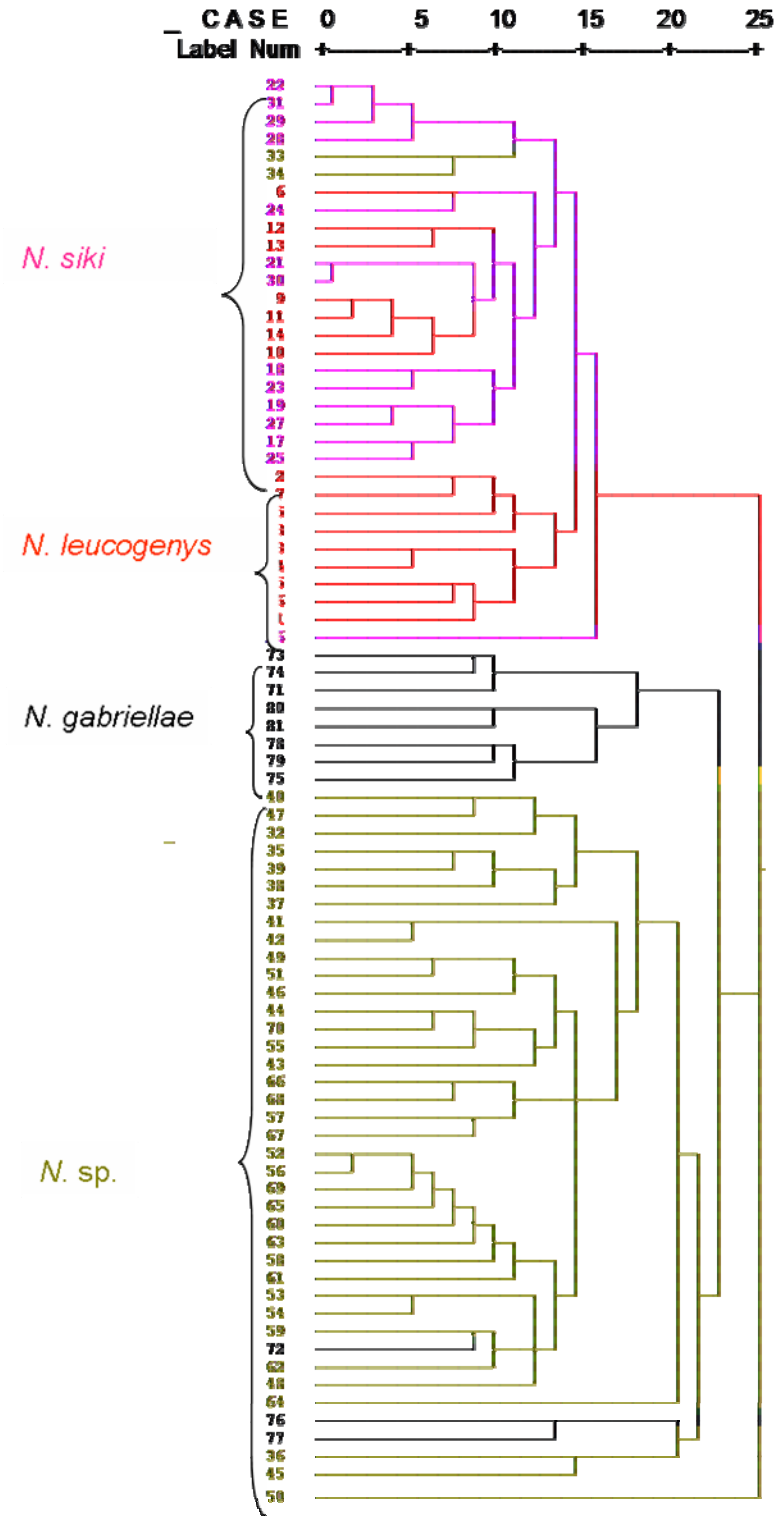
Location numbers refer to those shown in Figure 5.1; ** v: vocal samples, g: genetic sample; *** VN: Vietnam

Appendix C.2: Qualitative criteria to describe crested gibbon taxa.

Taxa	Male call	Great call	Assigned populations
N. nasutus	• Booms absent. • Trough part of first note missing in sweep up frequency. No roll spears and initial part of second note start with short sweep up before sweeping down, then rapid changes of frequency modulation up to the last note. • Repeated staccato notes with short and rapid up-down sweeps. • Multi-modulated phrase immediately after first few notes of the great call.	• 8-12 notes and except the first 2-3 very rapid vibrato sounds. • All fundamental frequencies < 2.8 kHz. • Great call elements sweep up-down as spiral spring.	1-Trung Khanh
N. concolor	• Single booms during inflation of throat sac, staccato phrases and multi-modulated phrases. • First note start at high frequency (>1 kHz) and is of ascending, followed by notes with fast up-down modulation.	• 9-14 notes and except the first, ascending frequency only. • From second note fast down-up modulation.	2-Che Tao 3-Muong La

Taxa	Male call	Great call	Assigned populations	
N. gabriellae	1a: Booms during inflation of throat sac.	6a: Series of 9-19 notes and Oo	4-Xuan Lien	1a, 2a, 3a, 4a, 5a, 6a, 7a, 8a
N. sp.	1b: Booms appears sometime during inflation of	notes <4.	5-Pu Huong	1a, 2a, 3b, 4a, 5b, 6b, 7b, 8b
N. siki	throat sac.	6b: Series of 8-15 notes.	6-Vu Quang	1b, 2a, 3b, 4a, 5b, 6b, 7a, 8a
N.	1c: Booms absent during inflation of throat sac.	6c: Series of 6-12 notes.	7-Nam Kading N	1a, 2a, 3a, 4b, 5b, 6b, 7b, 8a
leucogenys	2a: Stable frequency at the beginning with fast	7a: Start frequency of notes low	8-Nam Kading S	1b, 2a, 3a, 4b, 5b, 6b, 7b, 8a
	down-up sweep at the end.	(<600Hz).	9-Khe Ve	1b, 2a, 3b, 4a, 5b, 6b, 7b, 8a
	2b: Starts at low frequency then increasing with a	7b: Start frequency of notes medium	10-Phong Nha-Ke	1b, 2a, 3b, 4a, 5c, 6b, 7b, 8b
	fast down-up-sweep at the end.	(600Hz-700Hz).	Bang	
	2c: Starts low and holds to the end with stable	7c: Start frequency of notes high	11-Huong Hoa	1b, 2a, 3b, 4a, 5b, 6b, 7b, 8b
	frequency.	(>700Hz).	12-Huong Hoa	1b, 2a, 3b, 4a, 5b, 6b, 7b, 8b
	3a: Staccato regular.	8a: Start frequency across all notes	13-Da Krong	1b, 2b, 3b, 4a, 5c, 6b, 7a, 8b
	3b: Staccato not regular.	constant.	14-Phong Dien	1b, 2c, 3c, 4a, 5b, 6b, 7b, 8b
	3c: Staccato rare.	8b: Start frequency across all notes	15-Xe Sap	1b, 2b, 3c, 4b, 5c, 6b, 7b, 8b
	4a: Modulation of rolls slow.	ascending and descending of last	16-Sao La	1b, 2b, 3c, 4b, 5c, 6b, 7b, 8b
	4b: Modulation of rolls fast.	few notes.	17-Bach Ma	1b, 2a, 3c, 4b, 5c, 6b, 7b, 8b
	4c: Modulation of rolls very fast.	8c: Start frequency across all notes	18-Xe Pian	1b, 2b, 3c, 4b, 5c, 6c, 7c, 8b
	5a: Rolls on second and third note.	ascending.	19-Chu Mom Ray	1b, 2a, 3c, 4b, 5c, 6c, 7b, 8b
	5b: Rolls absent sometime.		20-Kon Ka Kinh	1b, 2b, 3c, 4b, 5c, 6c, 7c, 8b
	5c: Rolls only on second note.		21-A Yun Ba	1c, 2b, 3c, 4b, 5c, 6c, 7c, 8c
			22-Phnom Prich	1c, 2b, 3b, 4b, 5c, 6c, 7c, 8c
			23-Bi Dup-Nui Ba	1c, 2c, 3c, 4b, 5c, 6c, 7c, 8c
			24-Ta Dung	1c, 2c, 3c, 4c, 5c, 6c, 7c, 8c

Appendix C.3: Dendrogram showing the acoustic dissimilarity between the four southern crested gibbon species. Hierarchical cluster analysis of acoustic data based on the z-score value comprised from 13 stepwise in DFA which included 81 groups. The first cluster includes *N. siki* groups (pink), interspersed with *N. leucogenys* (red) and *N.* sp. (green) groups. The second and third cluster comprises only *N. leucogenys* and N. gabriellae (black) groups, respectively. The fourth cluster is mainly composed of *N.* sp., but also includes *N. gabriellae* groups.



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Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Desweiteren erkläre ich, dass ich mich nicht anderweitig einer Doktorarbeit ohne Erfolg unterzogen habe und dass diese Arbeit in gleicher oder ähnlicher Form noch keiner anderen Prüfungsbehörde vorgelegen hat.

Die Publikationen, wie sie in den Kapiteln 2-5 repliziert sind, wurden von mir selbst verfasst. Christian Roos leitete alle Arbeiten als Dissertationsbetreuer an und entwickelte die Idee zur molekularen Phylogenie von Gibbons. Kurt Hammerschmidt betreute die akustischen Analysen. Alle Koautoren wirkten bei der Finalisierung der Manuskripte mit.

Göttingen, den 6. Mai 2010

Van Ngoc Thinh