

# Molecular phylogeny and evolutionary history of Southeast Asian macaques forming the *M. silenus* group

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## Abstract

The 12 presently recognized taxa forming the *Macaca silenus* group represent the most diverse lineage within the genus *Macaca*. The present study was set up to clarify the phylogenetic relationships of the extant members of the *M. silenus* group and to explain their geographical distribution patterns seen today. A combined approach involving the analysis of one paternal (TSPY) and two maternal (cyt *b* and 12S–16S rRNA) molecular markers enabled us to resolve the phylogenetic relationships within this lineage. Our Y chromosomal marker is not informative enough to allow detailed conclusion. Based on our mitochondrial data, however, *M. pagensis*, endemic to the three southern Mentawai islands (Sipora, North- and South Pagai), split off early (2.4–2.6mya) and represents a sister clade to the macaques from the northern Mentawai island of Siberut and from those of the Southeast Asian mainland, which diverged in a radiation like splitting event about 1.5–1.7mya. By combining our new results with available data on behavioural as well as climate and sea level changes in Southeast Asia during the Plio- and Pleistocene, we have developed two scenarios for the evolutionary history of this primate group, which may help explain the current geographical distribution of its members.

## 1. Introduction

Having According to fossil data, the genus *Macaca* arose about 7mya in North Africa and subsequently spread throughout most of Asia during the past 5.5 million years (Delson, 1980, 1996; Delson and Andrews, 1975). Today, found in over 20 Asian countries and covering an area of more than 5 million km<sup>2</sup> (Fa, 1989), this genus of old world monkeys can be considered as the most successful of all the extant nonhuman primate radiations (Tosi et al., 2003). During its spread into Asia, the genus *Macaca* diversified into a number of distinct species groups which have been variously defined according to their geographical distribution, differences in male reproductive anatomy (Delson, 1980) and more recently, molecular genetic data (Hayasaka et al., 1996; Morales and Melnick, 1998; Tosi et al., 2003). Currently, five main species/phyletic groups are recognised as reflecting the major steps of radiation within the genus; these are the two monotypic groups *M. sylvanus* and *M. arctoides*, and the polytypic groups *M. fascicularis* and *M. sinica*, with four and five species, respectively, and the *Macaca silenus* group (Groves, 2001; Tosi et al., 2003; Sinha et al., 2005). With 12 currently recognized taxa exhibiting a range of distinct morphological and behavioural differences (Abegg and Thierry, 2002b; Fooden, 1975), the *M. silenus* group is the most diverse lineage within the macaque genus. The group is also characterised by showing a wide distribution, its range extending from Southwest India (*M. silenus*) through mainland Southeast Asia (*M. leonina*), peninsular Malaysia, Sumatra and Borneo (*M. nemestrina*) to Sulawesi (seven taxa) in the East and the Mentawai archipelago (*M. pagensis* and *M. siberu*) off Sumatra's coast in the West (Fig. 1).

Although the general classification of the *M. silenus* group seems widely accepted (Evans et al., 2003; Fooden, 1980; Groves, 2001; Roos et al., 2003), the phylogenetic relationships among the various taxa and the biogeographic processes leading to their current distribution are not well understood. One of the main reasons for this is the extent and complexity of the geographical and environmental changes that repeatedly occurred during the recent past throughout their current distribution range. Notably, in both the Plio- and Pleistocene, a series of dramatic climatic changes profoundly 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; Bird et al., 2005; also see Meijaard, 2003a; for an overview). In particular, periods of maximum glaciation reduced rainforest cover, resulting in the appearance of more open and deciduous vegetation types in many parts of Sundaland, (Bird et al., 2005; Kaars van der et al., 2001; Sun et al., 2000; Urushibara-Yoshino and Yoshino, 1997; Meijaard, 2003b). Moreover, due to the alternately falling and rising sea water levels during the several glacial and interglacial periods characterising the Plio- and Pleistocene periods (Molengraaff and Weber, 1920; Tjia, 1976; Jablonski and Whitfort, 1999; Meijaard, 2003a), connections and separations of landmasses were common, and repeated migration between habitats was possible (Verstappen, 1975; Tougaard, 2001; Woodruff, 2003).



**Fig. 1.** Distribution of the members of the *M. silenus* group. Origin of samples are indicated by black dots (see also Table 1).

It is therefore reasonable to assume that climatic and geological factors have played an important role in influencing the biogeographic patterns leading to the current distribution of the *M. silenus* group members (and other terrestrial animals) in Southeast Asia (Ferguson, 1993; Abegg and Thierry, 2002b; Evans et al., 2003). Nevertheless, the details remain unclear of how such factors operated to affect the process of macaque radiation through Southeast Asia. Existing hypotheses that have attempted to explain the occurrence of the different *M. silenus* group members in different habitats and regions (Abegg and Thierry, 2002b; Fooden, 1980) are based on incomplete data and cannot be considered to be comprehensive.

Thus, further clarification of this issue requires not only knowledge about former palaeogeographical and –climatic conditions in Southeast Asia but also (and in particular) more complete information on the phylogenetic relationships within the species group and the splitting times among its members. In contrast to the numerous studies on the radiation of the macaque genus as a whole (Deinard and Smith, 2001; Delson, 1980; Hayasaka et al., 1996; Morales and Melnick, 1998; Tosi et al., 2003), information about the phylogenetic relationships within the *M. silenus* group is scarce, and data that do exist are incomplete and at times contradictory (Evans et al., 2003; Roos et al., 2003; Tosi et al., 2003). For example, available molecular data indicate that the *M. silenus* group originated in the Sunda region and that the Sulawesi macaques and *M. silenus* probably stem from *M. nemestrina* like ancestors (Evans et al., 2003), contradicting the hypothesized ancestral position of these species based on morphological and behavioural data (Abegg and Thierry, 2002a,b). Moreover, it has recently been shown that Mentawai macaques are mitochondrially paraphyletic, with the northernmost form from Siberut being more closely related to *M. nemestrina* from Sumatra than they are to the form present on the three southern islands of the archipelago (Roos et al., 2003). This unexpected finding remains to be explained, but may indicate two independent colonisations of the Mentawai islands, as suggested by Roos et al. (2003).

However, all available hypotheses, whether based on genetic, morphological, or behavioural data are to some extent contradictory and consequently no consensus exists concerning the radiation of the *M. silenus* group. Our aim in the present study was to attempt an explanation of the evolutionary history of the *M. silenus* group and the biogeographic patterns leading to its current distribution by comparing information about past geological and climatic conditions with phylogenetic relationships and splitting times as revealed from molecular data. A comprehensive dataset is provided, in which sequences from one Y chromosomal locus and two mitochondrial loci have been generated for all species and major populations present in the group.

## 2. Materials and methods

### 2.1. Sample Collection

Faecal material from macaques was collected in zoos, from pet monkeys, or during field surveys. In order to obtain a broad geographic and taxonomic sampling of the *M. silenus* group, material was collected from specimens representing populations from Sumatra, peninsular Malaysia and Borneo (*M. nemestrina*), Vietnam and Laos (*M. leonina*), South Pagai and Sipora (*M. pagensis*) and Siberut (*M. siberu*). Faecal samples from liontailed macaques (*M. silenus*) were obtained from the captive colony of the German Primate Centre, while faecal samples from Assamese macaques (*M. assamensis*) were collected during a field survey in Vietnam. All study individuals were identified by fur colouration, and information about their origin, gender and approximate age was noted. In order to avoid repeated sampling of the same individual, only fresh material was collected and stored in 70%

ethanol at room temperature before further processing. Further details about analysed species, individuals and sample locations are shown in Fig. 1 and Table 1.

Species	Origin	Cytochrome <i>b</i>	12S–16S rRNA	TSPY
<i>Papio hamadryas</i>	—	Y16590 <sup>d</sup>	AF424974 <sup>a</sup>	—
<i>Macaca sylvanus</i>	—	AY151088 <sup>b</sup>	AF424971 <sup>a</sup>	—
<i>M. thibetana</i>	—	AY151089 <sup>b</sup>	AY224267 <sup>a</sup>	—
<i>M. assamensis</i>	Vietnam	DQ355482 <sup>c</sup>	AY224245 <sup>a</sup>	—
<i>M. fascicularis</i>	—	AY151090 <sup>b</sup>	AF424957 <sup>a</sup>	—
<i>M. tonkeana</i>	Sulawesi	AF349041 <sup>d</sup>	AY224269 <sup>a</sup>	AF284235 <sup>a</sup>
<i>M. maura</i>	Sulawesi	AF350397 <sup>d</sup>	AY224250 <sup>a</sup>	AF284257 <sup>a</sup>
<i>M. nigra</i>	Sulawesi	AF350386 <sup>d</sup>	AY224258 <sup>a</sup>	AF284267 <sup>a</sup>
<i>M. nigrescens</i>	Sulawesi	AF350383 <sup>d</sup>	AY224259 <sup>a</sup>	AF284268 <sup>a</sup>
<i>M. ochreata</i>	Sulawesi	AF350400 <sup>d</sup>	AY224260 <sup>a</sup>	AF284269 <sup>a</sup>
<i>M. brunnescens</i>	Sulawesi	AF350407 <sup>d</sup>	AY224247 <sup>a</sup>	—
<i>M. hecki</i>	Sulawesi	AF350376 <sup>d</sup>	AY224249 <sup>a</sup>	AF284256 <sup>a</sup>
<i>M. nemestrina</i>	Northeast Borneo	—	AY224253 <sup>a</sup>	AF284238 <sup>a</sup>
<i>M. nemestrina</i>	East Borneo	—	AY224254 <sup>a</sup>	AF284239 <sup>a</sup>
<i>M. nemestrina</i>	Samarinda, East Borneo	DQ355483 <sup>c</sup> , DQ355484 <sup>c</sup>	—	DQ355498 <sup>c</sup> , DQ355499 <sup>c</sup>
<i>M. nemestrina</i>	Sumatra	AY151094 <sup>b</sup> , AY151097 <sup>b</sup>	AY224255 <sup>a</sup>	AY224235 <sup>a</sup> , DQ355500 <sup>c</sup>
<i>M. nemestrina</i>	Malaysia	DQ355485 <sup>c</sup> , DQ355486 <sup>c</sup>	—	DQ355501 <sup>c</sup> , DQ355502 <sup>c</sup>
<i>M. pagensis</i>	Sipora	AY151124 <sup>b</sup> , AY151128 <sup>b</sup>	DQ355492 <sup>c</sup> , DQ355493 <sup>c</sup>	—
<i>M. pagensis</i>	South Pagai	AY151119 <sup>b</sup> , AY151120 <sup>b</sup> , AY151121 <sup>b</sup>	DQ355494 <sup>c</sup> , DQ355495 <sup>c</sup>	DQ355503 <sup>c</sup> , DQ355504 <sup>c</sup>
<i>M. siberu</i>	Siberut	AY151104 <sup>b</sup> , AY151109 <sup>b</sup> , AY151113 <sup>b</sup>	AY224256 <sup>a</sup> , AY224257 <sup>a</sup> , DQ355496 <sup>c</sup> , DQ355497 <sup>c</sup>	AF284264 <sup>a</sup> , AF284265 <sup>a</sup> , DQ355505 <sup>c</sup>
<i>M. leonina</i>	China	—	AY224251 <sup>a</sup>	AF284262 <sup>a</sup> , AF284263 <sup>a</sup>
<i>M. leonina</i>	Thailand	—	AY224252 <sup>a</sup>	AF284266 <sup>a</sup>
<i>M. leonina</i>	Laos	DQ355487 <sup>c</sup> , DQ355488 <sup>c</sup>	—	—
<i>M. leonina</i>	Vietnam	DQ355489 <sup>c</sup>	—	—
<i>M. silenus</i>	—	DQ355490 <sup>c</sup> , DQ355491 <sup>c</sup>	AY224263 <sup>a</sup> , AY224264 <sup>a</sup>	AF284237 <sup>a</sup> , AF284272 <sup>a</sup>

**Table 1.** Species, origin of samples, and accession numbers of analysed sequences [ <sup>a</sup> Tosi et al (2003); <sup>b</sup> Roos et al. (2003) ; <sup>c</sup> Newly generated sequences, <sup>d</sup> Unpublished, taken from GenBank ]

## 2.2 DNA extraction and PCR conditions

DNA from faeces was extracted with the Qiagen Stool Kit (Qiagen, Hildesheim, Germany) following procedures recommended by the supplier, with the exception that the DNA was diluted in HPLC quality water and stored at -20 °C before further processing. To study the evolution of the *M. silenus* group, we amplified and sequenced two mitochondrial regions and a portion of the Y chromosomal linked TSPY gene representing maternal and paternal inherited marker systems, respectively. The two mitochondrial loci represent a fragment of the Cytochrome *b* gene and a region spanning the 3' end of the 12S rDNA, tRNA<sup>Val</sup> and the 5' end of the 16S rDNA. The amplifications of the two loci were performed following PCR conditions and using oligonucleotide primers as described in Roos et al. (2003) and Tosi et al. (2002), respectively. A 420 bp long fragment of the TSPY gene was amplified using primers 5'- AAG CCA CCT GAA GAG GAA AC-3' and 5'-GGT AAG ATG ACT CCC CGT C-3'. Standard, wax-mediated hot start PCRs were carried out for 40 cycles, each with a denaturation step at 94 °C for 1 min, annealing at 58 °C for 1 min, and extension at 72 °C for 1 min, followed by a final extension step at 72 °C for 5 min. Aliquots of all PCRs were checked on 1% agarose gels. PCR products were subsequently cleaned with the Qiagen PCR Purification Kit and sequenced on an ABI3100-Avant sequencer using the Big-Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany) and the primers as indicated above or as described in Roos et al. (2003) and Tosi et al. (2002). All newly generated sequences were deposited in the GenBank database and are available under the accession numbers shown in Table 1.

### 2.3 Phylogenetic tree reconstruction (mitochondrial data)

To obtain a comprehensive overview of the phylogenetic relationships among members of the *M. silenus* group, the mitochondrial data sets were expanded using further sequences deposited at GenBank (Table 1). Both mitochondrial data sets comprise sequences from macaques representing all species and major populations of the *M. silenus* group, two representatives of the *M. sinica* group (*M. assamensis* and *M. thibetana*), one species of the *M. fascicularis* group (*M. fascicularis*), and the only species of the *M. sylvanus* group (*M. sylvanus*). As outgroup taxon, the hamadryas baboon (*Papio hamadryas*) was used. Sequence alignments were carried out with ClustalW (Thompson et al., 1994) and subsequently checked by eye. The final data sets for the Cytochrome *b* gene and the 12S–16S rDNA region comprised 31 and 27 sequences and have a length of 567 and 1509 bp, respectively. In the 12S–16S rDNA alignment, several indels were detected, which were removed with the Gblocks software (Castresana, 2000). After elimination of these poorly aligned positions, the final alignment of this locus comprised 1465 bp.

Phylogenetic tree reconstructions were carried out for both data sets separately, and by applying the maximum-parsimony (MP), neighbor-joining (NJ) and maximum-likelihood (ML) algorithms as implemented in PAUP 4.0 (SwoVord, 2002) and TREEPUZZLE 5.0 (Strimmer and von Haeseler, 1996), respectively. For MP analyses, all characters were treated as unordered and equally weighted throughout. NJ and ML trees for the Cytochrome *b* and 12S–16S rDNA data sets were constructed with the TrN+I+ $\Gamma$  and HKY+ $\Gamma$  models of sequence evolution, respectively, because they were selected as best fitting models with Modeltest 3.06 (Posada and Crandall, 1998). Statistical support for internal nodes was performed by bootstrap analyses (MP and NJ) with 1000 replications or by the quartet puzzling support values on the basis of 1000 puzzling steps (ML).

A Kishino–Hasegawa test in TREEPUZZLE was conducted to check the significance of the differences between the log likelihoods of alternative tree topologies. Therefore, all possible relationships among *M. silenus* taxa (excluding the Sulawesi macaques and *M. nemestrina* from Borneo) were tested.

### 2.4 Population aggregation analysis (TSPY data)

Of the amplified 420 bp of the TSPY gene, sequence information of 350 bp was used for further investigations. Since only a few informative sites were detected, we performed a population aggregation analysis (PAA) instead of reconstructing phylogenetic trees. The complete data set comprises 24 sequences from most members of the species group (Table 1). The PAA was performed according to the diagnostic character framework (Davis and Nixon, 1992). Accordingly, fixed nucleotide characters provided the unit for which aggregation of taxonomic units occurs. For diagnosis, attributes whose fixed unique states unite a group (populations, species), to the exclusion of other groups, were considered characters. Polymorphic attributes, or traits, were indicative of population frequency differences. To identify diagnostic sites, sequences were imported into MacClade 3.0 (Maddison and Maddison, 1992).

### 2.5 Estimation of the most recent common ancestors

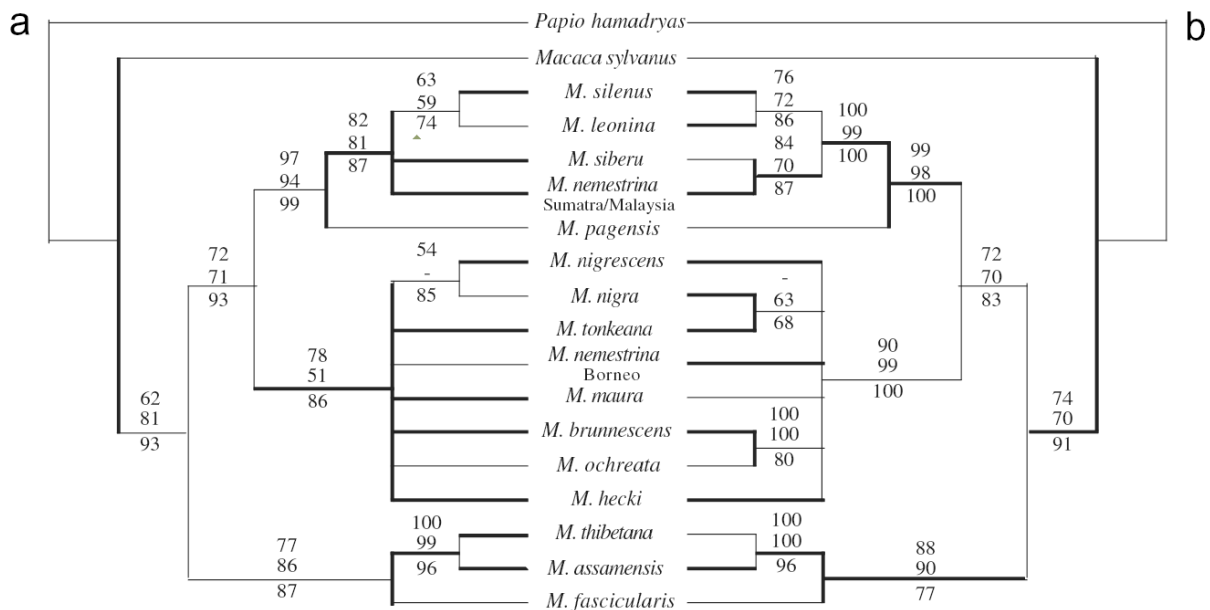
Divergence times between macaque species were estimated only from the two mitochondrial data sets, since only low variation was detected at the Y chromosomal locus. First, we examined the existence of significantly different lineage-specific evolutionary rates observable in the datasets by conducting a relative rate test for all possible pairwise comparisons and using *P. hamadryas* as

outgroup with the RRTree program (Robinson et al., 1998). Furthermore, we performed a molecular clock likelihood ratio test as implemented in TREEPUZZLE to check whether the sequences evolved in a clock-like manner. For the estimation of divergence times, the r8s program 1.7 (Sanderson, 2003) was used. Age calculations were conducted on the basis of estimated branch lengths as deduced from an *a priori* fixed tree topology, and Powell's optimisation, with all other settings set by default. As calibration point we used the proposed value of 5.5 million years ago (mya) for the divergence between the only African macaque species *M. sylvanus* and the Asian species (Delson, 1996).

### 3. Results

#### 3.1. Mitochondrial DNA

To determine the phylogenetic relationships among analysed species, phylogenetic tree reconstructions were conducted for each of the two mitochondrial data sets separately and by applying MP, NJ and ML algorithms. The phylogenetic relationships obtained from both data sets display in general the same tree topology and differ only in their support values for different branches (Fig. 2a and b). For several species, more than one individual was tested. In all these cases, the species monophyly was supported by high bootstrap or puzzling support values of at least 95% (data not shown).



**Fig. 2.** Phylogenetic relationships as obtained from the mitochondrial Cytochrome *b* (a) and 12S-16SrRNA (b) genes. Numbers on branches indicate bootstrap or puzzling support values for the NJ (top), MP (middle) and ML (bottom), respectively.

In all trees, *M. sylvanus* branched off first, confirming the sister group relationship of African and Asian macaques. Within the Asian clade, a major split occurred between the *M. silenus* group and representatives of the *M. sinica* (*M. assamensis* and *M. thibetana*) and *M. fascicularis* (*M. fascicularis*) group. The *M. silenus* group further diverged into a clade containing all Sulawesi macaques (*M. brunnescens*, *M. hecki*, *M. maura*, *M. nigra*, *M. nigrescens*, *M. ochreata*, *M. tonkeana*) and *M. nemestrina* from Borneo, and a clade comprising all the other members of the *M. silenus* group (*M.*

*pagensis*, *M. siberu*, *M. leonina*, *M. silenus*, *M. nemestrina* from Sumatra and peninsular Malaysia). Within the latter clade, *M. pagensis* split off first. The relationships among the remaining four species are not well resolved, although at least a *M. leonina*–*M. silenus* grouping is indicated. The relationships among the three lineages *M. nemestrina* from Sumatra and the peninsular Malaysia, *M. siberu*, and *M. leonina*–*M. silenus* are depicted as a trichotomy in the Cytochrome *b*-based trees. In contrast, in the 12S–16S rDNA-based trees, *M. nemestrina* and *M. siberu* form a weakly supported sister clade to *M. silenus* and *M. leonina*.

Since the relationships among the *M. silenus* group members (excluding the Sulawesi macaques and *M. nemestrina* from Borneo) are not well resolved and contradicting topologies were depicted by the two data sets, the log likelihoods of alternative tree topologies were determined (Table 2). For both data sets, the tree in which a monophyletic Mentawai clade (*M. siberu* and *M. pagensis*) forms a sister clade to *M. silenus*, *M. leonina* and *M. nemestrina* is rejected at a significance level of 5%; trees in which *M. pagensis* is sister to the other four species however cannot be rejected. Alternative relationships among *M. nemestrina*, *M. siberu* and a *M. silenus*–*M. leonina* clade are not rejected; hence, none of them can be excluded. Based on this information, *M. pagensis* is the sister species to the remainder. The relationships among *M. siberu*, *M. nemestrina* and *M. silenus*–*M. leonina* are not resolved and alternative relationships are possible, so that an unresolved trichotomy between the three lineages may be the best way to describe the relationships among them.

Tree topology	Cyt <i>b</i>			12S–16S rRNA		
	Log likelihood	SE	Different at <i>P</i> < 0.05	Log likelihood	SE	Different at <i>P</i> < 0.05
((( <i>silenus,leonina</i> ), <i>nemestrina,siberu</i> ), <i>pagensis</i> )	<b>–2812.75</b>	—	<b>Best</b>	–4830.99	2.66	No
((( <i>silenus,leonina</i> ),( <i>nemestrina,siberu</i> )), <i>pagensis</i> )	–2826.49	7.19	No	<b>–4828.63</b>	—	<b>Best</b>
((( <i>silenus,leonina</i> ), <i>nemestrina</i> ), <i>siberu</i> ), <i>pagensis</i> )	–2826.49	7.19	No	–4830.99	2.66	No
((( <i>silenus,leonina</i> ), <i>siberu</i> ), <i>nemestrina</i> ), <i>pagensis</i> )	–2823.79	7.83	No	–4831.00	2.66	No
(( <i>silenus,leonina,nemestrina,siberu</i> ), <i>pagensis</i> )	–2828.09	7.50	Yes	–4837.93	5.50	No
((( <i>silenus,leonina</i> ), <i>nemestrina</i> ),( <i>siberu,pagensis</i> ))	–2828.94	7.90	Yes	–4853.66	10.22	Yes

**Table 2.** Calculated log likelihoods of alternative tree topologies based on the two different mitochondrial loci

### 3.2 Y chromosomal DNA

The TSPY data set for the PAA comprised 24 sequences from most species and major populations of the *M. silenus* group (Table 1).

In the analysed 350 bp long fragment we detected only 10 polymorphic sites of which only four (at position 26, 67, 138 and 337) are informative in that they separate the Sulawesi macaques from the remaining members of the group (Table 3). The remaining sites were detected in different Sulawesi macaques or within different populations of Sumatran *M. nemestrina* or *M. siberu*. Since no informative sites were detected to distinguish between different pigtailed macaque species (*M. nemestrina*, *M. leonina*, *M. pagensis*, *M. siberu*) and liontailed macaques, the TSPY dataset allows no resolution of the phylogenetic relationships among them.

### 3.3. Estimation of the most recent common ancestors

Since only low variation was detected at the Y chromosomal locus, divergence ages were calculated only from the two mitochondrial data sets. Because significantly different evolutionary rates between several lineages were detected and an overall clock-like sequence evolution was rejected at a significance level of 5%, divergence times and their 95% confidence intervals were estimated by applying nonparametric methods that relax the stringency of the molecular clock assumption. Using

the fossil evidence of 5.5 mya for the main split between African (*M. sylvanus*) and Asian macaques as calibration point, we estimated the divergence times of splitting events within the macaques (Table 4). According to our analysis, after macaques colonised Asia, a first major split occurred between the *M. silenus* group and a proto-*M. fascicularis* group about 5.1 mya. The latter group diverged into the *M. fascicularis* and *M. sinica* group about 3.7–4.0 mya. Roughly at the same time, Sulawesi macaques and *M. nemestrina* from Borneo were separated from the remaining species of the *M. silenus* group. Afterwards, *M. pagensis* diverged about 2.4–2.6 mya from an ancestor of the other four species. The lineages leading to *M. siberu*/*M. nemestrina* and *M. leonina*/*M. silenus* were separated from each other about 1.5–1.7 mya. The split between *M. leonina* and *M. silenus* occurred about 1.1–1.5 mya.

	26	67	81	87	116	132	138	218	265	337
<i>M. hecki</i>	T	C	A	G	C	T	T	A	A	A
<i>M. maura</i>	T	C	G	C	C	T	T	C	A	A
<i>M. nigra</i>	T	C	A	C	C	T	T	A	A	A
<i>M. nigrescens</i>	T	C	A	C	C	T	T	A	A	A
<i>M. ochreata</i>	T	C	A	C	C	C	T	A	A	A
<i>M. tonkeana</i>	T	C	A	C	C	T	T	A	A	A
<i>M. pagensis</i>	G	A	A	C	C	T	C	A	A	G
<i>M. siberu</i> I	G	A	A	C	C	T	C	A	A	G
<i>M. siberu</i> II	G	A	A	C	G	T	C	A	A	G
<i>M. nemestrina</i> Sumatra I	G	A	A	C	C	T	C	A	C	G
<i>M. nemestrina</i> Sumatra II	G	A	A	C	C	T	C	A	A	G
<i>M. nemestrina</i> Malaysia	G	A	A	C	C	T	C	A	A	G
<i>M. nemestrina</i> Kalimantan	G	A	A	C	C	T	C	A	A	G
<i>M. leonina</i>	G	A	A	C	C	T	C	A	A	G
<i>M. silenus</i>	G	A	A	C	C	T	C	A	A	G

**Table 3.** Polymorphic characters in the 350 bp fragment of the TSPY gene based on the population aggregation analysis

#### 4. Discussion

The present study is the first to include all twelve members of the *M. silenus* group and as such, allows us to present important results with far reaching consequences for models explaining the distribution patterns during the evolutionary history of the *M. silenus* group. The combined approach involving the analysis of one paternal and two maternal molecular markers of all extant members of the *M. silenus* group has enabled us to resolve phylogenetic relationships within the species group. Moreover, the phylogenetic relationships and splitting times separating the different lineages obtained from the two mitochondrial loci are largely congruent with earlier studies (Deinard and Smith, 2001; Evans et al., 2003; Hayasaka et al., 1996; Morales and Melnick, 1998; Page et al., 1999; Roos et al., 2003; Tosi et al., 2000, 2003).

The main finding of the study is that, based on our mitochondrial tree, macaques from the three southern Mentawai islands (Sipora, North and South Pagai; *M. pagensis*) represent a sister clade to those from the northern Mentawai island of Siberut and the Southeast Asian mainland. This result is in stark contrast to previous assumptions that all Mentawai macaques share a common ancestor, which led to the traditional classification of both as subspecies of either *M. nemestrina* or *M. pagensis*

Divergence times of the most recent common ancestors calculated from the two mitochondrial data sets, calibrated with the fossil evidence (5.5 mya)

Most recent common ancestor	Cyt b divergence (mya)	12S–16S rRNA divergence (mya)
<i>Macaca</i>	5.5	5.5
<i>M. silenus</i> group–proto- <i>M. fascicularis</i> group	5.1 (±0.4)	5.1 (±0.3)
<i>M. fascicularis</i> –( <i>M. thibetana</i> / <i>M. assamensis</i> )	4.0 (±0.3)	3.7 (±0.3)
<i>M. silenus</i> group	3.7 (±0.3)	4.0 (±0.4)
Sulawesi macaques (including <i>M. nemestrina</i> from Borneo)	2.0 (±0.4)	1.9 (±0.8)
<i>M. pagensis</i> –( <i>M. siberu</i> / <i>M. nemestrina</i> / <i>M. leonina</i> / <i>M. silenus</i> )	2.6 (±0.1)	2.4 (±0.3)
<i>M. siberu</i> – <i>M. nemestrina</i> –( <i>M. leonina</i> / <i>M. silenus</i> )	1.7 (±0.2)	1.5 (±0.2)
<i>M. leonina</i> – <i>M. silenus</i>	1.5 (±0.2)	1.1 (±0.1)

**Table 4.** Divergence times of the most recent common ancestors calculated from the two mitochondrial data sets, calibrated with the fossil evidence (5.5mya)

(Brandon-Jones et al., 2004; Fooden, 1975, 1980; Fuentes and Olson, 1995; Groves, 2001). However more recently, they have been recognized as distinct species (Abegg and Thierry, 2002a; Kitchener and Groves, 2002). Although a paraphyletic origin of Mentawai macaques has previously been shown by Roos et al. (2003), it was highly unexpected that (at least based on mitochondrial data) *M. pagensis* actually forms a sister clade to all mainland forms as well as Siberut macaques, and not only to a clade containing *M.*

Incongruence between the two mitochondrial data sets was obtained for the relationships between *M. siberu* and *M. nemestrina* (from Sumatra and peninsular Malaysia) and the *M. leonina*–*M. silenus* clade. The sister grouping of *M. nemestrina* and *M. siberu* as depicted by the 12S–16S rRNA data set, however, is not significantly supported and alternative relationships cannot be excluded. Thus, an unresolved trichotomy as indicated by the Cytochrome *b* data may be the best alternative to describe the relationships among *M. siberu*, *M. nemestrina* and the *M. leonina*–*M. silenus* clade.

In order to detect possible introgression or hybridization events, we also analysed a portion of the paternal inherited TSPY gene. Although only low variation was detected at the Y chromosomal locus and no informative sites were found to allow discrimination between pigtailed macaques, the obtained grouping pattern are not in contrast to the mitochondrial data, with the exception that *M. nemestrina* from Borneo clusters with the other pigtailed macaques and not with the Sulawesi macaques. This result however is not unexpected, since it is in agreement with earlier studies (Evans et al., 2003; Tosi et al., 2003), and may be explained by later male-mediated gene flow.

Phylogenetic relationships and splitting time estimates presented here are mainly based on mitochondrial DNA since the analysed fragment of the TSPY gene shows only four diagnostic sites separating Sulawesi macaques from the remaining members of the group. Since macaques are mainly female philopatric, which leads to high mitochondrial variability between populations but not within populations (Melnick and Hoelzer, 1993), and no autosomal markers were analysed in the present study, we are not able to exclude events such as nuclear panmixia via male mediated gene flow as, for example, is seen in the case of Bornean *M. nemestrina*. Moreover, large differences between nuclear and mitochondrial splitting time estimates were calculated (Evans et al., 2003; Tosi et al., 2003), indicating that our estimates do not necessarily represent the definitive divergence times. As we will argue below, however, divergence estimates derived from this study do coincide with palaeogeographic events that are thought to have led to divergence in other species groups. This congruence gives us some confidence that our divergence estimates provide a relatively accurate picture of major events in macaque evolution.

Although further genetic data are required to definitively solve the phylogeny of the *M. silenus* group members, the present results on phylogenetic relationships and splitting times of the different lineages in combination with data on behaviour, as well as geological and climatic conditions during the last 5 million years, allow us to reconstruct possible scenarios leading to the distribution of the *M. silenus* group members seen today.

#### 4.1. Reconstructing the evolutionary history of the *M. silenus* group

The complicated zoogeography of Southeast Asia, which is subject to ongoing scientific discussion, is closely related to very active tectonics, changing climate and sea levels during the Plio- and Pleistocene and the periodic formation of landbridges (Holloway and Hall, 1998; Voris, 2000; Meijaard, 2003a,b). According to our mitochondrial data, the progenitor of the *M. silenus* group split off from the proto-*M. fascicularis* group at about the same time as an early-middle Pliocene sea level highstand (Woodruff, 2003), which effectively split the Sundaic fauna from mainland Asia along the Isthmus of Kra. Subsequently, the ancestor of the *M. silenus* group spread throughout Sundaland

around 4 mya, when extensive evergreen forests were promoted by a wet climate and there were no extensive tropical rainforest areas north of the Malay Peninsula (Morley, 2000). Later on, the species group split into an eastern (Bornean) and western (Sumatran, Malaysian) lineage most likely as a result of an early vicariance event in central Sundaland (Evans et al., 2003). It is likely that the break up of the land area connecting Borneo to the Malay Peninsula occurred around this time (see Meijaard, 2003b), and that it played a role in the separation of the two macaque lineages. Subsequent range expansion towards Sulawesi in the East and mainland Asia in the North, as well as towards the Mentawai islands in the West, was associated with further diversifications of these lineages. In the East, Bornean *M. nemestrina* reached Sulawesi most likely by natural rafting (Meijaard, 2003b) and diversified into numerous distinct species about 1.9–2.0 mya. In the West, the Mentawai islands were colonised about 2.4–2.6 and 1.5–1.7 mya (detailed discussion see below). The remaining pigtailed macaque population in the western Sunda region further expanded to the North into mainland Southeast Asia. The ancestor of *M. silenus* dispersed into India about 1.1–1.5 mya and was subsequently isolated, probably by the shrinkage of suitable habitat. Since only small numbers of individuals are likely to have formed the founder populations of *M. pagensis*, *M. siberu*, *M. silenus* and the Sulawesi macaques, these populations can be assumed to have differentiated into the respective species while still possessing several ancestral characteristics, such as the dark pelage coloration and certain behavioural components (Abegg and Thierry, 2002a). In contrast, comparatively large populations on Sumatra, Borneo and mainland Southeast Asia remained undifferentiated and later, male-mediated gene flow via migration across the Sunda region during lowered sea levels, homogenized variation in DNA and morphology in *M. nemestrina* (Evans et al., 2003).

Events leading to the occurrence of two forms of macaques on the Mentawai islands however, remain largely unexplained. As the shallow-water route between Sumatra and the Mentawai islands leads through the Batu islands in the north, dispersal into the Mentawais would be via Siberut [but we note that this is a tectonically very active area with ongoing local subsidence and uplift; present-day geographies may have little in common with palaeogeographies several million years ago]. From the evidence currently available, one possible scenario would be that the colonisation of the Mentawai islands by macaques occurred as two independent events (Roos et al., 2003). Following this scenario, we propose that in the first dispersal event about 2.4–2.6 mya, a *proto-M. nemestrina*, widespread over Sundaland, colonised the entire Mentawai region via landbridges which emerged during a major glacial in early Pleistocene when sea levels were 180 m below present (Worldbath, 2000). Subsequently, sea levels rose and these macaques, isolated in the Mentawai region, evolved into *proto-M. pagensis* forms. After this first colonisation of all Mentawai islands, a second invasion of Siberut may have been possible by rafting or with the emergence of another short-lived (non forested) landbridge about 900,000 years later (1.5–1.7 mya), which closed before macaques were able to invade the southern islands. Having arrived on Siberut, females from the mainland hybridized with the island form (leading to the fixation of the mainland mitochondrial haplotypes) or alternatively, the island form was completely replaced by the newcomers.

Although plausible, this “two colonisation theory” cannot be confirmed with available data, and alternative scenarios explaining the biogeographical patterns of the *M. silenus* group seen today need to be considered. One possible alternative, the “island refuge” scenario, can be described as follows:

Whereas members of an eastern population of *M. nemestrina* reached Sulawesi by natural rafting, western populations colonized the Mentawai Islands using a landbridge from Sumatra, which emerged during a major global cooling event about 2.4–2.6 mya (Shackleton et al., 1984; Brandon-Jones, 1998; Dennell, 2004). Subsequently, this progenitor became extinct over most of its distribution in Southeast Asia during a particularly cold and dry glacial of the late Pliocene that greatly reduced

forested habitat availability (Brandon-Jones, 1996, 1998; Heaney, 1991), but was able to survive in a number of equatorial islands (including the Mentawais) at the periphery of its former distribution (Meijaard, 2003b) were climatic (and ecological) conditions may have been more stable (Meijaard, 2004; Verstappen, 1975). After having colonized the Mentawai archipelago, these macaques became isolated from Sumatra at the advent of warmer climates, when sea levels remained above 160 m. Mentawai macaques differentiated while they were isolated on their respective islands and evolved independently for about 900,000 years when some macaques from Siberut were able to recolonise Sumatra during another period of sea water level low stand about 1.5–1.7 mya (Haq et al., 1987). Once established again in mainland Sumatra, these ancestors of the extant *M. silenus* lineage were able to disperse again, eventually as far as India (Brandon-Jones, 1998). Finally, during the Pleistocene (about 1.1–1.7 mya), they diversified into *M. nemestrina*, *M. leonina* and *M. silenus*. In such a way, the Mentawai islands, as well as Sulawesi and Borneo may have served as ecological refuges for the progenitor of the oldest macaque radiation in Asia. Although speculative, some support for this scenario is provided by the fact that all other Mentawai primates are believed to be ancestral to their mainland relatives based on morphological and behavioural data (Brandon-Jones, 1977; Fuentes, 1996; Meijaard, 2004) and, at least for *Simias concolor* also genetic data (Whittaker et al., 2006). For the other two species (*Hylobates klossii*, *Presbytis potenziani*), genetic data are missing or allow no definitive conclusions.

It is clear that our two proposed colonization scenarios to explain the phylogeographic pattern seen in macaques of the *M. silenus* species group are mainly based on mitochondrial data and that neither can be ultimately confirmed or rejected. Final resolution of this issue requires insights from genetic studies of the other primates endemic to the Mentawais, as well as the availability of further detailed information about macaque phylogenetic relationships and splitting times, including the use of nuclear markers.

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