Faecal DNA to the rescue: Shotgun sequencing of non-invasive samples reveals two subspecies of Southeast Asian primates to be Critically Endangered species

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Abstract

A significant number of Southeast Asian mammal species described in the 19th and 20th century were subsequently synonymized and are now considered subspecies. Many are affected by rapid habitat loss and there is thus an urgent need to re-assess the conservation status based on species boundaries established with molecular data. However, such data are lacking for many populations and subspecies. We document via a literature survey and empirical study how shotgun sequencing of faecal DNA is a still underutilized but powerful tool for accelerating such evaluations. We obtain 11 mitochondrial genomes for three subspecies in the langur genus *Presbytis* through shotgun sequencing of faecal DNA (*P. femoralis femoralis*, *P. f. percura*, *P. siamensis* cf. *cana*). The genomes support the

resurrection of all three subspecies to species based on multiple species delimitation algorithms (PTP, ABGD, Objective Clustering) applied to a dataset covering 40 species and 43 subspecies of Asian colobines. For two of the newly recognized species (*P. femoralis*, *P. percura*), the results lead to an immediate change in the IUCN status to Critically Endangered due to small population estimates and fragmented habitat. We conclude that faecal DNA should be more widely used for clarifying species boundaries in endangered mammals.

Introduction

Human impacts on the environment have rapidly accelerated species extinction via habitat degradation and climate change. Recent report by Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services (IPBES) predicts that climate change has already affected the distribution of nearly half (47%) of land-mammals[1]. Conservation efforts are urgently needed but are hampered by the lack of data for a large number of mammal species, subspecies, and populations which face imminent extinction[2,3,4]. A typical example is Asian primates for which 70% of the species are threatened with extinction[5]. Effective conservation programs are needed but they require a robust understanding of species numbers and boundaries based on up-to-date taxonomic information[6,7]. Unfortunately, this information is lacking for many rare, globally threatened, and elusive mammalian species. Many lack molecular data and collecting these data is difficult because invasive sampling that would yield fresh tissues is often not feasible.

This leaves only three alternative sources of DNA. The first is museum specimens, but the number of samples in museums tends to be small and many were collected in the 19th or early 20th century thus reflecting (historic) genetic diversity prior to extensive habitat loss. The second is tissue samples obtained from specimens that died of "natural causes" such as road accidents. The third source of genetic material is non-invasive samples such as hair and faeces. Arguably, faecal samples are still an underappreciated source of information although they could be collected in good numbers during routine field surveys. This can make faecal samples particularly useful for data-deficient taxa that are in urgent need for re-assessment of species boundaries. Faecal samples contain a complex pool of DNA including that of the host. The host DNA is particularly informative because it reflects the current genetic diversity of the species. However, many field research protocols still lack the collection of faecal samples although it is now straightforward to obtain complete mitochondrial genomes from

such samples using shotgun sequencing[8,9]. In this study, we document the power of faecal metagenomics for testing the species boundaries in two species of Asian primates that are listed as Data Deficient on the IUCN Red List of Threatened Species.

Asian colobines (langurs and odd-nosed monkeys) are a diverse group of mammals, with 55 recognized species (87 spp.) belonging to seven genera (*Nasalis*, *Presbytis*, *Pygathrix*, *Rhinopithecus*, *Semnopithecus*, *Simias*, *Trachypithecus*)[10]; i.e., nearly half of all primate species in Asia are colobines. Unfortunately, many of these species are dependent on habitats that are quickly disappearing. Thus, nine species are already considered Critically Endangered, 23 are Endangered, and nine Vulnerable according to IUCN threat criteria[5]. This also applies to the genus *Presbytis* which is one of the most species-rich primate genera[11]. The 17 recognized species are found in the tropical rainforests of Sundaland, including the Malay Peninsula and the western Indo-Malay Archipelago[12,13]. Eleven species within *Presbytis* (*chrysomelas*, *comata*, *femoralis*, *frontata*, *hosei*, *melalophos*, *natunae*, *potenziani*, *rubicunda*, *siamensis*, and *thomasi*) were recognized in the last IUCN assessment[14]. The assessment predated the elevation of six subspecies to species (see Roos et al.[10]: *bicolor*, *canicrus*, *mitrata*, *sabana*, *siberu*, *sumatrana*) which suggests that many of the *Presbytis* taxa currently ranked as subspecies are in urgent need for re-assessment with molecular data. Unfortunately, these data are lacking for many subspecies and species which has serious consequences for the proper conservation assessment of these taxa.

Meyer et al.[11] presented the most comprehensive phylogenetic reconstruction of *Presbytis* which included 13 of the 17 recognized species. The analysis was based on two mitochondrial markers (cyt-b and d-*loop*). Of interest in this study is the banded langur *P. femoralis*, the relationship within which has been addressed by several studies. *Presbytis femoralis* is found on the Malay Peninsula and the island of Sumatra[15] (Fig. 1). The species

currently consists of three subspecies that were originally described as species because they are distinguishable based on a combination of morphological characters. However, many primatologists currently considered these characters insufficient for recognizing the three taxa as species (see Table 1).

The nominal species, *Presbytis femoralis*, was described by Martin (1838) based on specimens collected by Raffles (1821) from Singapore[16,17]. Raffles' banded langur *P. f. femoralis* occurs in southern Peninsular Malaysia and Singapore. East Sumatran banded langur *P. f. percura* (Fig. 2) occurs only in eastern Sumatra and was described by Lyon (1908) based on specimens collected from near Siak Kecil River, Makapan, Kompei, Pulau Rupat, and Salat Rupat[18]. Robinson's banded langur *P. f. robinsoni* was described by Thomas (1910) based on white phenotypic variants collected in Trang, southern Thailand[19,20,21]. However, typical *robinsoni* specimens are uniformly dark brown to black, with the inner side of upper arms, lower abdomen following onto the inside of the thighs to the heel being white. *Presbytis f. robinsoni* is widespread and ranges from northern Peninsular Malaysia to southern Thailand and Myanmar.

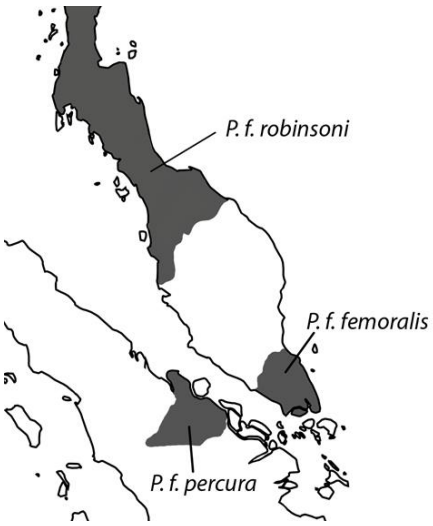


Figure 1.

Figure 2.

Table 1.

	femoralis	*percura*	*robinsoni*
Fur coat	Less dark; dusky greyish brown on the top of the head, the back, and the shoulders to the elbows	Less dark; upper parts of the head, the body, the feet, the hands, and the tail are black	Darkest; uniformly dark brown to black
Amount of white on body	The chin, a line down the chest and abdomen, the inside of the humeri from the axilla, and the inside of the thighs	The belly, a narrow line on the chest, the inner side of thighs extending to the heel, the inner side of arms from the axilla to the wrist, and the chin	The inner side of upper arms, lower abdomen following onto the inside of the thighs to the heel
Coloration of underparts	Pale of variable extent, but at least leaving the postumbilical area pale; the pale marking follows onto the inner side of the leg as a well-defined (femoral) stripe	Not uniform dark brown from umbilical region to chin; anterior margin of pale postumbilical region not sharply defined; outer side of thigh often with some trace of gray	Uniform dark brown from umbilical region to chin; anterior margin of pale postumbilical area sharply defined; outer side of thigh without trace of gray
Number of whorls on crown	A pair, with a long crest between, or one of the pair may be suppressed	One or (occasionally) a pair	One or a pair
Direction of hairs on chest	All directed backward	All directed backward	Directed more outward than backward on the sides of chest, and directed back in the midline of chest

Pale stripe on the underside of tail	Absent	Short and poorly developed	Very faint or absent
Condylo-basal length of skull	~65mm	at least 70mm	at least 70mm
Mandible	~60mm	~68mm	~68mm

References: Groves[15]; Martin[16]; Raffles[17]; Lyon[18]; Thomas[19]; Robinson and Kloss[20]; Weitzel et al.[21]; Miller[72]

Presbytis femoralis, encompassing all three subspecies, is listed as Vulnerable in the most recent IUCN Red List assessment, with the nominate subspecies considered Endangered because the known populations are restricted to small and isolated patches of forest. In addition, one population from Singapore showed low genetic variability[9,22]. *Presbytis f. robinsoni* is considered Near Threatened, while the least known and least studied subspecies is *P. f. percura* which is currently Data Deficient[23]. Genetic data suggest that at least *P. f. femoralis* and *P. f. robinsoni* are different species[24,25] which is also in agreement with the aforementioned morphological characters. However, resolving all species-level boundaries within banded langurs required data for *P. f. percura*.

For several reasons the species boundaries within *P. femoralis* remain poorly understood. The main problem is the lack of molecular data for *P. f. percura.* However, even if the data were available, a comprehensive analysis would still be difficult because the sequence data from three published analyses were not submitted to public sequence repositories (cyt-b, 12S rDNA, and d-*loop*)[26,27,28]. This means that currently the only publicly available molecular data are for *P. f. femoralis* from the type locality in Singapore (KU899140)[9] and *P. f. robinsoni* from Redang Panjang, Malaysia (DQ355299)[29]. Some additional molecular data can be reconstructed based on a table published in Abdul-Latiff et al.[28] that lists the variable d-*loop* sites for several species and subspecies (see Nijman[25]). The last complication is the confusing nomenclatural changes. The authors proposed to replace the type species *P. femoralis* (Martin 1838) with a junior synonym (*P. neglectus neglectus* Schlegel 1876)[30] without considering the detailed information in Low and Lim[31] that explains why Martin is

the author of the name *femoralis* and Singapore the type locality of the species. Abdul-Latiff et al.[28]'s study furthermore violated its own proposed nomenclatural changes by retaining *P. f. percura* and *P. f. robinsoni* (see Nijman[25]).

Here, we solve these problems by providing the first mitogenomes of *P. f. percura* and thus addressing the taxonomic position of all three subspecies of banded langurs. We also obtain the first mitogenome of the Riau pale-thighed langur *P. siamensis* cf. *cana* from Sumatra which helps with resolving species limits within this species. Lastly, we provide an updated dated phylogenetic tree for Asian colobines based on mitochondrial genomes and survey the mammal literature to illustrate that faecal DNA is currently still an underutilized source of genetic information.

Results

Survey of Zoological Record

In order to investigate to what extent faecal samples have been used in addressing taxonomic problems, we surveyed the literature as captured in Zoological Record. We retrieved 1,852 articles that mentioned faecal samples, but only a subset of 43 articles were also classified under Systematics/Taxonomy. Inspection of these records revealed only two studies that used faecal DNA for resolving species limits[32,33].

Sequence Data

Illumina sequencing of faecal metagenomes yielded 60.3-69.7 million sequences for each sample from Sumatra (ESBL1-8, Pres2; Table 2). The data were combined with the Hi-Seq data for six samples from Singapore (BLM1-6)[9]. All data were quality trimmed using Trimmomatic[34] and complete mitochondrial genomes were obtained. One sample of

150 *Presbytis femoralis percura* ESBL_7 had a low average coverage of <5X for the

151 mitochondrial genome and was not analysed further.

152

153 Table 2.

Sample ID	Location	Organism	Raw/trimmed (millions)	Average mitochondrial coverage (X)
ESBL_1b	Kampar	*Presbytis femoralis percura*	60.33/58.81	25.205
ESBL_5	Bengkalis	*Presbytis femoralis percura*	63.38/61.74	5.155
ESBL_6a	Bengkalis	*Presbytis femoralis percura*	67.04/66.61	8.721
ESBL_8b	Bengkalis	*Presbytis femoralis percura*	69.69/67.98	13.275
Pres2	Kampar	*Presbytis siamensis* cf. *cana*	67.37/65.69	9.358
BLM1	Central Catchment Nature Reserve	*Presbytis femoralis femoralis*	107.68/92.35	21.794
BLM2	Central Catchment Nature Reserve	*Presbytis femoralis femoralis*	72.66/60.18	19.716
BLM3	Central Catchment Nature Reserve	*Presbytis femoralis femoralis*	85.96/74.05	15.029
BLM4	Central Catchment Nature Reserve	*Presbytis femoralis femoralis*	66.99/56.88	37.606
BLM5	Central Catchment Nature Reserve	*Presbytis femoralis femoralis*	68.19/56.15	104.315
BLM6	Central Catchment Nature Reserve	*Presbytis femoralis femoralis*	76.44/65.11	12.708

154

155 *Species Delimitation*

156 Pairwise comparison of cyt-b, hypervariable region HV1 of the d-*loop* and mitochondrial

157 genomes (CDS+rDNA+d-*loop*) revealed minimum genetic divergence of 7.1%, 6.1% and

158 5.3% between *P. f. femoralis* and *P. f. percura.* On the other hand, the minimum pairwise

159 distance between either these taxa with *P. f. robinsoni* is 6.0% for HV1, 10.3% for cyt-b and

160 7.6% across the mitochondrial genome. For the two subspecies of *P. siamensis*, we were only

161 able to compare HV1 sequences. The HV1 sequence of *P. s.* cf. *cana* is 11.1% diverged from

162 *P. s. siamensis* and 5.1% from *P. melalophos* (KY117602), while cyt-b and complete

163 mitochondrial genomes show divergence of 2.8% and 2.5% between sequences from *P. s.* cf.

164 *cana* and *P. mitrata/P. melalophos.* Overall, these results suggest that *P. s.* cf. *cana*

165 represents a genetically distinct *Presbytis* lineage.

166 The high genetic distances are consistent with results of species delimitation using Poisson

167 Tree Processes (PTP)[35], Automated Barcode Gap Discovery (ABGD)[36] and Objective

168 Clustering[37]. PTP consistently split *P. f. femoralis* and *P. f. percura* into different molecular

169 Operational Taxonomic Units (mOTUs) across the three datasets examined (Asian colobine
170 mitogenome and *Presbytis* mitogenome+cyt-b+HV1, and *Presbytis* HV1-only datasets)
171 (Supplementary Figs. S1-3). ABGD and Objective Clustering (thresholds 2-4%) similarly
172 split these two subspecies across different datasets (Asian colobine mitogenome, *Presbytis*
173 HV1 and cyt-b) and a range of parameters (Table 3). For ABGD, these subspecies would
174 only lump if unusually high priors for intraspecific divergences were used (priors>=0.0215).
175 These parameters are not likely to be appropriate because they also led to collapse of many
176 recognized *Presbytis* species into a single mOTU. All three species delimitation methods also
177 placed *P. f. robinsoni* as a distinct species from *P. f. femoralis* and *P. f. percura.* Lastly, *P. s.*
178 cf. *cana* was placed as a distinct species using PTP and ABGD unless inappropriately high
179 priors for intraspecific divergence are used in ABGD. Objective Clustering based on HV1
180 also identified *P. s.* cf. *cana* as a distinct species. However, the mitogenome and cyt-b
181 datasets lumped *P. s.* cf. *cana* with *P. melalophos* and *P. mitrata* at 3% and 4% thresholds.

182 We observed further species-level splitting when species delimitation was based on only HV1
183 data for the subspecies of *P. femoralis*. This dataset included sequences for multiple
184 individuals of *P. f. femoralis, P. f. robinsoni* and *P. s. siamensis* from the Malay Peninsula
185 (reconstructed in Nijman[25], based on Abdul Latiff et al.[28]). At low prior intraspecific
186 divergences, ABGD split some haplotypes of *P. f. femoralis* from the Malay Peninsula as
187 separate mOTUs from other haplotypes of the same subspecies from the same region. These
188 however consistently grouped together as single mOTU at higher thresholds. Similarly, PTP
189 based analyses of only HV1 data split haplotypes of *P. f. robinsoni* into multiple mOTUs
190 (Fig. S2) while ABGD consistently placed them as a single species.

191 Table 3.

	cyt-b, N =16				HV1, N=17				Mitogenome, N=33		
Prior intraspecific divergence	X=0.1	X=0.5	X=1		X=0.1	X=0.5	X=1		X=0.1	X=0.5	X=1
0.0010	21	21	21		26	26	24		33	33	33

0.0017	21	21	21		26	26	24		33	33	33
0.0028	20	20	20		26	26	24		33	33	33
0.0046	20	20	20		23	23	21		33	33	33
0.0077	20	20	20		21	21	21		31	31	22
0.0129	20	20	20		21	21	21		19	19	21
0.0215	18	18	NA		11	1	NA		19	19	21
0.0359	8	NA	NA		NA	NA	NA		12	11	7

Mitochondrial Phylogeny of Asian Colobines and Genus Presbytis

The phylogenetic reconstruction based on mitochondrial genomes of Asian colobine dataset revealed that *P. femoralis* is polyphyletic (Fig. 3). The reconstructions based on Maximum Likelihood (ML) and Bayesian Inference (BI) are congruent and reveal that *P. f. femoralis* and *P. f. percura* are sister taxa. Divergence time estimates dated the split of *P. f. femoralis* and *P. f. percura* at 2.6 Mya (CI: partitioning by codon: 1.96-3.35 Mya, partitioning by gene 1.90-3.37 Mya (Supplementary Fig. S4)). This clade is sister to clade comprising of *P. mitrata, P. comata, P. siamensis* cf. *cana*, and *P. melalophos*. *Presbytis f. robinsoni* diverged from these species at 4.5 Mya (CI: partitioning by codon: 3.49-5.48 Mya, partitioning by gene 3.46-5.62). Overall, the mitochondrial phylogeny reveals high support for clade comprising of *Presbytis* and *Trachypithecus* as well as all the relationships within the (((*Simias*+*Nasalis*)+*Pygathrix*)+*Rhinopithecus*) clade. Only the placement of *Semnopithecus* remains uncertain, as revealed by low support for its relationship to the clade comprising of *Nasalis*, *Simias*, *Pygathrix* and *Rhinopithecus* on the ML tree. This result is different from Wang et al.[38], who found high support for a sister group relationship of *Semnopithecus* to all the other genera of Asian colobines. However, a combined analysis of nuclear and mitochondrial data placed *Semnopithecus* differently thus suggesting our mt-genome phylogeny correctly reflects that the placement of *Semnopithecus* remains uncertain.

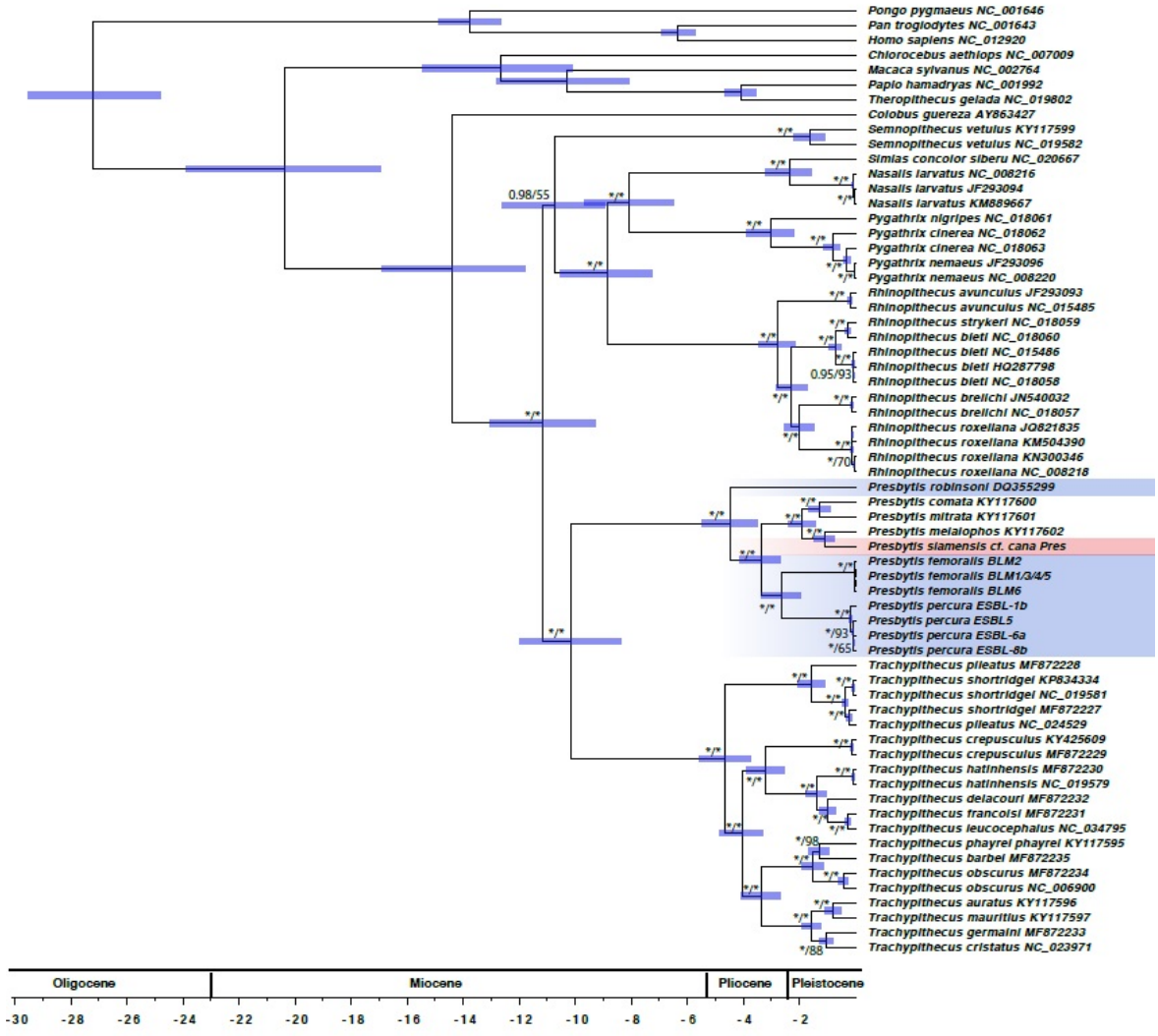


Figure 3.

With regard to the phylogenetic relationships within *Presbytis*, our results on *Presbytis* mitogenome+cyt-b+HV1 dataset (Fig. 4) are largely consistent with the reconstruction by Meyer et al.[11]. The only differences are as follows: low support for a clade comprising of *P. comata, mitrata, melalophos, bicolor, sumatrana, rubicunda* and *P. siamensis* cf. *cana* but resolution for *P. rubicunda, melalophos, mitrata* and *bicolor* which formed a trichotomy in Meyer et al.[11]. Here, we found *P. rubicunda* to be sister to *P. bicolor*. *Presbytis f. femoralis* and *P. f. percura* remain sister taxa. Both taxa combined are more closely related to *P. mitrata, P. comata* and several other taxa of *Presbytis* than *P. f. robinsoni.* The split between

222 *P. f. femoralis* and *P. f. percura* is again deeper than for most recognized taxa of *Presbytis.*

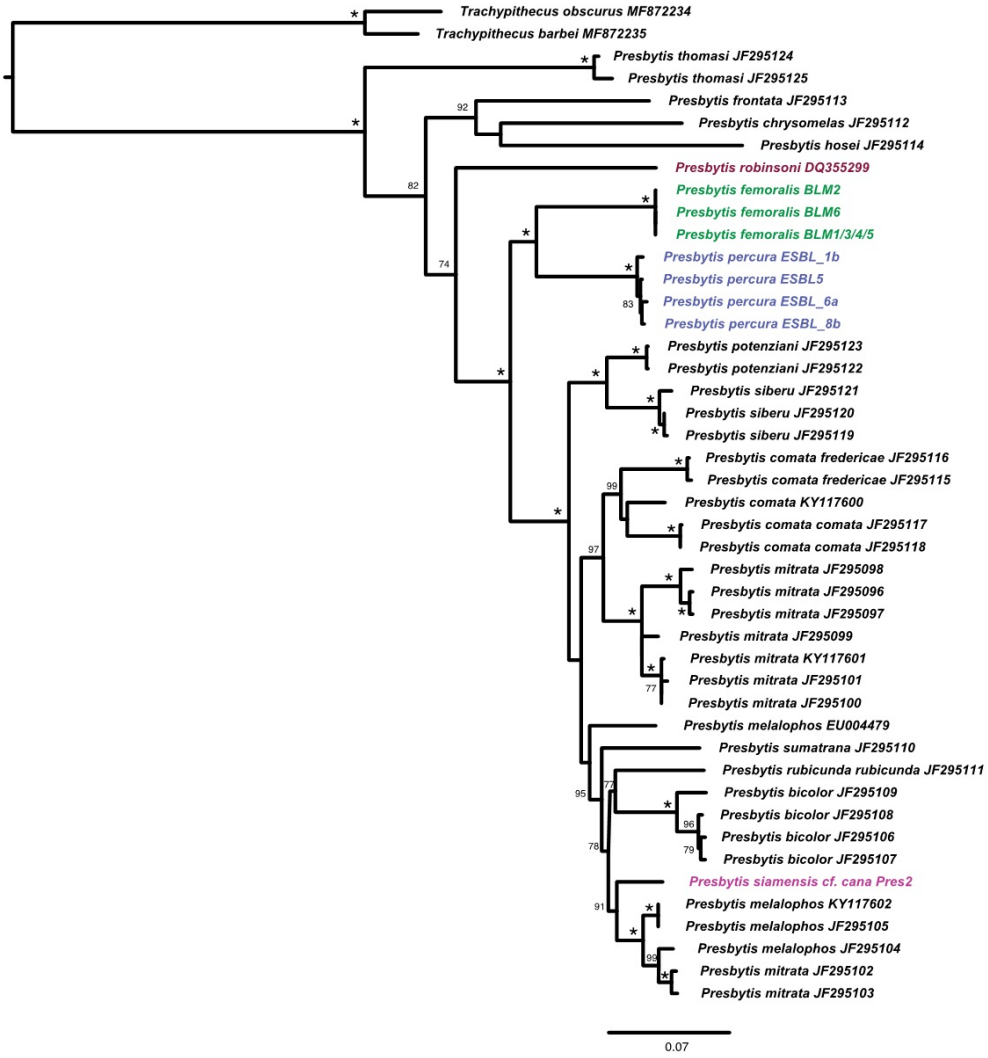
223 Divergence estimates based on cyt-b for this taxon set revealed deeper divergence times as

224 compared to mitochondrial genomes, but with overlapping confidence intervals. *Presbytis f.*

225 *femoralis* and *P. f. percura* split 2.93 Mya (2.09-3.78 Mya) (Supplementary Fig. S5), while *P.*

226 *f. robinsoni* diverged from the clade comprising of *P. femoralis, potenziani, mitrata,*

227 *melalophos, bicolor, sumatrana* and *P. siamensis* cf. *cana* at 5.47 Mya (CI: 4.28-6.66 Mya).



228

229 Figure 4.

230

231 **Discussion**

232 The species limits of many Southeast Asian mammal taxa remain unclear which interferes

233 with a timely conservation assessment although many populations, subspecies, and species

face extinction. We here demonstrate how such taxonomic uncertainty can be addressed rapidly through shotgun sequencing of faecal DNA. We document the power of the approach by studying langur species in *Presbytis* Eschscholtz, 1821 which continue to undergo many changes that significantly affect the conservation status of many taxa. At one point all Asian langurs and leaf monkeys in *Presbytis*, *Semnopithecus*, and *Trachypithecus* were included in *Presbytis* and only five widespread species were recognized (*P. aygula*, *P. melalophos*, *P. frontata*, *P. potenziani*, *P. rubicunda*)[39,40,41]. This has dramatically changed over the last 20 years and currently three genera and 45 species are recognized (17 spp. in *Presbytis*; eight spp. in *Semnopithecus*; 20 spp. in *Trachypithecus*)[5,10]. Many of these changes in species boundaries were based on newly-obtained genetic data which allowed for the application of explicit species delimitation methods. These new data and analyses revealed that many taxa that were initially described as species and later downgraded to subspecies diverged well before the Pleistocene and should be recognized as species; i.e., the morphological characters that were used for the initial species descriptions were appropriate for the delimitation of species and the subsequent lumping was not justified.

Resurrection of Presbytis femoralis, P. percura and P. robinsoni

Based on multiple species delimitation methods, high genetic divergence, placement in the mitochondrial phylogenies, as well as distinct morphological differences, we here resurrect the three species of *P. femoralis* from their current subspecific status (Table 4). The Raffles' banded langur *P. femoralis* is only known from southern Peninsular Malaysia (states of Johor and Pahang) and Singapore. The East Sumatran banded langur *P. percura* only occurs in Riau Province of east-central Sumatra. Lastly, Robinson's banded langur *P. robinsoni* has the widest distribution and ranges from northern Peninsular Malaysia (states of Kedah and Perak) through southern Thailand (provinces of Surat Thani, Phetchaburi, and Prachuap Khiri Khan)

259 to southern Myanmar (Tanintharyi Region). These changes to species status mean that
260 *Presbytis* now comprises 19 species (Fig. 5).
261

262
263 Figure 5.
264
265 A >5% genetic difference and divergence estimates of 2.6-2.9 Mya between *P. femoralis* and
266 *P. percura* suggest that these two species radiated prior to the Pleistocene, while several other
267 species of *Presbytis* diverged more recently. These results are particularly intriguing because
268 the changing sea levels during Pleistocene would have increased connectivity between the
269 land masses of Sumatra and Malay Peninsula. However, the Malacca Straits River flowing
270 northwards with tributaries in what is now Sumatra and the Malay Peninsula[42] may have been

a substantial barrier between *P. femoralis* and *P. percura*, as it would have been significantly wider than the rivers that currently form the geographic barriers between some of the *Presbytis* species in Sumatra. Furthermore, it has been argued that the land bridge had coarse sandy and/or poorly drained soils which may have limited plant growth in central Sundaland. Unsuitable vegetation may have acted as a dispersal barrier for rainforest plants and animals[43]. These barriers could have kept the langur populations separate and it thus remains unclear whether *P. femoralis* and *P. percura* would have formed a hybrid population if they had encountered each other. Note that it is known that recognized primate species in *Trachypithecus* that radiated ~0.95-1.25 Mya can interbreed[44,45], but the genetic divergence between *P. femoralis* and *P. percura* is considerably higher.

Table 4.

Reference	***femoralis***	***percura***	***robinsoni***
Martin 1838 (16)	*Semnopithecus femoralis*	-	-
Lyon 1908 (18)	-	*Presbytis percura*	-
Thomas 1910 (19)	-	-	*Presbytis robinsoni*
Elliot 1913 (73)	*Pygathrix femoralis*	*Pygathrix percura*	*Pygathrix robinsoni*
Miller 1913 (74)	*Presbytis femoralis*	-	*Presbytis keatii*
Miller 1934 (72)	*Presbytis femoralis*	*Presbytis percura*	*Presbytis keatii*
Pocock 1934 (75)	*Presbytis femoralis femoralis*	*Presbytis melalophos percura*	*Presbytis femoralis keatii*
Raven 1935 (76)	*Presbytis femoralis*	*Presbytis percura*	*Presbytis robinsoni*
Chasen 1940 (77)	*Pithecus femoralis femoralis*	*Pithecus femoralis percura*	*Pithecus femoralis robinsoni*
Hooijer 1962 (78)	*Presbytis melalophos femoralis*	-	-
Medway 1970 (79)	*Presbytis melalophos femoralis*	-	*Presbytis melalophos robinsoni*
Thorington and Groves 1970 (80)	*Presbytis melalophos femoralis*	*Presbytis melalophos percura*	*Presbytis melalophos robinsoni*
Wilson and Wilson 1977 (81)	-	*Presbytis femoralis percura*	-
Medway 1983 (82)	*Presbytis melalophos femoralis*	-	*Presbytis melalophos robinsoni*
Brandon-Jones 1984 (83)	*Presbytis femoralis femoralis*	*Presbytis femoralis percura*	*Presbytis femoralis robinsoni*
Napier 1985 (84)	*Presbytis melalophos femoralis*	*Presbytis melalophos percura*	*Presbytis melalophos robinsoni*
Weitzel et al. 1988 (21)	*Presbytis femoralis femoralis*	-	*Presbytis femoralis robinsoni*
Aimi and Bakar 1992 (85)	-	*Presbytis femoralis percura*	-
Oates et al. 1994 (12)	*Presbytis melalophos femoralis*	*Presbytis melalophos percura*	*Presbytis melalophos robinsoni*
Groves et al. 2001 (15)	*Presbytis femoralis femoralis*	*Presbytis femoralis percura*	*Presbytis femoralis robinsoni*
Brandon-Jones et al. 2004 (86)	*Presbytis femoralis femoralis*	*Presbytis femoralis percura*	*Presbytis femoralis robinsoni*
Md.-Zain 2001 (87)	*Presbytis melalophos femoralis*	-	*Presbytis melalophos robinsoni*
Meyer et al. 2011 (11)	*Presbytis femoralis femoralis*	-	*Presbytis femoralis robinsoni*
Vun et al. 2011 (27)	*Presbytis melalophos femoralis*	-	*Presbytis melalophos robinsoni*
Roos et al. 2014 (10)	*Presbytis femoralis femoralis*	*Presbytis femoralis percura*	*Presbytis femoralis robinsoni*
Abdul Latiff et al. 2019 (28)	*Presbytis neglectus neglectus*	*Presbytis femoralis percura*	*Presbytis femoralis robinsoni*
This study	*Presbytis femoralis*	*Presbytis percura*	*Presbytis robinsoni*

Grey boxes indicate the recognition of species rank for *Presbytis femoralis*, *P. percura*, and *P. robinsoni*.

Conservation Status of P. femoralis, P. percura, and P. robinsoni

In the most recent IUCN Red List assessment conducted in 2015, *Presbytis femoralis* (comprising *femoralis*, *percura* and *robinsoni*) was listed as Vulnerable A2cd A3cd A4cd (population size reduction of at least 30% over three generations based on a decline in area of occupancy, extent of occurrence and habitat quality, and actual or potential levels of exploitation). As part of this assessment the status of the three subspecies were also evaluated against the IUCN Red List criteria. *Presbytis f. femoralis* was considered Endangered A2cd A3cd A4cd, *P. f. percura* Data Deficient, and *P. f. robinsoni* Near Threatened (unpublished data from a Red List re-assessment in 2015). With their resurrection to species rank, the conservation status of each of the taxa requires re-assessment. *Presbytis femoralis* has a small global population size which continues to decline mainly due to habitat loss. There are 60 individuals (48 mature individuals) in the Singapore population of *P. femoralis*[46]. There are no precise population estimates available for the conspecifics in the Malaysian states of Johor and Pahang, but it is believed that only a few hundred individuals remain (see Abdul-Latiff et al.[28]); i.e., the overall population of *P. femoralis* could well be <250 mature individuals. Furthermore, the extensive habitat loss especially to industrial oil palm plantations in southern Peninsular Malaysia is unlikely to cease in the near future (see Shevade et al.[47]; Shevade and Loboda[48]). Hence, based on a small population size and decline, we propose to list *P. femoralis* as Critically Endangered C2a(i) (<250 mature individuals, continuing population decline, and ≤50 mature individuals in each subpopulation).

Presbytis percura is only found in a number of isolated forests and faces extinction in the wild based on large-scale forest loss in Riau Province[49]. Riau experienced the highest rate of deforestation in Sumatra such that 63% of natural forest have been lost between 1985 and 2008[50]. Additionally, forest fires linked to the ENSO events, and open burning of forest land for agricultural purposes destroy millions of hectares of land in Indonesia on an annual basis, and Riau is often one of the worst impacted areas, owing in part to its high concentration of

peatland[51]. We thus infer that the area of occupancy, extent of occurrence and quality of habitat of *P. percura* have declined such that their population size has reduced by ≥80% over the last three generations since 1989 (30 years approximately; see Nijman and Manullang[52] for the closely-related *P. melalophos*), thus fulfilling the IUCN criteria for Critically Endangered A2cd A3cd A4cd.

Presbytis robinsoni ranges from northern Peninsular Malaysia through southern Thailand to southern Myanmar. There are no population estimates (either recent or in the past), but some of the species' habitat continues to be converted for agriculture (primarily oil palm) and it is also targeted for illegal pet trade. Overall, it cannot be evaluated on small population size and/or restricted population criteria, but *P. robinsoni* is certainly a taxon of conservation concern and is here considered as Near Threatened.

An Urgent Need for Molecular Data for Additional Presbytis Populations

Our results highlight the need for sampling multiple populations of *Presbytis* species and subspecies because even our limited fieldwork already provided strong evidence for the widespread presence of cryptic diversity or inappropriate synonymization within *Presbytis.* Additional data are also needed in order to be able to precisely assign samples. We collected one faecal sample that was suspected to come from an individual of *P. siamensis* cf. *cana.* However, its placement in the phylogeny reveals that it belongs to a genetically distinct lineage that is more closely related to *P. melalophos*+*mitrata* than *P. s. siamensis* (Supplementary Fig. S2). If the sample was indeed from *P. s. cana*, then the taxonomy of the pale-thighed langur *P. siamensis,* which currently comprises of four subspecies, needs to be revisited unless the unexpected signal is due to introgression via the hybridization of two species. Regardless of the explanation, the taxon represented by the sample deserves species status, but the correct scientific name and range limits remain unclear because *P. s. paenulata*

335 and *P. s. rhionis* still lack molecular data. Even if the faecal sample originated from
336 individual of *P. melalophos/mitrata*, its genetic distinctness suggests that these species
337 require more attention from taxonomists. In addition, it would mean that the geographic
338 ranges of the species need to be revised because the species are unknown from the place of
339 collection. Overall, either explanation is reasons for concern. Geographically, only *P. s.*
340 *siamensis* (Fig. 6; left photo) has a wide distribution on the Malay Peninsula while the
341 remaining three subspecies have narrow distributions. *Presbytis s. cana* (Fig. 6; right photo)
342 occurs in eastern Sumatra and on Kundur Island, *P. s. paenulata* is found mainly in a small-
343 wedge of coastal forest in east-central Sumatra, and *P. s. rhionis* has only been found on the
344 islands of Bintan and Batam (but may also be found on Galang Island) in the Riau
345 Archipelago[10]. Given the extensive habitat loss to oil palm plantations in Sumatra and large-
346 scale economic development in Bintan and Batam, these taxa are likely highly threatened.
347 Study is urgently needed and we submit that faecal samples would be the best way to rapidly
348 address the species limits and distribution of these undersampled *Presbytis* species. We lack
349 genetic data, even COI barcodes, for many subspecies of *Presbytis* and their distributions are
350 poorly understood. Clearly, Southeast Asian langurs have received insufficient attention and
351 broad surveys are needed that estimate population sizes while collecting faecal samples for a
352 re-assessment of species boundaries.
353

Figure 6.

Mitochondrial Phylogeny of Presbytis

In the process of delimiting species, we re-examined the phylogenetic relationships within *Presbytis* by combining the data for mitochondrial genomes with the data generated by Meyer et al.[11] for cyt-b and d-*loop*-HV1. This led to the resurrection of *P. femoralis* and *P. percura* which are here revealed to be sister species and more closely related to several other *Presbytis* species from Sumatra (and *P. rubicunda* in Borneo) than *P. robinsoni* from Malay Peninsula. This placement of *P. femoralis* is in conflict with relationships proposed by Abdul-Latiff et al.[28], who obtained a clade comprising of *P. femoralis* + *P. robinsoni* + *P. siamensis* which was sister species of the *Presbytis* species from Sumatra + *P. rubicunda*.

One limitation of our study is the lack of nuclear data for species delimitation and reconstructing relationships (e.g. Wang et al.[38]). This is because mitochondrial data represents matrilineage only. However, obtaining nuclear data from faecal samples remains

challenging partially due to the low concentration of primate DNA in faecal samples. We assessed the primate nuclear DNA content in these metagenomes and found that it was only 0.09-3.13% of total DNA in the faecal samples (Table 5). However, we do not think that the lack of nuclear data seriously challenges our conclusions. Firstly, we reveal deep mitochondrial splits of >2 million years between *P. femoralis* and *P. percura*. We think that it would be more perilous to argue for the presence of one species based on identical mitochondrial sequence because it could be caused by introgression. Secondly, these splits are consistent with morphological data that allow for assigning specimens unambiguously to one of the lineages that are here re-corrected as species. Lastly, whatever limited information is available for Asian colobines does not point to widespread conflict between nuclear and mitochondrial signals. Wang et al.[38] presents phylogenetic reconstructions based on mitochondrial and nuclear markers for Asian colobines. Four of the six congeneric nodes are congruent between nuclear and mitochondrial data although they came from different individuals (i.e., study used data from multiple sources). Nonetheless, it is essential to develop new approaches for obtaining nuclear data from faecal samples (see Chiou and Bergey[53]).

Table 5.

Sample ID	% host	% host after removal of human reads
ESBL_1b	0.51	0.49
ESBL_5	0.62	0.59
ESBL_6a	0.23	0.21
ESBL_7	2.00	1.95
ESBL_8b	3.15	3.13
Pres_2	0.11	0.09

Non-invasive Samples for Species Discovery

Seventeen (61%) of the 28 taxa of *Presbytis* are threatened (Vulnerable, Endangered, or Critically Endangered) while five (18%) taxa are Data Deficient[5,10]. Many taxa continue to be

affected by habitat loss. This means that there is an urgency to resolve species limits in order to better assess their conservation status and needs. Molecular data play a critical role in resolving species limits, and here faecal samples are particularly valuable because they allow for accelerating data collection. Yet, our literature survey suggests that faecal DNA remains underutilized for taxonomic research with only two other published studies explicitly using faecal DNA as evidence for justifying decision on species status: nine members from a brown lemur complex were given species rank[33] and two subspecies of otters were elevated to species[32]. Faecal samples yield DNA that are valuable not only for taxonomic purposes but also population genetics, diet analyses, microbiome and parasite research, and should be routinely collected during field surveys. In groups such as *Presbytis,* faecal samples would be particularly useful as these animals are shy and many populations are highly threatened.

Conclusions

We here demonstrate the value of non-invasive faecal samples for addressing taxonomic questions that are of significant conservation importance. Based on mitochondrial DNA (mitogenomes, cyt-b and d-*loop*), we resurrected three species within the *Presbytis femoralis* group. The new species limits also led to a change in the conservation status of *P. femoralis* and *P. percura* which now have to be considered Critically Endangered. We further urge researchers to include the collection of non-invasive faecal samples into their field protocols.

Materials and Methods

Literature Survey

In order to assess how frequently faecal samples are used for addressing taxonomic questions, we conducted a survey of mammalian literature for the last 41 years (between 1978-2018). We downloaded the records pertaining to Supertaxon Mammalia (ST=Mammalia) from

Zoological Record (articles only). We then identified a subset containing valid mammalian species names by using the checklist by Burgin et al.[54]. This was done by searching the binomial name of 6,399 species in the list. We also searched for the genus and species names separately to include records that utilize genus abbreviations. We then retrieved the studies that involved DNA/molecular work on faecal samples by searching for the terms (feces/faeces/fecal/faecal/scat/scats) and (DNA/barcod/sequenc/molecul/genom/genetic/microsatellite). We lastly retrieved the records that were classified under Systematics/Taxonomy in the "Broad terms" field and examined the records manually.

Sample Collection, DNA Extraction, and Sequencing

Five faecal samples of *Presbytis femoralis percura* were collected during an eight-day (25 April - 2 May 2018) survey in Riau Province[49]. We also collected one faecal sample believed to come from *P. siamensis cana* (herein referred as *P. siamensis* cf. *cana*) as the monkeys were seen on the same tree below which the fresh faecal sample was found. The samples were preserved following a two-step ethanol-silica method[55] and subsequently stored at a -20°C freezer at the Andalas University in Sumatra. Genomic DNA was extracted at Andalas University from 50 mg of faeces using QIAamp® Fast DNA Stool Mini Kit (QIAGEN, Singapore). DNA was recovered in 30 µl of elution buffer (instead of 200 µl) in order to obtain a higher concentration of DNA. Each sample was also extracted 2-3 times and later pooled to recover more genomic DNA. Genomic DNA of these six samples were sent from Andalas University for Illumina HiSeq 4000 (Illumina Inc., San Diego, CA) sequencing (150 PE) by a commercial provider (NovogeneAIT). A library was constructed for each faecal sample (fragment size 350 bp) using NEBNext Ultra II DNA Library Prep Kit.

Bioinformatics for Obtaining Mitochondrial Genomes

Raw reads generated for *P. f. percura* and *P. s. cana* in this study as well as those for *P. f. femoralis* from Srivathsan et al.[9] were trimmed using Trimmomatic v.0.33[34] under the following parameters: LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:50, and the ILLUMINACLIP parameter was set at 2:30:10[34]. New mitochondrial reference genomes were assembled for *P. f. percura and P. s. cana* using MITObim v.1.9[56] using the available mitogenome of *P. f. femoralis* (KU899140) from one sample each (ESBL1b and Pres2). Any redundancy due to circular nature of mitochondrial genome was removed in the resulting assembly. We then obtained mitochondrial genomes per faecal sample by mapping the quality-trimmed reads for the sample to the reference genome (ESBL samples to mitochondrial genome from ESBL1b, Pres2 to Pres2 and BLM1-6 to KU899140). Reads were mapped using Bowtie2[57] under paired end and -end-to-end mode. The resulting SAM files were converted to BAM files using SAMtools[58]. Variants were detected using Lofreq[59] using parameters similar to Isokallio and Stewart[60] with a few more stringent criteria: we included mapping quality in LoFreq's model and also retained base-alignment quality. We furthermore applied a minimum allele frequency of 0.2 while filtering the vcf to accept heteroplasmy only if it was at a frequency of >0.2. Alternate mitochondrial genomes were reconstructed from the resulting vcf file using gatk[61] FastaAlternateReferenceMaker and heteroplasmic sites were modified as ambiguous nucleotides using custom script. All mitochondrial genomes were annotated using MITOS[62].

Phylogenetic Reconstructions and Species Delimitations

The newly reconstructed mitochondrial genomes were combined with publicly available data from GenBank. For the latter, we downloaded all the mitochondrial sequences for colobine primates and then retained only the data for Asian colobines. We then curated the GenBank

records by consulting the source publication and assessing the locality information provided in order to update the taxonomic names given that many subspecies are now considered species. We excluded those sequences for which the source information was incomplete. This curated set of sequences was used for downstream distance based (Automated Barcode Gap Discovery, ABGD[36] and Objective Clustering[37] and tree-based species delimitation analyses (Poisson Tree Processes or PTP)[35]. We also included data for d-*loop* HV1 obtained by Abdul-Latiff et al.[28] for *P. f. femoralis, P. f. robinsoni* and *P. siamensis siamensis* from Malaysia. Given that these sequences were not submitted to GenBank, we used Nijman[25]'s reconstruction of the sequences based on a table that lists all variable sites relative to a reference sequence.

For analyses with PTP, we used three datasets: (1) The Asian colobine mitogenome dataset based on genomes for Asian colobines (minimum length >10,000 bp). The sequences for the 13 mitochondrial CDS, two ribosomal genes, and complete d-*loop* sequences were extracted, aligned, and concatenated. Here, *Colobus guereza* and *Macaca sylvanus* were selected as the outgroups. (2) A second dataset included the *Presbytis* mitogenome+cyt-b+HV1 dataset. This dataset covers more samples because *Presbytis* has been well sampled for cyt-b and the hypervariable region I (HV1) of d-*loop* (see Meyer et al.[11]). For the analyses of this dataset, we used *Trachypithecus obscurus* and *T. barbei* as outgroups. (3) The last dataset comprised HV1 sequences only (*Presbytis* HV1-only dataset). This included HV1 sequences obtained by Abdul-Latiff et al.[28] for *P. f. femoralis, P. f. robinsoni* and *P. s. siamensis*.

All coding sequences were aligned in MEGA X[63] based on amino acid translations (using Clustal). The ribosomal genes and d-*loop* sequences were aligned using MAFFT LINSI[64]. We ensured that only distinct haplotypes were retained and only retained the longest sequence if identical sequences were found. The alignments were concatenated in SequenceMatrix

1.7.8[65]. Maximum Likelihood reconstructions were carried out using RAxML v8[66]. For the mitogenome and the *Presbytis* mitogenome+cyt-b+HV1 datasets, we determined best partitioning scheme by providing 42 different partitions to PartitionFinder[67] corresponding to codon position for the 13 coding regions and 3 separate partitions for 12S, 16S and d-*loop*. RAxML was run using GTRGAMMA with the resulting partitioning scheme (no partitioning was done for the HV1 dataset) with 20 independent searches for the best tree. Multiparametric bootstrapping was conducted applying the automatic bootstopping criterion (autoMRE). The resulting trees were subjected to PTP-based species delimitation after excluding outgroups.

Distance-based species delimitation utilized ABGD based on uncorrected distances[68]. We assessed species delimitations under different parameters by varying the slope X=0.1, X=0.5 and X=1 (X=1.5 was not applicable to the dataset) and prior intraspecific divergences. Species delimitation was carried out based on the Asian colobine mitogenome dataset described above and the *Presbytis* cyt-b and HV1 alignments. The same datasets were also clustered using Objective Clustering as implemented in Species Identifier (Taxon DNA 1.6.2)[37] at genetic distances of 2.0, 3.0 and 4.0%.

Divergence Dating

Divergence dates between Asian colobine lineages were determined using BEAST v 2.6.0[69]. We used Asian colobine mitogenome dataset but excluded d-*loop* for this analyse due its differing mutational patterns as done for previous studies[11,70]. For divergence estimates, we included the following genomes to the Asian colobine mitogenome dataset: *Pongo pygmaeus* (NC_001646), *Pan troglodytes* (NC_001643), *Homo sapiens* (NC_012920), *Chlorocebus aethiops* (NC_007009), *Macaca sylvanus* (NC_002764), *Papio hamadryas* (NC_001992), *Theropithecus gelada* (NC_019802). We also did a second analysis for *Presbytis* cyt-b as

done by Meyer et al.[11] and used a similar strategy for the various steps. Here, representative sequences from different Asian colobine genera were included: *Trachypithecus obscurus* (NC_006900), *Nasalis larvatus* (NC_008216), *Rhinopithecus avunculus* (NC_015485), *Semnopithecus vetulus* (NC_019582) in addition to the above-mentioned sequences for fossil-based calibration. The fossil calibration dates used in this study also followed the dates used by Meyer et al.[11]. For mitochondrial genomes, we analysed the data using the following partitioning schemes: by codon (5 partitions: 1,2,3 codon for coding genes, 12S, 16S) and by gene. We also tested partitioning by both gene and codon, but found the Effective Sample Size (ESS) to be low for multiple parameters. For cyt-b dataset, we used the 1+2 and 3 codon partitioning scheme[11].

Divergence estimates were based on a relaxed log normal clock and a Yule prior. Site models were unlinked across partitions, and a model-averaging approach was used as implemented in bModelTest[71]. Two independent runs were conducted with 25 million generations with sampling at every 1000 generations. Tracer v 1.7.1 was used to assess convergence, LogCombiner 2.6.1 was used to combine the results and 10% burn-in removal was applied. TreeAnnotator v 2.6.0 was used to summarize the trees.

Estimation of Host DNA Content in Faecal Metagenomes

In order to estimate the amount of host nuclear DNA in the faecal metagenomes, we mapped the metagenomic reads to a colobine reference genome (*Rhinopithecus roxellana*: GCF_000769185.1) using bowtie-2 under --end-to-end and ---very-sensitive mode. We next excluded potentially contaminated reads that could correspond to humans. For this, the mapped reads were retrieved from the resulting bam files using samtools. These were mapped back to a combined reference dataset of *R. roxellana* and human genome (GCF_000001405.39, GRCh38) using Bowtie2. Resulting BAM file was filtered to exclude

reads with any mismatches, and we then excluded all sequences that matched to human genome only.

Ethics Statement

We followed the Code of Best Practices for Field Primatology (2014). All genetic material were obtained non-invasively through faecal samples; no animals were harmed in the process. We followed the rules and regulations of the Government of Indonesia and RISTEKDIKTI (research permit no. 3051/FRP/E5/Dit.KI/IX/2018).

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751

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760 **Author Contributions**

761 A.A., A.S., R.M., V.N. wrote the main manuscript text; A.A., A.S., R., R.M., V.N. designed
762 the study; A.A., R. conducted the field work; A.A., D.R. extracted DNA samples in D.R.'s

763 laboratory; A.S., D.R. conducted bioinformatics analyses; and A.S. reconstructed the time-
764 tree. All authors reviewed and edited the manuscript and gave final approval for submission.
765
766 **Additional Information**
767 The authors declare no competing interests.
768
769 **Legends**
770 Figure 1. Distribution of the banded langur *Presbytis femoralis* on the Malay Peninsula and
771 eastern Sumatra. Map: Ang Yuchen
772
773 Figure 2. Three subspecies of *Presbytis femoralis*; clockwise from East Sumatran banded
774 langur *P. f. percura* (1), Raffles' banded langur *P. f. femoralis* (2), to Robinson's banded
775 langur *P. f. robinsoni* (3). Photos: Andie Ang
776
777 Figure 3. Dated phylogeny of Asian colobine primates based on mitochondrial genomes. The
778 values at nodes represent posterior probability (codon partitioning)/ML bootstrap support for
779 relationships between Asian colobines. Values are omitted if both BI and ML support values
780 are <0.7/70, while * represents support of 1/100. The bars represent the 95% confidence
781 intervals for divergence times estimates.
782
783 Figure 4. ML reconstruction of relationships between *Presbytis* species based on
784 mitogenome+cyt-b+HV1 dataset. Node values represent bootstrap support, values <70 are
785 excluded, while node support of 100 is represented by *.
786

787 Figure 5. Nineteen species of *Presbytis* langurs recognised in this study. Photo credits:
788 Wilson Novarino (1. *P. bicolor*); Brent Loken (2. *P. canicrus*); Chien Lee (3. *P.*
789 *chrysomelas*); Andie Ang (4. *P. comata*); Andie Ang (5. *P. femoralis*); Milan Janda (6. *P.*
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797 Figure 6. *Presbytis siamensis siamensis* (left; photo: Lee Zan Hui) and *P. s. cana* (right:
798 Andie Ang).
799
800 Table 1. Morphological characters of three subspecies of *Presbytis femoralis*
801
802 Table 2. Summary of sequencing data and mitochondrial genomes obtained from eleven
803 *Presbytis* langurs from Singapore and eastern Sumatra.
804
805 Table 3. Summary of results of ABGD-based species delimitation. Values represent number
806 of molecular Operational Taxonomic Units (mOTUs). N represents number of species
807 (include those resurrected in this study), X the slope parameter. Colours: green: all
808 resurrected species are valid. Purple: Supports splitting of the three subspecies of *Presbytis*
809 *femoralis* and splitting of *P. siamensis* cf. *cana*. However, some sequences of *P. f. femoralis*
810 from Peninsular Malaysia are split into multiple mOTUs. Orange: Resurrection of all three

811 subspecies of *P. femoralis* is valid, but *P. s.* cf. *cana* is lumped with other *Presbytis* species.

812 Red: Not valid.

813

814 Table 4. Taxonomic classifications of the type specimens of *femoralis*, *percura*, and

815 *robinsoni* followed by later authors since their first descriptions (non-exhaustive).

816

817 Table 5. Estimation of host DNA content in faecal metagenomes