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Mitochondrial DNA Sequence Phylogeny of 4 Populations of the Widely Distributed Cynomolgus Macaque (*Macaca fascicularis fascicularis*)

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Abstract

We studied the mitochondrial DNA (mtDNA) polymorphism of 304 *Macaca fascicularis fascicularis* (*M. f. fascicularis*) individuals, representative of 4 cynomolgus macaque populations (Indochina, Indonesia, Philippines, and Mauritius). By sequencing a 590-bp fragment in the hypervariable II region of the D-loop region, we defined 70 haplotypes. The homologous region was also characterized in 22 Chinese *Macaca mulatta* and 2 *Macaca sylvanus*. The phylogenetic analysis confirms the monophyly of *M. f. fascicularis* and defines 2 haplotype groups inside the *M. f. fascicularis* clade: one "insular," encompassing 6 Philippines, 2 Mauritius, and 31 Indonesian haplotypes, the other "continental" that contains all Indochinese and 6 Indonesian haplotypes. Continental and insular group divergence time was estimated to be approximately 10^6 years before present (BP). Among Indonesian haplotypes, some have a continental origin. This suggests either direct migration from mainland to Indonesia or that remnant lineages from an ancient population genetically close to the mainland (i.e., in the Sunda Shelf, $<$550 000 years BP) were subsequently brought southward to Indonesia. The low nucleotide diversity in the Philippines population suggests a bottleneck following colonization by Indonesian individuals, around 110 000 years BP. mtDNA and further observations of nuclear genetic data corroborate the mixed origin (Indonesian/continental) hypothesis of Mauritius individuals and a population bottleneck.

Cynomolgus macaque (*Macaca fascicularis*) is used as a non-human primate model for biomedical research in various domains such as tissue allograft rejection in case of kidney (Borie et al. 2002; Wieczorek et al. 2006) or cardiac (Schroder et al. 2007) transplantation, bone marrow graft (Lau et al. 2004), immune response against pathogens (Sato et al. 2008) or new vaccines (Kita et al. 2005), and infectious diseases and particularly Simian Immunodeficiency Virus (SIV)-induced AIDS or emergent pathogens (Kuiken et al. 2003; Reed et al. 2005; Lawler et al. 2006). In all these models, the genetic background of the animals could be of great importance; in some cases, it was reported that animal geographical origin can influence the response to drugs or the sensibility to the experimental disease (Schmidt et al. 1977; Menninger et al. 2002). Numerous reports have been published on the geographical variations of the cynomolgus macaque. Blood groups (Terao et al. 1981), blood proteins (Nozawa et al. 1977; Kondo et al. 1993), mitochondrial DNA (mtDNA) (Harihara et al. 1988; Lawler et al. 1995; Tosi et al. 2002, 2003; Smith et al. 2007; Tosi and Coke 2007), and Y chromosome DNA (Tosi et al. 2002, 2003; Tosi and Coke 2007) were studied. The phylogenetic relationships among the populations of *M. fascicularis* have been discussed, and discrepancies in population divergence times between mtDNA and nuclear markers have been found. Moreover, the analysis of paleoclimate in South Asia has led some authors to propose various hypotheses concerning the geographical dispersal of macaques in

Southeast Asia (Fooden 1995; Delson 1980; Eudey 1980; Abegg and Thierry 2002). The cynomolgus macaque is characterized by its habitat, the mangroves (Fittinghoff and Lindburg 1980; Wheatley 1980), which may have favored a dispersal mode through the estuaries and by sea rafting. Thus, both habitat characteristics and past sea level changes in Southeast Asia could explain the wide geographic distribution of the species. This study focuses on the common subspecies *Macaca fascicularis fascicularis* (*M. f. fascicularis*), excluding all other subspecies that are either restricted to some small islands or morphologically different (i.e., the dark pelage subspecies *Macaca fascicularis philippinensis*), as extensively described in Fooden (1991, 1995).

Maternally inherited mtDNA has provided valuable data for investigating intraspecific variation, population structure, phylogeography, and demography in macaque species (Harihara et al. 1988; Melnick and Hoelzer 1992; Lawler et al. 1995; Tosi et al. 2002; Marmi et al. 2004; Modolo et al. 2005; Smith and McDonough 2005; Kawamoto, Shotake, et al. 2007; Smith et al. 2007). Harihara et al. (1988), through restriction fragment length polymorphism (RFLP) data on *M. fascicularis* populations using 5 restriction enzymes applied to 149 individuals, described 2 population groups: "insular" (Philippines and Indonesia) and "continental" (Malaysia and Indochina). The genetic distance between the haplotypes of these 2 groups suggests a deep split between the continental and the insular groups. The dichotomy between insular and continental populations was confirmed by Tosi et al. (2002, 2003) and Tosi and Coke (2007). However, the mtDNA lineage heterogeneity found for the Indonesian population suggests a complex origin.

Lawler et al. (1995) showed that cynomolgus macaque from Mauritius have only a few mitochondrial RFLP haplotypes, all possibly deriving from a single one. This observation indicates a recent founding of the Mauritius macaque population by an artificial introduction between 300 and 500 years ago (see Sussman and Tattersall 1986). Sailors imported a few animals most probably from Indonesia, as confirmed by the genetic proximity of 1) Mauritius and Java populations using nuclear and mtDNA markers (Kondo et al. 1993; Kawamoto Y, Kawamoto S, et al. 2008, Tosi and Coke 2007) and of 2) Mauritius and Sumatra by means of Y chromosome sequences (Tosi and Coke 2007).

In the present study, we investigate the molecular genetic relationships between cynomolgus macaque populations and one rhesus macaque (*Macaca mulatta*) population, using mtDNA sequence data. The aim of this study is to refine more precisely the phylogeographic structure of the cynomolgus macaque, to make inferences about the dispersal scenario of this species, and to confront mtDNA polymorphism to nuclear genetic diversity (Bonhomme et al. 2007) in the same samples. Herein, we sequenced around 590 bp in hypervariable segment 2 (HVII) of the D-loop of a large number of individuals (304) from 4 cynomolgus macaque populations (Indochina, Indonesia, Philippines, and Mauritius island). The artificially established Mauritius population was studied to trace the most probable ancestral population and the signature of the founding effect. The phylogenetic relationships of the 3 natural populations (Indonesia, Indochina, and Philippines) are discussed with regard to the colonization of Southeast Asia by *M. f. fascicularis*.

Materials and Methods

Animals and DNA Samples

Genomic DNA was extracted from peripheral blood of 304 unrelated *M. f. fascicularis* using either QIA amp Blood Kit (Qiagen, Courtaboeuf, France) or phenol-chloroform extraction. DNA blood samples of 137 animals captured from the Indonesian wild population were obtained from the Tsukuba Primate Center through K.T. Other animals are F_1 derived from breeders that had been captured in the wild on various locations in Mauritius ($N = 74$), Philippines ($N = 25$), and Indochina ($N = 68$) by Noveprim (Mauritius), Siconbrec (Philippines), and Nafovanny (Vietnam), respectively. *Macaca mulatta* blood samples ($N = 22$) were obtained from the Bioprim quarantine center (Bioprim, Baziège, France). The rhesus monkeys were F_1 imported from a Chinese breeding center (South China Primate Research Development Center, Jiufu center, Guangzhou, China). *Macaca sylvanus* blood samples from 2 unrelated individuals were provided by Dr. Ducos de Lahitte (Ecole Nationale Vétérinaire, Toulouse, France).

Polymerase Chain Reaction and Sequencing

A 590-bp fragment of the HVII of the mtDNA D-loop was amplified by using the pair of primers: Saru-4F (5′-ATCACGGGTCTATCACCCTA-3′) and Saru-5R (5′-GGCCAGGACCAAGCCTATTT-3′). This primer pair has been successfully used for Japanese macaque mtDNA studies (Hayasaka et al. 1991; Kawamoto, Shotake, et al. 2007) and is also known to produce no amplification of nuclear-inserted mtDNA sequences in *M. f. fascicularis* (Dr. Kawamoto Y, personal communication). This pair amplifies positions 22–606 in the complete sequence of *M. mulatta* mitochondrial genome (DNA Databank of Japan-DDBJ/European Molecular Biology Laboratory-EMBL/GenBank accession number AY612638).

Amplified material was separated on agarose gels, purified by using QIAquick PCR Purification Kit (Qiagen, Courtaboeuf, France), and directly sequenced on both strands by the fluorescent Dye Terminator method on an ABI 373 automatic sequencer (Applied Biosystems Japan, Tokyo, Japan) or an CEQ 2000 automatic sequencer (Beckman Coulter, Paris, France). In all population samples, we paid special attention to sequences differing from the most frequent and checked all these sequences by sequencing again the target fragment.

Sequence Data Analysis

The *M. f. fascicularis* sequences characterized were aligned with macaque mtDNA sequences obtained from 2 *M. sylvanus* and 22 *M. mulatta* (this study) and homologous sequences available in DDBJ/EMBL/GenBank International Nucleotide Sequence Database (2 *Macaca fuscata* and 1 *Papio hamadryas*, accession numbers M80209, M80210, and

Y18001). Nucleotide diversity π (Nei 1987), Tajima's *D* (Tajima 1989), and Fu's (1997) F_s were calculated using the ARLEQUIN ver. 2.000 package (Schneider et al. 2000) on the HVII region dataset reported here and on the HVI region dataset of Smith et al. (2007). We constructed phylogenetic trees, based on 590 nucleotides, using 3 different methods. First, a neighbor-joining tree (Saitou and Nei 1987) was constructed with the Kimura's 2-parameter distance (Kimura 1980), using the computer program MEGA ver. 3.0 (Kumar et al. 2004), and 10 000 bootstraps were performed to determine the significance of tree branching. We then performed maximum likelihood (ML) and Bayesian phylogenetic analyses using the most likely substitution model inferred using a likelihood framework implemented in the software MODELTEST 3.7 (Posada and Crendall 1998). This software tests 56 different substitution models and estimates the most likely one using a likelihood ratio test. ML inference was performed using the PHYML software (Guindon and Gascuel 2003), and 10 000 bootstraps were performed on the ML tree. Finally, a Bayesian analysis was performed using the software MrBayes (Ronquist and Huelsenbeck 2003), where 2 Markov Chains were run on 10×10^6 generations with a sampling each 100 generations. Such a run length allowed the standard deviation of allelic frequencies to pass below 0.01 and the potential scale reduction factor to reach a value of 1, as suggested by the authors. The first 25 000 trees (25%) were discarded from the analysis as a burn-in.

Results

Determination of mtDNA Sequences

Primate mtDNA is known to be often inserted into nuclear DNA—"numts"—(Bensasson et al. 2001). Numts were shown to hinder the evolutionary history inference of primate species (Thalmann et al. 2004, 2005). Thus, we carefully considered the putative amplification and sequencing of numts, although caution was done when choosing the polymerase chain reaction (PCR) primers (see Materials and Methods). With all *M. fascicularis* samples, we obtained a single band by PCR amplification, and most direct sequencing results showed unique nucleotide sequence for each individual. As for *M. mulatta* samples, about 1/3 of PCR products did not show unique sequence, so we chose ones that were very close to the complete sequence of *M. mulatta* mitochondrial genome (DDBJ/EMBL/GenBank accession number AY612638). Our 2 newly determined *M. sylvanus* sequences were also compared with the complete sequence of *M. sylvanus* mitochondrial genome (DDBJ/EMBL/GenBank accession number AJ309865), and they were found to be very close with each other. Furthermore, phylogenetic analyses of these sequences produced trees consistent with the known macaque phylogeny (see below). In conclusion, we believe that our nucleotide sequences were derived from authentic mtDNA. Nucleotide sequences determined in this study were deposited to the DDBJ/EMBL/GenBank under accession numbers AB261898–AB261974.

Macaca fascicularis fascicularis mtDNA Sequence Diversity

In total, 120 positions are polymorphic in 304 individuals, including 8 positions with indels (Figure 1), for a total of 70 haplotypes. Among the 112 positions with substitutions, 109 are dimorphic with transitions being much more frequent (65 C ↔ T and 38 A ↔ G) than transversions (6 in total). Only 2 populations, Indonesia and Mauritius, share one haplotype that is present in all Mauritius animals but one and in 6 out of 137 animals from Indonesia.

The intrapopulation diversity is shown in Table 1(a). Nucleotide diversity (π) was the highest (0.032) for Indonesia, followed by Indochina (0.017) and Philippines population (0.009). The Mauritius population has only 2 haplotypes differing by a single-base substitution. Accordingly, the nucleotide diversity (4.6×10^{-5}) was extremely low for this population. The gene diversity (*h*) in Indochina and Indonesia is similar in our study (HVII) and in that of Smith et al. (2007) based on HVI, as shown in Table 1 (a, b). However, gene diversity is higher with HVI than with HVII in Philippines and Mauritius. The nucleotide diversity is at least 5 times higher for HVI than for HVII in all populations. This confirms, in *M. f. fascicularis*, the higher mutation rate in the HVI region than in the HVII region of the D-loop, found in humans (Meyer et al. 1999). Except for Mauritius, both neutrality tests for HVI and HVII reveal that polymorphism is neutral, as expected for the mtDNA D-loop region. In the case of the Mauritius population, only HVI (and not HVII) analysis revealed significantly negative F_S and *D* values. The greater polymorphism of HVI may thus indicate a recent expansion in the Mauritius population.

Phylogenetic Analysis

The neighbor-joining, ML, and Bayesian trees have the same topology. We thus show only the Bayesian tree (Figure 2). All the *M. f. fascicularis* sequences clustered together (posterior value = 91%), showing that *M. f. fascicularis* is monophyletic.

Inside the *M. f. fascicularis* clade, 2 main groups of sequences can be detected. Group I encompasses all haplotypes from the Philippines and Mauritius samples and most of the Indonesian sample haplotypes (29 out of 35). The sequences from the Philippines population and that from one Indonesian individual constitute a subgroup I-1 (posterior value = 85%). There are 3 other subclusters in group I (I-2, I-3, and I-4), all supported with posterior values higher than 77% (see Figure 2). Two Mauritius haplotypes belong to subcluster I-3 with 14 Indonesian haplotypes (posterior value = 98%), and the remaining 3 subclusters (I-2 and I-4) consist of 2 and 12 Indonesian haplotypes, respectively. Group II includes all Indochinese haplotypes and 6 haplotypes from Indonesia, and we divided it into 3 subclusters (II-1, II-2, and II-3), as shown in Figure 2. Subclusters II-1 and II-3 include haplotypes only found in Indochina (2 and 25, respectively), whereas subcluster II-2 consists of 6 Indonesian haplotypes that are closer to Indochinese haplotypes than to Indonesian haplotypes of group I (posterior value = 97%).

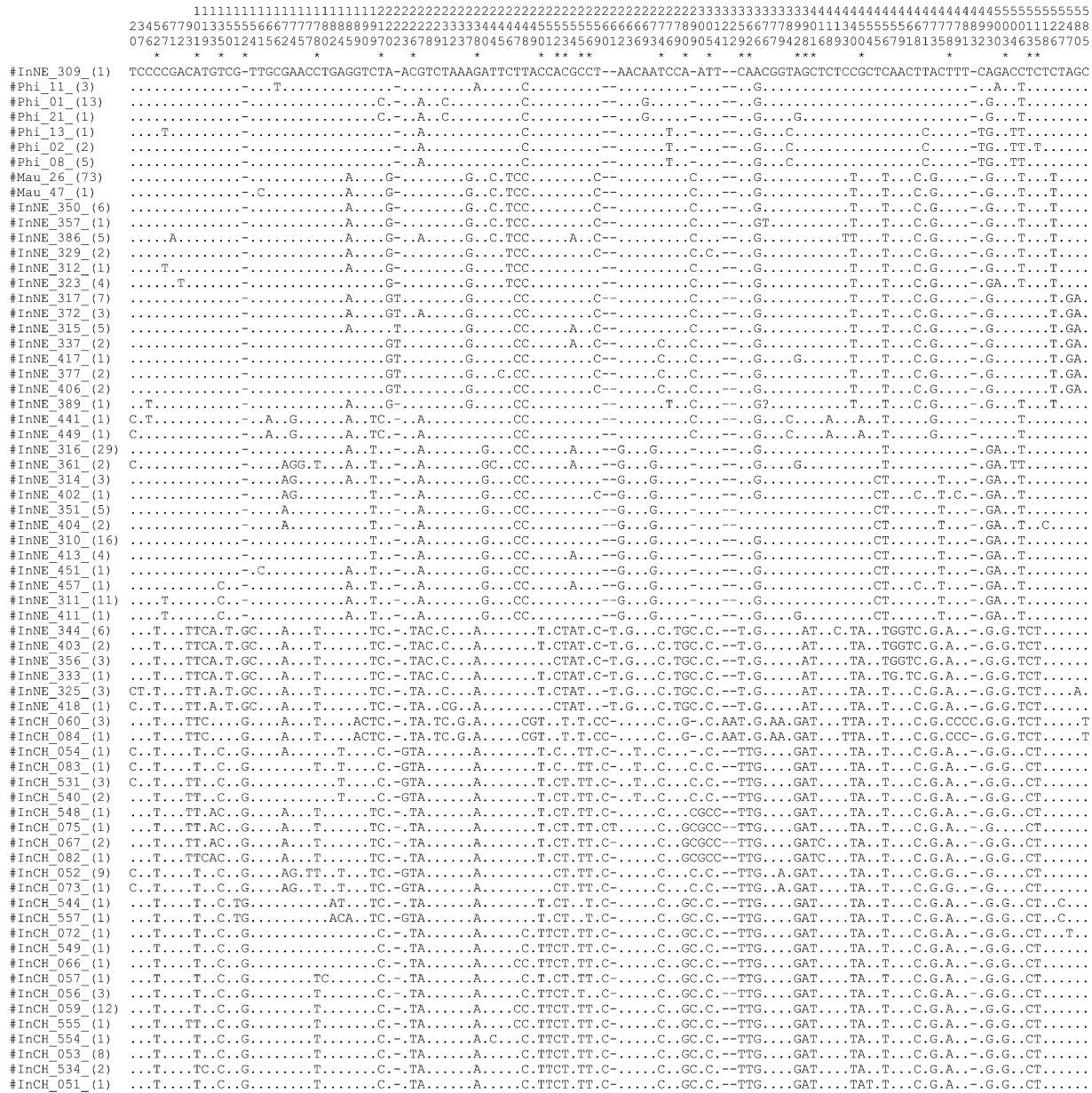

Figure 1. Variant sites of the mtDNA control region (HVII) in *Macaca fascicularis fascicularis* determined in this study. The sequence names begin with letters corresponding to the 4 populations (InCH = Indochina; InNE = Indonesia; Mau = Mauritius; and Phi = Philippines), followed by a code number identifying the sequence and a number in brackets corresponding to the number of animals sharing the sequence. Stars in the fourth line indicate the positions that differentiate Indochinese sequences from those of other populations.

Dating the Divergence of *Macaca fascicularis fascicularis* Populations

The Bayesian tree showing the length of branches (Figure 2) was transformed under the assumption of a constant rate of mutation, while keeping its tree topology, and the resulting tree is shown in Figure 3. In the absence of *M. f. fascicularis* fossil data allowing us to date interpopulational divergences, calibration of the tree is difficult. Therefore, we decided to calibrate it at node 0, which separates *M. fascicularis* and *M. mulatta* clusters. Among all the dates proposed, we chose 1.6 million years before present (My BP) as the most probable divergence time between the 2 macaque species. This divergence time was deduced from a composite estimate of primate divergence times (Purvis 1995). In that

Table 1. Summary statistics for mtDNA and nuclear genetic diversity in *Macaca fascicularis fascicularis* populations

(a) Based on HVII region sequences observed in 4 *M. f. fascicularis* populations (this study)

	Diversity parameters			Neutrality tests	
Population	N_{hap} (poly sites/indel)	$\pi \times 1000$ (±SE)	h (±SE)	D (P value)	F_s^* (P value)
Indochina (68)	27 (59/6)	17.1 (±8.7)	0.932 (±0.016)	−0.33 (0.42)	−3.14 (0.18)
Indonesia (137)	35 (88/4)	32.4 (±16)	0.926 (±0.013)	0.58 (0.21)	1.68 (0.28)
Philippines (25)	6 (16/0)	8.85 (±4.93)	0.693 (±0.082)	0.82 (0.18)	3.66 (0.07)
Mauritius (74)	2 (1/0)	0.046 (±0.164)	0.027 (±0.026)	−1.06 (0.11)	−1.97 (0.04)

(b) Based on HVI region sequences observed in 4 *M. f. fascicularis* populations (Smith et al. 2007)

	Diversity parameters			Neutrality tests	
Population	N_{hap} (poly sites)	$\pi \times 1000$ (±SE)	h (±SE)	D (P value)	F_s^* (P value)
Indochina (89)	48 (107)	83.9 (±41.4)	0.975 (±0.0064)	−0.04 (0.57)	−6.29 (0.12)
Indonesia (70)	58 (179)	183.7 (±89.3)	0.994 (±0.0035)	0.80 (0.16)	−10.39 (0.03)
Philippines (83)	34 (124)	59.5 (±29.8)	0.78 (±0.05)	−1.36 (0.067)	−2.23 (0.31)
Mauritius (68)	9 (15)	2.9 (±2.4)	0.298 (±0.073)	−2.24 (0.001)	−5.21 (0.005)

(c) Based on the microsatellite dataset from Bonhomme et al. (2007)

	Diversity parameters		Diversity parameters	
	MHC		non-MHC	
Population	n_A	H_e	n_A	H_e
Indochina (70)	12	0.80	12	0.81
Java (38)	11	0.76	9	0.83
Philippines (65)	8	0.68	8	0.74
Mauritius (81)	8	0.70	7	0.69

The number of individuals studied follows the population name; N_{hap} = Numbers of haplotypes in each population; poly sites = number of polymorphic sites; indel: number of insertion–deletions in each population sample; π = mean number of pairwise differences; SE = standard error for the sampling process; h = gene diversity; D = Tajima's D; F_S = Fu's F_S; n_A = mean number of alleles; and H_e = mean expected heterozygosity.

* Fu's F_S should be considered as significant at the 5% level if $P < 0.02$. NB: MHC and non-MHC datasets each consists of 7 microsatellite markers.

study, the baboon–macaque divergence time was proposed at around 9.25 My BP (mean value), a date compatible with more recent estimates (Raaum et al. 2005; Steiper and Young 2006). Moreover, the 1.6 My estimate for *M. mulatta* and *M. fascicularis* divergence time is in the range of dates proposed by Hayasaka et al. (1996) for the radiation of Asian macaques. With node 0 of Figure 3 being calibrated at 1.6 My BP, nodes 1, 2, and 3 become 1.2, 0.55, and 0.11 My BP, respectively. The divergence observed at node 1 corresponds to the separation between groups I and II, namely between the continental and insular groups of *M. f. fascicularis*, whereas nodes 2 and 3 correspond to the divergence time of Indochinese and Philippines populations, respectively.

Discussion

Monophyly of *Macaca fascicularis fascicularis*

This study, based on 4 *M. f. fascicularis* populations, clearly confirms that this subspecies is supported as a monophyletic mtDNA lineage by reference to other macaques (*M. mulatta*, *M. fuscata*, and *M. sylvanus*). This is in accordance with the mtDNA phylogenies of macaque species in Tosi et al. (2002, 2003) and Smith et al. (2007). In contrast to mtDNA, the paraphyly of sequences from the Y chromosome and autosomal genes led to suspect a possible male introgression from *M. mulatta* to *M. fascicularis* in Indochina (Tosi et al. 2002, 2003). *Macaca nemestrina* is also distributed in

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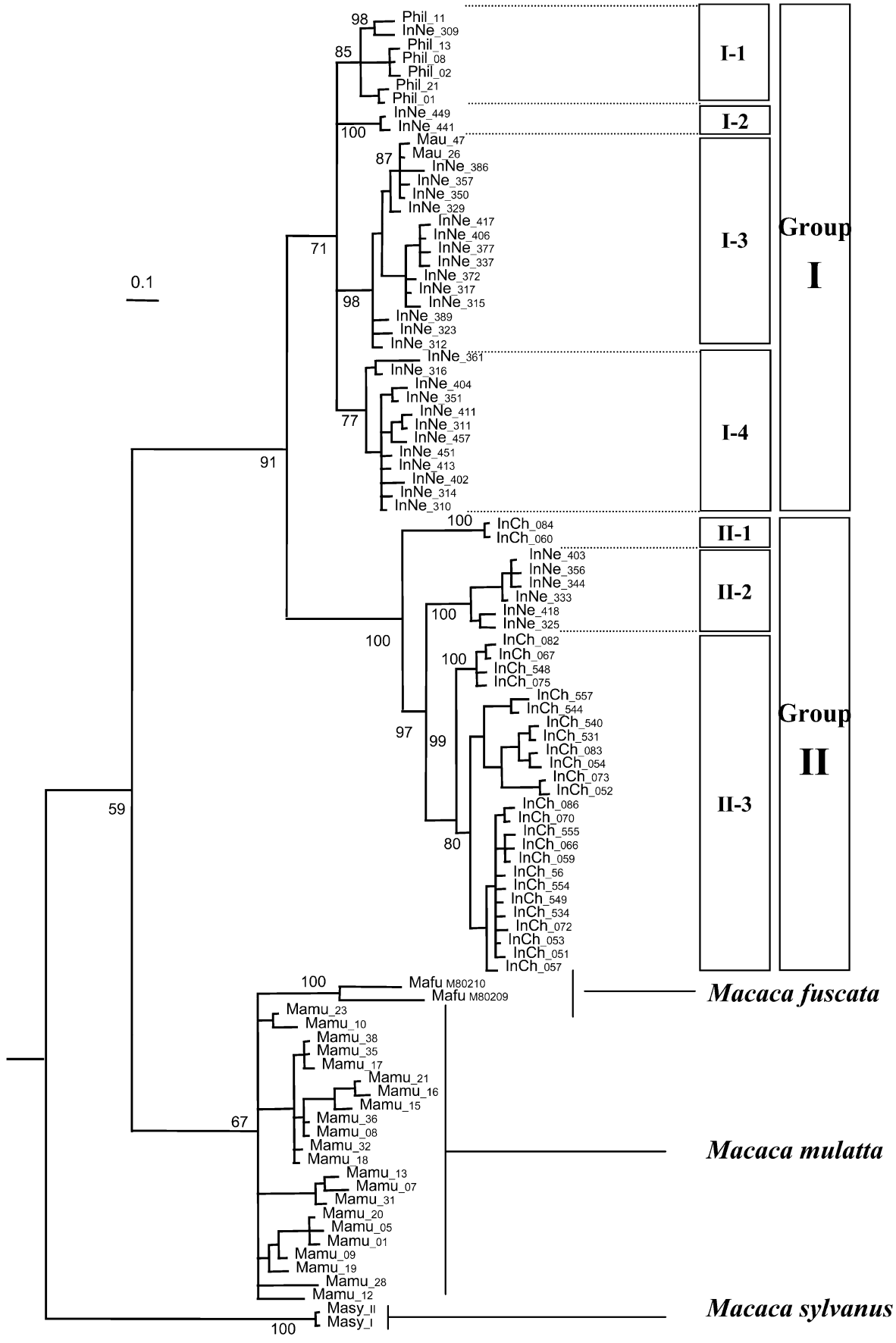
Figure 2. Bayesian phylogenetic tree of mitochondrial sequences of *Macaca fascicularis fascicularis*, with branch length. The sequence names begin by letters corresponding to the 4 populations (InCH = Indochina; InNE = Indonesian; Mau = Mauritius; and Phi = Philippines), followed by a code number identifying the sequence. When there is no number in brackets the sequence was observed in only one animal. Numbers at nodes of the tree correspond to posterior confidence values. Sequences from other macaque species (*Macaca sylvanus*, *Macaca mulatta*, and *Macaca fuscata*) are included in the tree, rooted with a baboon (*Papio hamadryas*) sequence (the DDBJ/EMBL/GenBank accession number Y18001) as outgroup. The 2 *M. fuscata* sequences in this tree were from Hayasaka et al. (1991), and all other sequences were newly determined in this study.


0.1
Phil_11
InNe_309
Phil_13
Phil_08
Phil_02
Phil_21
Phil_01
InNe_449
InNe_441
Mau_47
Mau_26
InNe_386
InNe_357
InNe_350
InNe_329
InNe_417
InNe_406
InNe_377
InNe_337
InNe_372
InNe_317
InNe_315
InNe_389
InNe_323
InNe_312
InNe_361
InNe_316
InNe_404
InNe_351
InNe_411
InNe_311
InNe_457
InNe_451
InNe_413
InNe_402
InNe_314
InNe_310
InCh_084
InCh_060
InNe_403
InNe_356
InNe_344
InNe_333
InNe_418
InNe_325
InCh_082
InCh_067
InCh_548
InCh_075
InCh_557
InCh_544
InCh_540
InCh_531
InCh_083
InCh_054
InCh_073
InCh_052
InCh_086
InCh_070
InCh_555
InCh_066
InCh_059
InCh_56
InCh_554
InCh_549
InCh_534
InCh_072
InCh_053
InCh_051
InCh_057
Mafu M80210
Mafu M80209
Mamu_23
Mamu_10
Mamu_38
Mamu_35
Mamu_17
Mamu_21
Mamu_16
Mamu_15
Mamu_36
Mamu_08
Mamu_32
Mamu_18
Mamu_13
Mamu_07
Mamu_31
Mamu_20
Mamu_05
Mamu_01
Mamu_09
Mamu_19
Mamu_28
Mamu_12
Masy_II
Masy_I
I-1
I-2
I-3
I-4
II-1
II-2
II-3
Group I
Group II
Macaca fuscata
Macaca mulatta
Macaca sylvanus

Indonesia, as well as *M. fascicularis* and this could again be a possible source of introgression. Therefore, when inferring the phylogenetic relationships of macaque species, caution should be taken as for the choice of the molecular markers and for the sampling of species and populations.

Origin and Genetic Diversity of the Mauritius Macaque Population

Among the 4 populations studied here, that from Mauritius clearly shows distinct polymorphism patterns. We found that most animals (73 out of 74) have the same sequence, whereas the remaining animal differs by only one nucleotide position. The latter sequence was validated twice by sequencing the DNA obtained from 2 independent PCRs. In 6 animals of the Indonesian sample, we found sequences strictly identical to the most frequent Mauritius haplotype, confirming that Indonesia is a very probable source of the Mauritius population founders. This result is compatible with the Javanese origin previously suggested by different studies (Sussman and Tattersall 1981; Kondo et al. 1993; Kawamoto Y, Kawamoto S, et al. 2008). However, recently, Tosi and Coke (2007) clearly demonstrated a mixed origin of the Mauritius population, with a Javanese or Sumatran maternal lineage, and a continental Y chromosome lineage currently found in Sumatra. In the same way, recent results obtained on DRB exon 2 polymorphism show that the Mauritius population shares several DRB sequences with not only Indonesian but also Indochinese cynomolgus macaques (9 and 6 sequences, respectively), and only 2 sequences with the Philippines (Blancher et al. 2006, and additional unpublished data from A. Blancher). Thus, the study of Tosi and Coke (2007) and our observations lead to propose the mixed origin of the Mauritius population as the most probable hypothesis.

Most probably, the low-frequency Mauritius haplotype (1%), not observed in the Indonesian sample, may have arisen by mutation (T → C) from the main haplotype, after *M. f. fascicularis* became established in Mauritius 300–500 years ago. These results are in agreement with the study of Kawamoto Y, Kawamoto S, et al. (2008) who described 2 haplotypes (S and L) of an HVII fragment sequenced on the same number of individuals, with the low-frequency haplotype at 9%. The extremely low nucleotide and genetic diversities found in the Mauritius sample is most probably the current molecular signature of a founding effect, with few females, that was firstly deduced by Lawler et al. (1995) using RFLP. Our results, which clearly demonstrate that the Mauritius population is much less polymorphic ($h = 0.03$) than the Philippines one ($h = 0.69$) for mtDNA, differ from those recently observed for DRB exon 2 polymorphism in these 2 insular populations (Blancher et al. 2006). From this DRB dataset, the expected heterozygosity values were similar between the Mauritius ($h = 0.78$) and Philippines samples ($h = 0.81$). In addition, genetic data from other studies of the nuclear genome emphasize the discrepancy observed between mtDNA and nuclear genomes in Mauritius: Average H_e from microsatellite data equaled 0.66 (Kawamoto Y, Kawamoto S, et al. 2008), 0.70, and 0.69 for MHC and non-MHC microsatellite data (Table 1c, Bonhomme et al. 2007). This discrepancy between mtDNA and nuclear (microsatellite and DRB) markers in Mauritius could have several levels of explanation: 1) the effective size (N_e) of the mtDNA is 1/4 the nuclear genes, so the probability for the Mauritius population to retain and/or maintain polymorphism after a recent founding event (~100 generations) is much lower for mtDNA than for nuclear genes; 2) the approximate date of the Philippines foundation is very much older than that of Mauritius (0.11 My BP instead of 400 years BP) and after such a long period following the colonization, mtDNA polymorphism in the Philippines population had enough time to be restored; and 3) a biased sex ratio toward males in the Mauritius founders may explain the rapid loss of mtDNA lineages. Under this hypothesis, the male founders (continental/Sumatran origin, Tosi and Coke 2007) could have strongly contributed to Mauritius cynomolgus macaque nuclear polymorphism.

Dispersal Scenario of *Macaca fascicularis fascicularis* Populations

Based on the mtDNA sequence analysis of 3 natural *M. f. fascicularis* populations, we propose the following dispersal scenario (see Figure 4). First, the ancestral population of *M. f. fascicularis* diverged (see also Harihara et al. 1988) into 2 subpopulations: one called continental, colonizing the Indochinese peninsula and the other, called insular, almost 1 My BP. This estimate is close to that proposed by Tosi et al. (2003; see also Tosi and Coke 2007).

A more precise past history of the cynomolgus macaque is given by the phylogenetic tree: 6 Indonesian haplotypes (group II-2 of Figure 2) cluster in the continental group of sequences (group II of Figure 2). This suggests the current presence in Indonesia of mtDNA lineages coming directly from the Indochinese peninsula via migrant individuals. Alternatively, those lineages may originate from a remnant population that was localized in the Sunda Shelf and closely connected to the Indochinese peninsula, during the complex dynamics of sea level change in this region at Pleistocene glacial periods (<550,000 years BP). Figure 4 shows the putative location on the Sunda Shelf of such an ancient population and gives possible migrations routes explaining the paraphyly of Indonesian haplotypes.

More recently, around 110 000 years BP, the Philippines population derived from an Indonesian *M. f. fascicularis* stock (see Figure 3). Invasion of the Philippines may have been possible by terrestrial access through Borneo during periods of low sea level in Southeast Asia. An alternative scenario is

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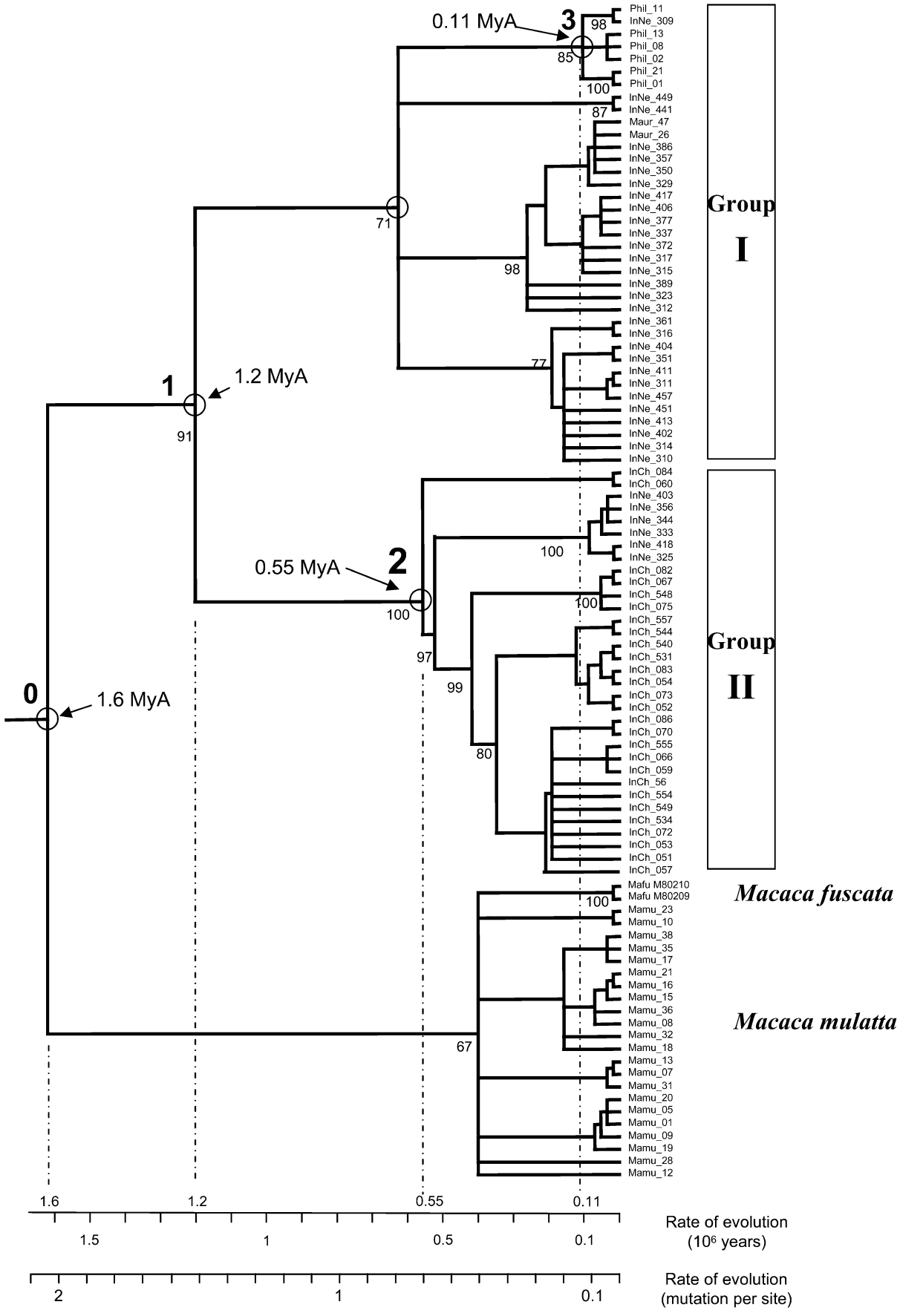
Figure 3. Bayesian phylogenetic tree of *Macaca fascicularis* populations, under the assumption of a constant mutation rate. Circles represent the nodes 0, 1, 2, and 3 that are dated at 1.6, 1.2, 0.55, and 0.11 My, respectively.


0.11 MyA
3
98
85
100
87
71
98
77
1
1.2 MyA
91
0.55 MyA
2
100
100
100
97
99
80
0
1.6 MyA
67
100
Phil_11
InNe_309
Phil_13
Phil_08
Phil_02
Phil_21
Phil_01
InNe_449
InNe_441
Maur_47
Maur_26
InNe_386
InNe_357
InNe_350
InNe_329
InNe_417
InNe_406
InNe_377
InNe_337
InNe_372
InNe_317
InNe_315
InNe_389
InNe_323
InNe_312
InNe_361
InNe_316
InNe_404
InNe_351
InNe_411
InNe_311
InNe_457
InNe_451
InNe_413
InNe_402
InNe_314
InNe_310
InCh_084
InCh_060
InNe_403
InNe_356
InNe_344
InNe_333
InNe_418
InNe_325
InCh_082
InCh_067
InCh_548
InCh_075
InCh_557
InCh_544
InCh_540
InCh_531
InCh_083
InCh_054
InCh_073
InCh_052
InCh_086
InCh_070
InCh_555
InCh_066
InCh_059
InCh_56
InCh_554
InCh_549
InCh_534
InCh_072
InCh_053
InCh_051
InCh_057
Mafu M80210
Mafu M80209
Mamu_23
Mamu_10
Mamu_38
Mamu_35
Mamu_17
Mamu_21
Mamu_16
Mamu_15
Mamu_36
Mamu_08
Mamu_32
Mamu_18
Mamu_13
Mamu_07
Mamu_31
Mamu_20
Mamu_05
Mamu_01
Mamu_09
Mamu_19
Mamu_28
Mamu_12
Group I
Group II
Macaca fuscata
Macaca mulatta
1.6
1.2
0.55
0.11
1.5
1
0.5
0.1
Rate of evolution (10⁶ years)
2
1
0.1
Rate of evolution (mutation per site)

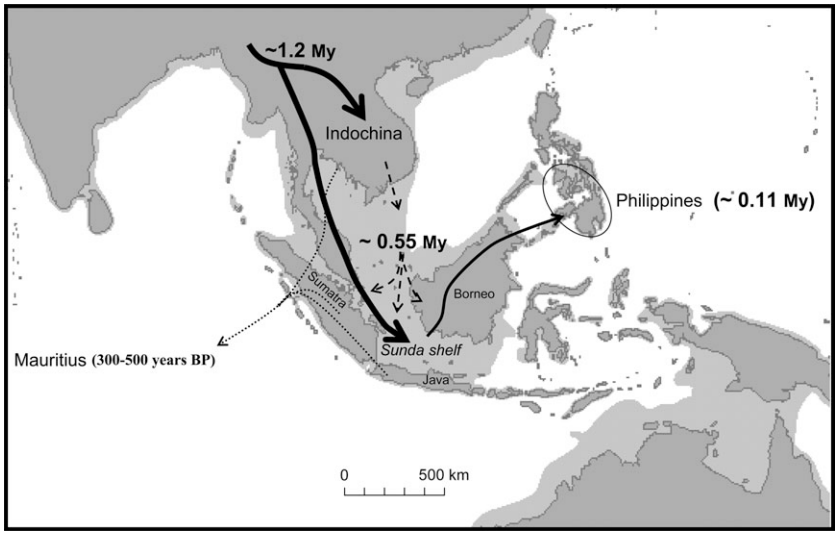


Figure 4. Map depicting the dispersal of *Macaca fascicularis fascicularis* in Southeast Asia. The figure was adapted from the original map from Voris (2000) where the limits of −120 m below present sea level are shown in light gray. Solid arrows depict the initial split between continental and insular groups and the colonization event of the Philippines. Dashed arrows depict hypothetic dispersal subsequent to the initial split. Dotted arrows represent the putative origins of the Mauritius population.

that, given that *M. fascularis* live in mangroves near the coast, it could have colonized the Philippines from Indonesia via Borneo by sea rafting (Abegg and Thierry 2002).

Most probably, all current Philippines haplotypes reported here have derived from a single common ancestral haplotype. The low genetic and nucleotide diversities suggest that a small group of Indonesian animals colonized the Philippines. Although precise dating of Philippines colonization cannot be assessed from our results, the phylogenetic tree suggests that after colonization of the Philippines, the population essentially remained isolated from the Indonesian population, allowing divergence of Philippines haplotypes that are not observed in the Indonesian sample. Although most Philippines sequences cluster in a specific group, this group encompasses one sequence from Indonesia. The latter could derive from the same ancestral haplotype, which gave rise to the Philippines population, but we cannot exclude back migration from the Philippines to Indonesia (Harihara et al. 1988).

Conclusions

The analysis of mtDNA D-loop sequences and further observations of nuclear genetic data confirms the founding of the Mauritius population by a small number of animals imported from Indonesia and perhaps also Indochina, as previously suggested by Tosi and Coke (2007). Our results also support the previously suggested ancient differentiation of *M. f. fascicularis* into 2 subgroups: 1 continental, another insular. In addition, our data raise the complexity of the Indonesian population. Indeed, the similarity between some sequences from Indonesia (subgroup II-2) and Indochina suggests either subsequent migrations southward, from Indochina to Indonesia, or an early colonization of the Sunda Shelf by the Indochinese population and then migrations southward, to Indonesia. The Philippines were most probably colonized by a small number of Indonesian *M. f. fascicularis* individuals. Unfortunately, the paucity of *M. f. fascicularis* fossil data in South East Asia and the uncertainty in calibrating the phylogenetic tree make divergence times dating difficult to assess in the light of complex and periodic changes in paleoclimates in Southeast Asia.

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