



Mitogenomics of macaques (*Macaca*) across Wallace's Line in the context of modern human dispersals

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ABSTRACT

Wallace's Line demarcates a biogeographical boundary between the Indomalaya and Australasian ecoregions. Most placental mammalian genera, for example, occur to the west of this line, whereas most marsupial genera occur to the east. However, macaque monkeys are unusual because they naturally occur on both western and eastern sides. To further explore this anomalous distribution, we analyzed 222 mitochondrial genomes from ~20 macaque species, including new genomes from 60 specimens. These comprise a population sampling of most Sulawesi macaques, *Macaca fascicularis* (long-tailed macaques) specimens that were collected by Alfred R. Wallace and specimens that were recovered during archaeological excavations at Liang Bua, a cave on the Indonesian island of Flores. In *M. fascicularis*, three mitochondrial lineages span the southernmost portion of Wallace's Line between Bali and Lombok, and divergences within these lineages are contemporaneous with, and possibly mediated by, past dispersals of modern human populations. Near the central portion of Wallace's Line between Borneo and Sulawesi, a more ancient dispersal of macaques from mainland Asia to Sulawesi preceded modern human colonization, which was followed by rapid dispersal of matrilineal and was subsequently influenced by recent interspecies hybridization. In contrast to previous studies, we find no strong signal of recombination in most macaque mitochondrial genomes. These findings further characterize macaque evolution before and after modern human dispersal throughout Southeast Asia and point to possible effects on biodiversity of ancient human cultural diasporas.

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1. Introduction

Huxley's modification of Wallace's Line lies between multiple Southeast Asian islands on the eastern margin of the Sunda continental shelf, including Bali and Lombok in the south, Borneo and Sulawesi in the middle, and Palawan/Busuanga and the oceanic islands of the Philippines in the north. This line demarcates a

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formidable biogeographical filter that impedes faunal migration between Asia and Australia via the intervening region of Wallacea, which includes Sulawesi, the Lesser Sunda Islands, and the Molucca Islands (Fig. 1; Huxley, 1868; Wallace, 1863). Huxley's modification of Wallace's Line and Wallace's Line (the northern portion of which runs between Sulawesi and the Philippines) demarcate distinct biodiversity hot spots (Myers et al., 2000) and ecoregions (Olson et al., 2001). For example, most terrestrial placental mammalian genera occur naturally only to the west of Wallace's Line (Raven, 1935; Holt et al., 2013). Turnover of bird species is also marked across Wallace's Line, although less so for amphibians (Holt et al., 2013). Even in modern humans, genetic variation is strongly structured east and west of this line, with a greater Australo-Melanesian ancestry component in the east, a greater Asian ancestry component in the west, and a gradient of admixture of these components as one moves east or west away from this line (Xu et al., 2012; Hudjashov et al., 2017).

Given these sharp biogeographical boundaries, it is notable that macaque monkeys (*Macaca* spp.) managed to successfully disperse across Huxley's modification of Wallace's Line at least three separate times (Fig. 1; Fooden, 1969, 1975, 1995). For each of these three macaque dispersal events, higher diversity and divergence of genetic variation are observed in populations west of this line (Liedigk et al., 2015; Evans et al., 2017; Yao et al., 2017), indicating that dispersal occurred in an eastward direction. The earliest of these three dispersal events occurred ~3 million years ago (Ma) or more, when a population (presumably from Borneo) ancestral to extant

Macaca nemestrina (Sunda pigtail macaques) on Borneo dispersed to Sulawesi (Evans et al., 2010, 2017; Roos et al., 2019), ultimately giving rise to as many as eight endemic species: *Macaca nigra*, *Macaca nigrescens*, *Macaca hecki*, *Macaca tonkeana*, *Macaca togeanus*, *Macaca maura*, *Macaca ochreata*, and *Macaca brunnescens* (Fooden, 1969; Froehlich and Supriatna, 1996); *M. togeanus* is sometimes considered to be a population of *M. tonkeana*, and *M. brunnescens* is sometimes considered to be a subspecies of *M. ochreata* (Groves, 2001; Roos et al., 2014). Another dispersal occurred further north (again presumably from Borneo) to the oceanic islands of the Philippines, possibly during the penultimate glacial maximum ~190 thousand years ago (ka) and led to a distinct subspecies of *Macaca fascicularis*, *M. f. philippinensis* (Fooden, 1991; Yao et al., 2017; but see Smith et al., 2014). A third dispersal event, the timing of which is less clear, occurred further south with the dispersal of *M. fascicularis* to the Lesser Sunda Islands (Fig. 1; Fooden, 1995, 2006; Yao et al., 2017) and established the easternmost extent of the genus *Macaca* (see Supplementary Online Material [SOM] for more background with regard to the diversification of *Macaca*).

Macaque dispersal to the Lesser Sunda Islands has been interpreted by some as a relatively recent event (~4–3 ka), possibly the direct result of translocation by modern humans (Fooden, 2006; Heinsohn, 2001; Long, 2003), in part owing to similarities in dorsal pelage color among populations from Java and the Lesser Sunda Islands and a lack of archaeological or fossil evidence of macaques on the Lesser Sunda Islands before the mid-Holocene (Fooden,

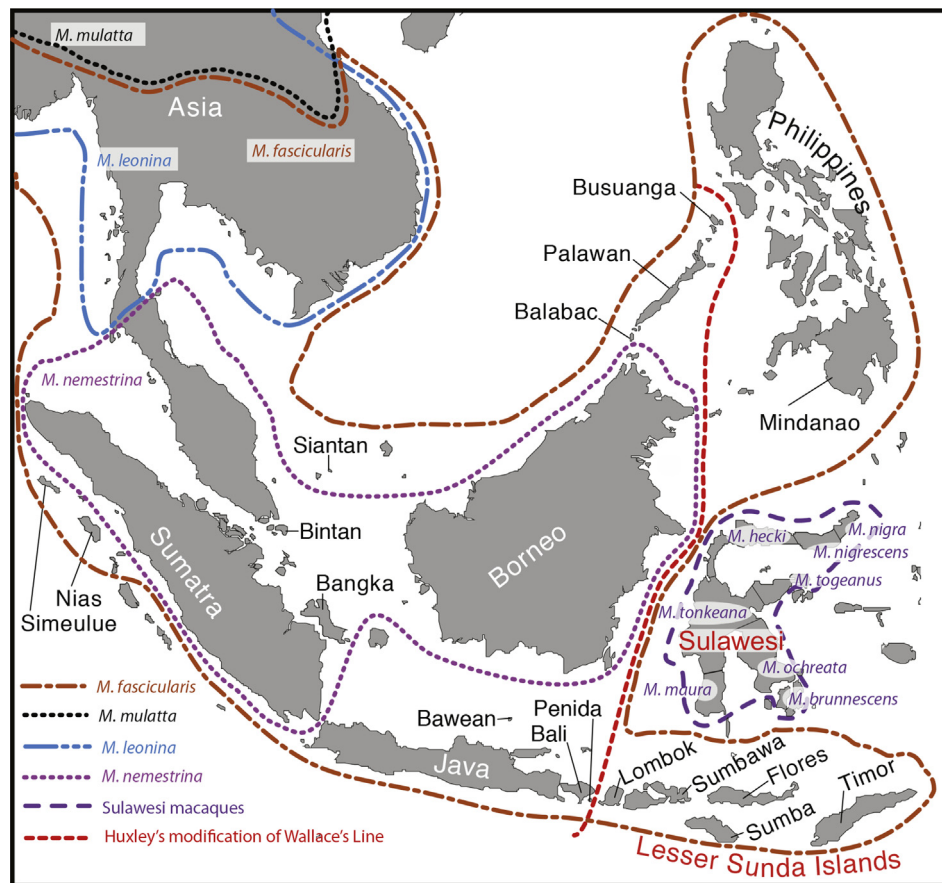


Figure 1. Macaque distributions in Southeast Asia. In bright red are three focal features of this study: Huxley's modification of Wallace's Line, Sulawesi Island, and the Lesser Sunda Islands. Bali and Penida are part of the Lesser Sunda Islands but here and elsewhere these islands are grouped or shaded with the Sunda Region because it lies west of Wallace's Line. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

1995). Consistent with this hypothesis, partial mitochondrial sequences from *M. fascicularis* specimens from Flores and Bali, which lie to the east and west of Wallace's Line, respectively (Fig. 1), are very similar to one another (Klegarth et al., 2017). However, the most recent common ancestor of other mitochondrial lineages from the Lesser Sunda Islands (specifically from Timor) and lineages from the Sunda region is ~1 Ma (Liedigk et al., 2015; Yao et al., 2017), opening the possibility that the Lesser Sunda population of *M. fascicularis* was initially established long before modern humans reached this region. One possibility is that the deep ~1 Ma mitochondrial divergence time observed in previous studies is due to unsampled (or extinct) lineages whose divergences across Wallace's Line are actually more shallow (Schillaci et al., 2017; Yao et al., 2017). Thus, although exactly when and how *M. fascicularis* dispersed to the Lesser Sunda Islands remains unresolved, these questions could potentially be addressed with increased sampling.

1.1. Molecular ecology of the silenus group of macaques

In macaques, interspecies hybridization in combination with incomplete lineage sorting frequently contributes to discordant phylogenetic affinities between mitochondrial and nuclear genomes (Evans et al., 2001; Melnick and Hoelzer, 1992; Stevison and Kohn, 2009; Tosi and Coke, 2007; Tosi et al., 2002, 2003). For example, partial mitochondrial DNA sequences support a closer relationship between *M. nemestrina* from Borneo and macaque species from Sulawesi than between *M. nemestrina* from Borneo and Sumatra (Evans et al., 2010). However, reduced representation genome sequences of nuclear DNA strongly support monophyly of *M. nemestrina* with respect to Sulawesi macaques (Evans et al., 2017). These nuclear data also strongly support reciprocal monophyly of Sulawesi macaques from the northern peninsula with respect to those from the rest of Sulawesi (Evans et al., 2017), but strong support for this relationship has not been recovered from partial mitochondrial DNA sequences (Evans et al., 1999, 2010; Tosi et al., 2003). Thus, it also remains an open question whether full mitochondrial genome sequences from *M. nemestrina* and Sulawesi macaques, including a diversity of intraspecific variation and increased geographic sampling, would provide results more consistent with those of nuclear DNA.

1.2. Recombination in mitochondrial DNA

Recombination in mitochondrial DNA has been directly illustrated in humans and flies (Kraytsberg et al., 2004; Ma and O'Farrell, 2015), and several studies suggest it occurs in other organisms as well, including macaques (Piganeau et al., 2004; Tsaousis et al., 2005; Ujvari et al., 2007). What is not clear, however, is how frequently recombination occurs and the degree to which detection of recombination in public sequence data reflects biological phenomena as opposed to statistical or data-related artifacts (White et al., 2013). Simulations indicate that analysis of recombined data using phylogenetic methods that assume no recombination has the potential to lead to phylogenetic misinference, exaggeration of terminal branch lengths, overestimation of substitution rate heterogeneity, and inference of non-clock-like evolutionary rates and star-like topologies (Posada and Crandall, 2002; Schierup and Hein, 2000a, b). Given its potential implications for phylogenetic inference, tests for recombination in mitochondrial DNA are a valuable prelude to phylogenetic analysis, with the caveat that the performance of methods to detect recombination varies under different conditions, such as variation in levels of polymorphism and demographic fluctuations (Posada and Crandall, 2001; White et al., 2013).

1.3. Goals of the present study

In this study, we explore macaque mitogenomics across Wallace's Line using 162 previously published mitochondrial genomes and those of 60 additional specimens. These new samples include two specimens collected by Alfred R. Wallace (one each from Lombok and Flores), three recovered during archaeological excavations at Liang Bua (Flores), all Sulawesi macaque species except *M. ochreata*, and novel geographic representation of *M. nemestrina* and *M. fascicularis*. Our aim is to resolve two long-standing questions about macaque evolution and dispersal: (1) when did *M. fascicularis* reach the Lesser Sunda Islands, and was this dispersal natural or mediated by modern human behavior? and (2) what are the phylogenetic relationships among mitochondrial genomes of *M. nemestrina* and Sulawesi macaques, and are they congruent with inferences from nuclear DNA? To accomplish these goals, we also test an important assumption of the phylogenetic methods that we deploy (Posada and Crandall, 2002), namely, that recombination does not occur among these mitochondrial genomes.

2. Methods

2.1. Samples

We obtained 60 new macaque mitochondrial genome sequences from either whole-genome shotgun (WGS) sequencing of DNA extractions from 29 blood samples (including six samples of *M. nemestrina* and 23 from seven species of Sulawesi macaque) or from targeted capture sequencing of DNA extractions from 31 museum or archaeological specimens (including 23 *M. fascicularis* and eight *M. nemestrina* specimens). All but one of these samples (a museum specimen labeled as *M. nemestrina* from 'Asia') have specific sampling locality information, minimally by island. The WGS samples generally have even more precise locations (usually to the nearest town or city). All of the museum specimens were collected within the past 150 years. The two *M. fascicularis* specimens from Lombok and Flores that were collected by Alfred R. Wallace were registered in the Natural History Museum, London (then known as the British Museum of Natural History), in 1864 and 1920, respectively, but their actual field collection dates remain unknown to us. Except as noted in the following paragraphs, species identification for the blood and museum specimen samples was unambiguous and based on provenance and external characteristics recorded during sampling. Additional information on samples analyzed in this study is presented in SOM Table S1.

Four bone and tooth fragments recovered during archaeological excavations of Liang Bua, a cave site on Flores, and identified based on morphology as *Macaca* sp. were selected for the screening of ancient DNA preservation and potential species identification. Portions of these same fragments were also sent for direct accelerator mass spectrometry (AMS) radiocarbon dating at the Research Laboratory for Archaeology and the History of Art at the University of Oxford, using chemical pretreatment, target preparation, and measurement protocols described elsewhere (Brock et al., 2010; Bronk Ramsey et al., 2002, 2004). DNA analysis of the archaeological and museum specimens was carried out at the Max Planck Institute for Evolutionary Anthropology (Leipzig, Germany).

Detailed information on DNA extraction, targeted capture methodology, sequencing, and genome assembly of WGS and targeted capture data is provided in SOM (Fig. S1 and Table S1). We combined our sample of 60 new mitochondrial genomes with 162 genomes published previously. One genome sequence from *Macaca mulatta* that was probably incorrectly annotated as *M. nigra* (GenBank accession KP072068.1) and one from *M. fascicularis* (FJ906803.1) were initially considered, but were subsequently

excluded based on tests for recombination discussed further in section 2.3. Provenance information and GenBank accession numbers for all data analyzed in this study are provided in SOM Table 1.

2.2. Phylogenetic analysis

We aligned the combined sample of 222 mitochondrial genomes from GenBank with a program for multiple alignment using fast Fourier transform (MAFFT) version 7.205 (Katoh and Standley, 2013) and then modified this alignment manually. Mitochondrial genomes from a gelada (accession FJ785426.1) and two hamadryas baboons (accessions NC001992.1, JX946201.2) were used as outgroups. We included in our analysis regions with some missing data, but excluded from all samples two regions of ambiguous alignment — a portion of the nongenic region that corresponds to positions 7697–7761 in KJ567054.1, and a portion of the D-loop, that corresponds to positions 15686–15741 in KJ567054.1 — leaving a total of 16,575 base pairs (including some positions with gaps) in the alignment of 222 sequences. Four sets of sequences in our analysis were identical: KM851028.1 + KM851029.1; KM851027.1 + KM851019.1; the new *M. nemestrina* mitochondrial genomes from samples PM665 and PM1206; and the new *M. maura* mitochondrial genomes from samples PF615, PM614, and PM616. All uncertain positions were converted to missing data before phylogenetic analysis. A maximum likelihood phylogeny was estimated using a model of evolution (TIM3+I+G4) favored by the Bayesian Information Criterion (BIC), as implemented by IQTREE version 1.4.0 (Nguyen et al., 2015). The TIM3 model uses empirical base frequencies; the reversible transition rate parameter between A and C is equal to that between C and G, and the same parameter between A and T is equal to that between G and T. This model also has a parameter that represents the proportion of invariant sites (I) and model heterogeneity in the evolutionary rate of different sites with a gamma distribution approximated by four rate categories (G4). We evaluated support for each node using 1,000 replicates of ultrafast bootstrap as implemented by IQTREE.

We used BEAST version 2 (Drummond and Rambaut, 2007) to estimate a time-calibrated phylogeny, using a random starting tree and assuming a relaxed log-normal clock with an estimated rate and a birth-death model for the tree prior. Six independent Markov chain Monte Carlo runs were performed, each for ~15 million generations and sampling every 10,000 generations. We used previously published estimated relationships among fossil and extant species and estimated dates for fossils to establish priors for the ages of two nodes in our phylogeny. We set the upper (youngest) boundary for the age of extant macaques at 5.3 Ma (Alba et al., 2014) based on the upper age range of the oldest fossil European macaques, which are considered to be closely related to *Macaca sylvanus* (Delson, 1980; Roos et al., 2019). We set the lower (oldest) boundary for the age of extant macaques at 6.2 Ma based on the earliest age for the first appearance of an unambiguous macaque, *Macaca libyca*, from Wadi Natrun, Egypt (Werdelin, 2010). *Macaca libyca* may have diverged before the diversification of African and Asian macaques rather than being ancestral to this clade (Delson, 1980); however, this lower boundary for extant macaque diversification is also consistent with the proposal that African and Asian macaque lineages diverged in association with the Messinian Salinity Crisis between 5.9 Ma and 5.3 Ma (Alba et al., 2014), while also allowing for the possibility (~0.3 million years) of a pre-Messinian divergence (Roos et al., 2019). The timing of divergence between geladas and baboons was recently estimated to be between 4.5 and 4.2 Ma (Pugh and Gilbert, 2018); however, we conservatively used a slightly lower limit of 5.5 Ma (Pugh personal communication), which is closer to but not as old as other estimates

(Leakey, 1993; Delson, 2000; Roos et al., 2019). A uniform prior was set for each of these calibrations.

For the BEAST analyses, we used a more parameterized model (GTR+I+G4, with empirical base frequencies) than the one that was selected by the BIC as implemented by IQTREE, as detailed previously. The GTR model has an independently estimated reversible transition rate between each of the four nucleotides. Preliminary analyses recovered no well-supported differences when a model with fewer parameters than the BIC-selected model was used (TN93). Fifty percent of the Markov chain Monte Carlo run was discarded as burn-in based on inspection of the posterior likelihood trace using Tracer version 1.71 (Drummond and Rambaut, 2007), enabling quantification of the effective sample sizes of model parameter estimates. The effective sample size values for these analyses exceeded 200. Well-supported relationships (> 95% posterior probabilities) within the BEAST analysis were identical to the maximum likelihood analysis.

2.3. Recombination

Previously, a signature of recombination was detected in partial mitochondrial sequences from *M. nemestrina* from Borneo and Sulawesi macaques (Piganeau et al., 2004; Tsaousis et al., 2005). To evaluate whether this signature was present in the mitochondrial genomes studied here, we analyzed these data using nine methods (RDP, GENECONV, Bootscan, Maxchi, Chimaera, SiScan, PhylPro, LARD, 3Seq) as implemented by the Recombination Detection Program (Martin and Rybicki, 2000), version 4.97.

3. Results

As the bulk of the sequences analyzed here have been published elsewhere (Liedigk et al., 2015; Yao et al., 2017; Roos et al., 2019), we present our results in terms of the new insights gained from the additional 60 new genomes we collected. Specifically, these new data provide novel perspectives into (1) the timing and nature of *M. fascicularis* dispersal across Wallace's Line into the Lesser Sunda Islands and (2) phylogenetic resolution among and within *M. nemestrina* and Sulawesi macaques using mitochondrial genomes.

3.1. *Macaca fascicularis*

Ancient DNA and radiocarbon ages of archaeological macaque samples from Liang Bua All four archaeological samples from Liang Bua tested positive for ancient DNA belonging to Cercopithecidae and yielded direct AMS radiocarbon ages (Table 1); however, only three of these samples had DNA preservation suitable for subsequent sequencing of mitochondrial genomes (see SOM and Table S2). The resulting three genome sequences belong to *M. fascicularis*. The AMS radiocarbon ages were calibrated using the CALIB 7.1 program (<http://calib.org>) with the offset for the Southern Hemisphere (SHCal13; Holt et al., 2013) and converted into a median probability age estimate with conventional calendar-year age ranges using 95% confidence intervals (CIs) (Table 1; Stuiver et al., 2020). Hereafter, we refer only to the calibrated ¹⁴C ages and their 95% CI ranges. Because the ¹⁴C ages for these archaeological *M. fascicularis* samples were all within the past 2,500 years, these ages were not used as tip dates in the BEAST analysis.

Intraspecific mitogenomics Several recent studies have used molecular markers, including mitochondrial genomes, to explore the mitochondrial phylogeography of macaques, especially *M. fascicularis* in Southeast Asia (Blancher et al., 2008; Evans et al., 2017; Liedigk et al., 2015; Mohd Salleh et al., 2017; Schillaci et al.,

Table 1¹⁴C ages for bone and tooth fragments of long-tailed macaques recovered from Liang Bua (Flores, Indonesia).

Sample code	Sequence code	Excavated sector	Depth (cm)	Stratigraphic unit	Methods ^a	Age		
						¹⁴ C (yr BP)	Calibrated median probability (yr cal. BP)	Calibrated range, 95% CI ^b (ka)
OxA-37730	D1638	XXVI	~40	8C	AMS	190 ± 23	188	0.23–0.14
OxA-37731	D1647	XXVI	~70	8C	AMS	1,605 ± 24	1,457	1.53–1.40
OxA-37732	D1541	XXVI	~100	8C	AMS	1,619 ± 25	1,470	1.54–1.41
OxA-39515	SP3918 ^c	XXVI	~130	8C	AMS	2,482 ± 19	2,483	2.54–2.36

^a AMS, accelerator mass spectrometry; for details of the chemical pretreatment, target preparation, and AMS measurement, see Bronck Ramsey et al. (2002, 2004).^b CI, confidence interval.^c Complete genome sequencing not attempted for this sample.

2017; Smith et al., 2014; Tosi and Coke, 2007; Tosi et al., 2002, 2003; Yao et al., 2017). Similar to this previous research, we find that the mitochondrial DNA of *M. fascicularis* consists of two major clades, clade A and clade B (Figs. 2 and 3; Liedigk et al., 2015; Yao et al., 2017). Our results suggest that these two clades diverged from one another ~1.67 Ma (this and all subsequent dates are median estimates from the posterior distribution), with a 95% CI of 1.95–1.41 Ma (Fig. 2, SOM Table S3), similar to previous results (Liedigk et al., 2015; Yao et al., 2017; Roos et al., 2019). Clade A is distributed across mainland Asia, northern Sumatra, and the surrounding islands, except for one mitochondrial genome that putatively originated in Mindanao, Philippines, which may be a recent introduction or mislabeled sample (Yao et al., 2017). Clade B, which includes the 23 new *M. fascicularis* genomes from this study, is distributed across southern Sumatra, Borneo, Java, the Philippines, and the Lesser Sunda Islands. Clade B diversified 0.90 Ma (95% CI = 1.05–0.77 Ma), again similar to previously published estimates (Liedigk et al., 2015; Yao et al., 2017).

Within clade B, there is strong support for five distinct subclades (labeled B1 thru B5; Figs. 2 and 3) and weak support for a clade containing mitochondrial genomes from the islands of Nias and Simeulue (west of north Sumatra), but the relationships among these clades are not well resolved. Clade B5 comprises specimens from southern Sumatra, Borneo, and the Philippines, whereas clade B4 comprises specimens from Java and Mauritius. The other three subclades (B1–B3) included specimens that span the southern portion of Wallace's Line from Java, Bawean, Bali, and Penida Islands in the west and the Lesser Sunda Islands of Lombok, Sumbawa, Flores, and Timor in the east. Although previously known only in specimens from Timor (eight sequences; Liedigk et al., 2015; Schillaci et al., 2017; Yao et al., 2017), our results show that clade B1 is also found on Flores (eight sequences, including one specimen collected by Alfred R. Wallace and all three archaeological specimens from Liang Bua), Sumbawa (three sequences), and Java (four sequences; Fig. 3). Similarly, mitochondrial genome sequences within subclades B2 and B3 were formerly known only from west of Wallace's Line, on Bali and the nearby Sundaic island of Penida (B2), and on Java, Bali, and the nearby Sundaic island of Bawean (B3). However, we found that mitochondrial DNA of a *M. fascicularis* specimen that was sampled by Alfred R. Wallace on Lombok, which is immediately east of Wallace's Line, is from clade B2, and mitochondrial DNA from another museum specimen from Lombok is from clade B3.

Our results suggest that extant sequence divergence within clade B1 began 80 ka (95% CI = 110–60 ka), within clade B2 began 140 ka (95% CI = 180–100 ka), and within clade B3 began 210 ka (95% CI = 270–160 ka). Within clade B1, there are two subclades (B1-a, B1-b; Fig. 3). Clade B1-b began diversifying 70 ka (90–50 ka) and is restricted to the Lesser Sunda Islands based on the currently available data. Clade B1-a comprises three clades that are respectively exclusive to the islands of Flores, Flores + Sumbawa, or Timor, although support for the Flores + Sumbawa clade differed between the Bayesian and maximum likelihood analyses (46% and 100% posterior

probability and bootstrap support, respectively). Clade B1-a includes four sequences from Java and two from Flores. Within clade B1, subclade B1-a1 (Fig. 3) contains genomes from Java and Flores.

The divergence times between closely related genomes from either side of Wallace's Line provide estimates for the timing of possible eastward dispersal events of *M. fascicularis*: (i) the most recent common ancestor of subclade B1-a1 is estimated to have lived 30 ka ago (95% CI = 40–20 ka); (ii) within clade B2, subclade B2-a includes two sequences from Penida, which is west of Wallace's Line, and from Lombok, which is east; the most recent common ancestor of this clade is 60 ka (90–40 ka); and (iii) within clade B3, subclade B3-a includes four sequences from Bali and one from Lombok; the most recent common ancestor of subclade B3-a is 10 ka (20–0 ka). Dispersal scenarios associated with these divergence times and also the divergence between subclades B1-a, and B1-b (Fig. 3) are presented in the Discussion section.

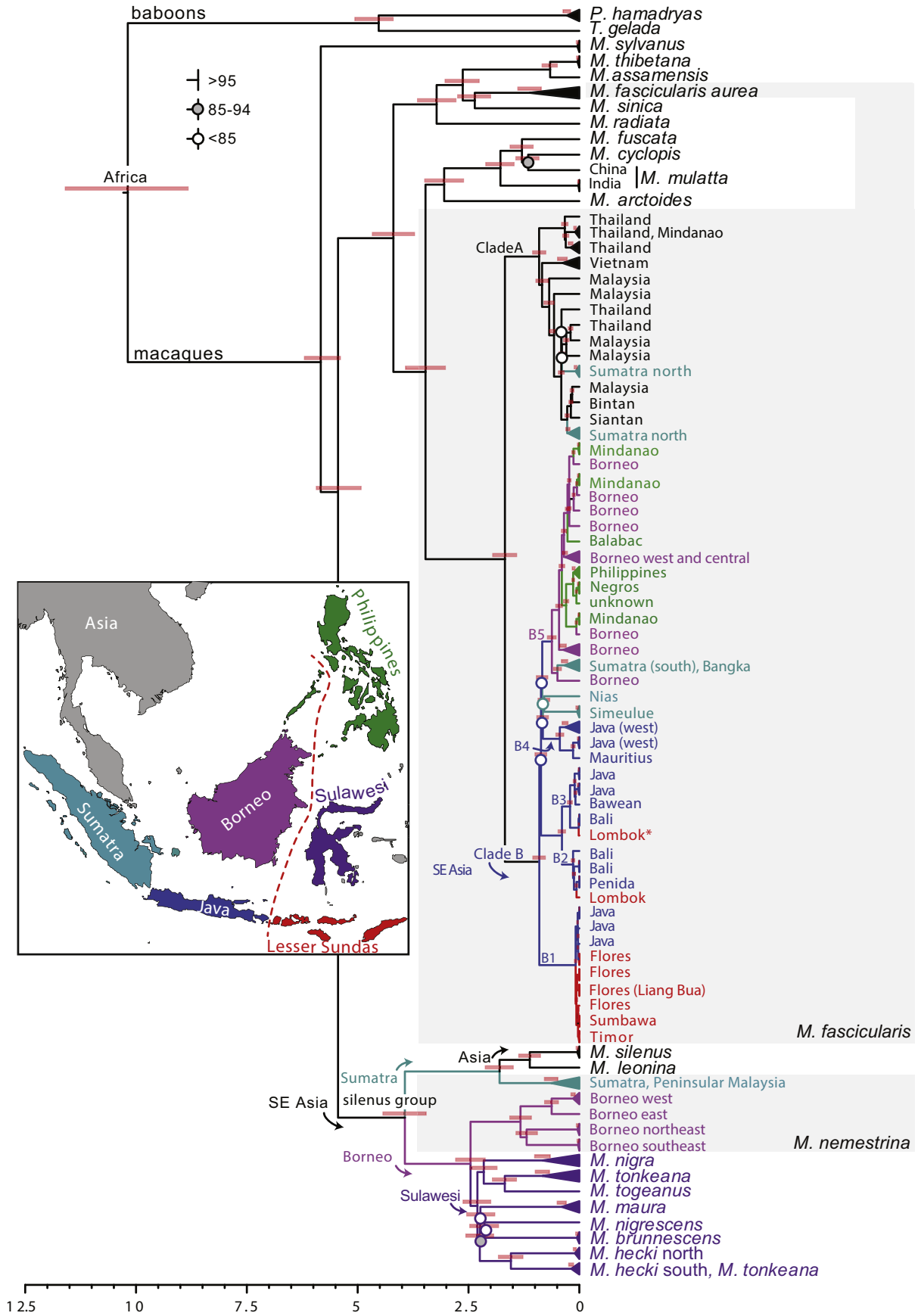
3.2. *Macaca leonina* and *Macaca nemestrina*

Consistent with several previous studies based on partial mitochondrial DNA sequences, our results support mitochondrial DNA paraphyly of *M. nemestrina* (Figs. 2 and 4; Evans et al., 2010). Mitochondrial genomes of macaques from mainland Asia that belong to the *silenus* group (*Macaca silenus*, which is from the Western Ghats of India, and *Macaca leonina*, which is from portions of mainland Southeast Asia including northeastern India, Bangladesh, Thailand, and Vietnam) form a clade that is sister to a *M. nemestrina* clade made up of specimens from Sumatra and Peninsular Malaysia as well as a museum specimen of unknown origin (SMF16836, from 'Asia'). Other *M. nemestrina* mitochondrial haplotypes from Borneo ($n = 12$) form a clade that is sister to a clade of Sulawesi macaques.

At least four major mitochondrial lineages are present in *M. nemestrina* from Borneo. The provenance of captive specimens in three of these clades suggests these lineages are represented by *M. nemestrina* from northeast, southeast, and east Borneo, respectively (Figs. 2 and 4). A fourth clade may be present in west Borneo, based on sequence similarity to a partial sequence in GenBank (AY206515.1) of another captive specimen from Sanggau, Kalimantan Barat, in Indonesia. Within *silenus* group macaques, our results suggest sequence divergence between Sulawesi and Borneo macaques and those from Asia and Sumatra occurred 3.93 Ma (95% CI = 4.43–3.46 Ma). Our results also suggest the mitochondrial genomes of *M. nemestrina* from Borneo diverged from those of Sulawesi macaques 2.45 Ma (95% CI = 2.79–2.13 Ma), which provides an estimate for when macaques (presumably from Borneo) dispersed east across Wallace's Line to Sulawesi.

3.3. *Sulawesi macaques*

Within Sulawesi macaques, mitochondrial DNA lineages are monophyletic and cluster by species, with one exception wherein a



mitochondrial genome from a *M. tonkeana* specimen (PF511) clusters with mitochondrial genomes from *M. hecki* (Figs. 2 and 4). This female macaque was sampled from near the hybrid zone between *M. tonkeana* and *M. hecki* (Bynum et al., 1997; Evans et al., 2003a). Based on photographs that were taken at the time of sampling, this specimen displayed narrow, kidney-shaped ischial callosities and relatively modestly sized brow ridges, which are morphological features of *M. tonkeana* and not *M. hecki*, in which these features are more oval and more prominent, respectively (Fooden, 1969). Preliminary analyses of WGS data from the nuclear genome of this specimen are consistent with this species' identification; at this time, however, we do not attempt to quantify the extent of *M. hecki* ancestry in the nuclear genome of this specimen. The uncorrected pairwise distance between the mitochondrial genome of sample PF511 and the most closely related *M. hecki* mitochondrial genome (PF643) is 0.0024.

Relationships among mitochondrial lineages of each Sulawesi species generally have short internal branches, and some are poorly supported (Figs. 2 and 4). However, support in the BEAST analysis for some early-branching relationships among Sulawesi macaques is surprising from a phylogeographical perspective. For example, a clade containing the mitochondrial genomes of *M. nigra* and *M. tonkeana* + *M. togeanus* is well supported (99% posterior probability) although *M. nigra* and *M. nigrescens* have a strongly supported sister relationship based on nuclear DNA and are distributed in parapatry on the distal end of the northern peninsula (Figs. 1, 2, and 4; Evans et al., 2017). *Macaca tonkeana* occurs in central Sulawesi, with the range of *M. hecki* between it and *M. nigra* + *M. nigrescens* (Fig. 1; Evans et al., 2017). Likewise, a clade containing the mitochondrial genomes of *M. nigrescens*, *M. maura*, *M. brunnescens*, and *M. hecki* has modest support (88% posterior probability) but is incongruent with supported relationships among nuclear DNA sequences (Evans et al., 2017). These nodes were present with modest support in the maximum likelihood analysis: the *M. nigra*, *M. tonkeana*, *Macaca togeanus* clade has a bootstrap support value of 77%, and the *M. nigrescens*, *M. maura*, *M. brunnescens*, *M. hecki* clade has a bootstrap support value of 61%. To explore the effect of analyzing only closely related mitochondrial genomes, we estimated a phylogeny from only the *silenus* group and Sulawesi mitochondrial genomes using BEAST. All nodes with >85% posterior probability in the *silenus* group in the main analysis (depicted in Figs. 2 and 4) were also present in the consensus topology of this reduced analysis (SOM Fig. S2). With the exception of the *M. nigra*, *M. tonkeana*, *M. togeanus* clade, which had a 96% posterior probability, other early-branching relationships among Sulawesi lineages were not strongly supported in the *silenus* group and Sulawesi-only analysis, which is similar to results of the main analysis.

Consistent with inferences based on partial mitochondrial sequences (Evans et al., 2003a), substantial intraspecific divergence is observed within *M. hecki* between the population in the southern portion of its distribution and that in the northeastern region. By contrast, very little intraspecific variation is observed between the two *M. nigrescens* samples.

3.4. Recombination tests

A total of 29 putatively recombined regions in 40 genomes were detected in the data by at least one of the nine recombination

methods implemented by RDP (SOM Table S4). Twenty-five of these 29 regions were flagged as possibly being 'caused by an evolutionary process other than recombination,' including four of the new mitochondrial genomes reported here: *M. hecki* sample PF644 (one 335 base pair region detected by five tests), *M. nigrescens* sample PM1011 (two regions that were 191 and 343 base pairs in size and detected by two tests and one test, respectively), and two *M. fascicularis* samples SP3942 (two regions that were 496 and 1099 base pairs in size and detected by three tests and one test, respectively) and SP3934 (one region that was 1099 base pairs in size and detected by one test). Recombination tests generally have a high rate of false positives and low power (Posada and Crandall, 2001), and none of these four putatively recombined regions were particularly convincing based on visual inspection. For example, the putatively recombined region of PF644 included un-called bases at 13 positions and unique SNPs in this genome at 14 positions that were not present in any putative parental genomes; this pattern is suggestive of false positives owing to rapid evolution of patches of sequence in a subset of individuals (patchy tachy; Sun et al., 2011). Both of the putatively recombined regions in the *M. fascicularis* genomes from Liang Bua (SP3942 and SP3934) largely comprised missing data. Visual inspection identified no discernable sign of recombination in the putatively recombined region of the *M. nigrescens* PM1011 genome.

Four recombination regions that were not flagged by RDP as possibly being caused by an evolutionary process other than recombination were all within two GenBank genomes that we excluded from phylogenetic analysis. One of these genomes was KP072068.1, which is annotated as a *M. nigra* genome, but phylogenetically positioned within a clade containing *M. mulatta* genomes based on preliminary analyses (not shown). One putatively recombined region in KP072068.1 was 1203 base pairs long and was identified by seven of nine tests. Visual inspection supports the inference of recombination based on high similarity with the minor parental sequences. Another region of KP072068.1 was identified by six tests, but recombination was not strongly evident based on visual inspection.

The second GenBank genome with a significant signature of recombination in seven tests was FJ906803.1, which is annotated as *M. fascicularis* of unknown origin. Visual inspection of two putatively recombined regions in this genome did not identify a marked indication of recombination because the regions were substantially diverged from the putative parental sequences. Because recombination can influence phylogenetic reconstruction, these two GenBank genomes were not included in the phylogenetic analyses presented here, and to our knowledge, neither has been included in a publication elsewhere. The signal of recombination in KP072068.1 and FJ906803.1 could have a technical explanation (e.g., contamination of nuclear insertions of mitochondrial origin, mapping or genotyping errors) or a biological basis. For KP072068.1, a technical explanation for inferred recombination event 1 (SOM Table S4) is supported by the geographically nonoverlapping distributions and relatively distant phylogenetic relationships of putative major (*M. mulatta*, *Macaca cyclopis*) and minor (*M. nigra*) parental sequences that are inferred to have contributed the larger and smaller fractions of sequence to the putative recombined sequence. The major parent of recombination event 3 (KP072068.1) and events 2 and 5 (FJ906803.1) was unknown, and the minor parents were *M. mulatta* (event 3) and *M. fascicularis* (events 2 and 5).

Figure 2. Inferred evolutionary relationships among mitochondrial genomes offer insights into macaque evolution in Southeast Asia. Consensus tree from Bayesian (BEAST) analysis of mitochondrial genome sequences; 95% confidence intervals for key divergence times are indicated with red bars above each node and also provided in SOM Table S3. Posterior probabilities of each node are depicted based on the legend. Scale bar indicates Ma, and branches and labels are colored according to the geographic localities in the inset. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

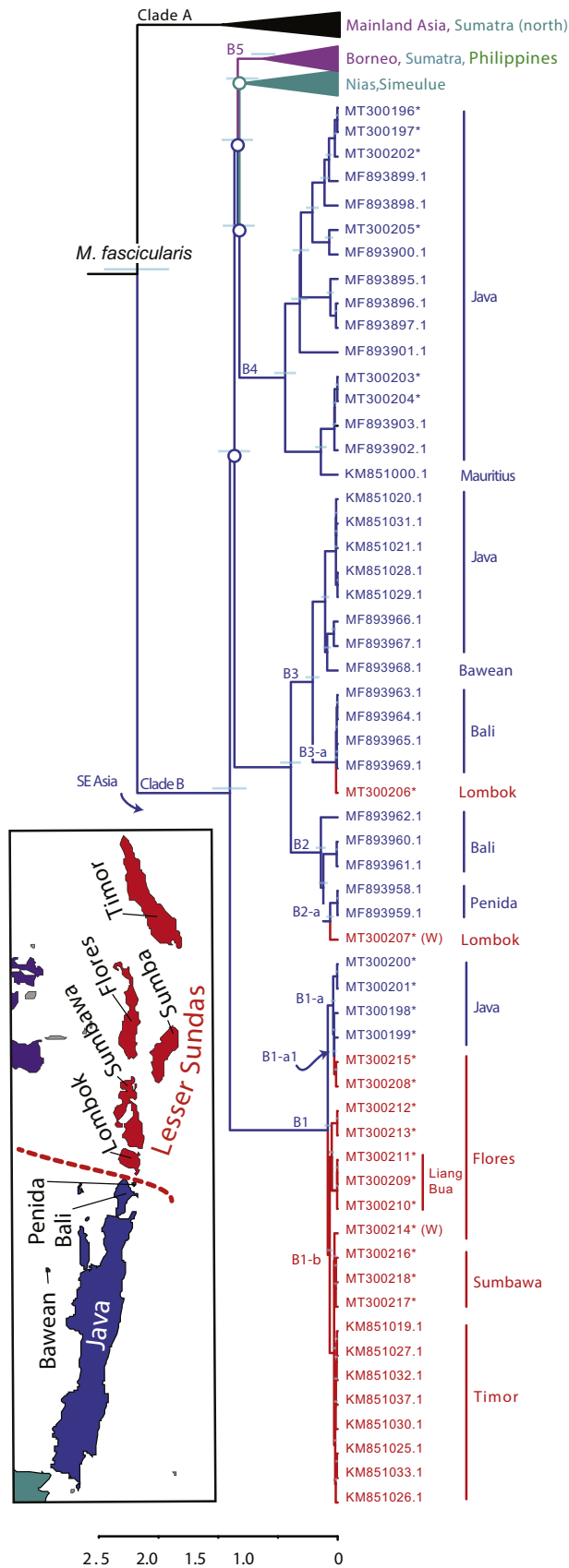


Figure 3. Detailed relationships among clade B of *M. fascicularis* demonstrate that multiple lineages occur on both sides of the southern portion of Wallace's Line. Samples collected by Wallace (W) and from Liang Bua are labeled. Except for very

4. Discussion

In this study, we conducted a phylogenetic analysis of 222 mitochondrial genomes from papionin monkeys, 219 of which are from the genus *Macaca* and 60 of which are previously unpublished. These 60 new mitochondrial genomes are novel in the sense that they include (i) species not previously sampled for whole mitochondrial genome sequencing (*M. nigrescens*, *M. hecki*, *M. togeanus*, *M. brunescens*), (ii) novel geographical regions for which full genomes and/or intraspecific variation were not previously available (*M. fascicularis* from several islands in the Lesser Sunda Islands, *M. nemestrina* from several locations on Borneo Island, all Sulawesi macaques except *M. ochreata* and *M. brunescens*), and (iii) prehistoric specimens of *M. fascicularis* as old as ~2 ka that were recovered during archaeological excavations on Flores. The increased taxon sampling and broader geographic and temporal coverage afforded by these new mitochondrial genomes provide insights into macaque evolution, particularly as it relates to macaque dispersals east across Wallace's Line.

4.1. Recombination in macaque mitochondria?

One concern about previous surveys of recombination in mitochondria using Sanger sequences (e.g., Piganeau et al., 2004; Tsaousis et al., 2005) is that these data may be affected by nuclear insertions of mitochondrial sequence (NUMTs). The very high coverage of our WGS data (SOM Fig. S1) suggests that the influence of NUMTs is almost certainly small in the new data analyzed here. We identified a convincing signature of four recombination events in two mitochondrial genomes from GenBank that were excluded from our analysis, but not in the other 222 genomes we included. For one of these four events (event 1), there is a lack of geographical proximity in extant ranges and diverged phylogenetic relationships of the putative parental sequences. In three others (events 2–3, 5) a major sequence was not identified in the data despite a relatively robust sample of extant variation in terms of interspecific and intraspecific variation for some macaque species. The characteristics thus cast doubt on a biological (as opposed to a technical) basis for the recombination signatures in these two GenBank sequences. In the 222 other genomes we analyzed, any signal of recombination that is present was insufficiently strong to be consistently detected by the methods we deployed. This could be because recombination is rare or absent or because demographic factors, such as rapid expansion of matrilineal lineages on Sulawesi, limited our ability to detect recombination.

4.2. Dispersal of *M. fascicularis* to the Lesser Sunda Islands

There are competing hypotheses for explaining the timing and nature of the dispersal of *M. fascicularis* to the Lesser Sunda Islands (Fooden, 1995; Heinsohn, 2001; Liedigk et al., 2015; Long, 2003; Schillaci et al., 2017; Yao et al., 2017). One of these is based on divergence in mitochondrial genomes and posits that this dispersal occurred naturally ~1 Ma, which is before modern humans and possibly even other hominins reached this region (Liedigk et al., 2015; Yao et al., 2017). *Macaca fascicularis* can swim (van Schaik et al., 1996), and the capacity of macaques to cross significant marine barriers is evidenced by the Sulawesi macaques, whose ancestors arrived on the island long before hominins reached this region (Fig. 2; Evans et al., 2010, 2017). Evidence of archaic

recent relationships, posterior probabilities are depicted following Fig. 2. Asterisks denote new mitochondrial genomes reported here; 95% confidence intervals for key divergence times are indicated with blue bars above each node. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

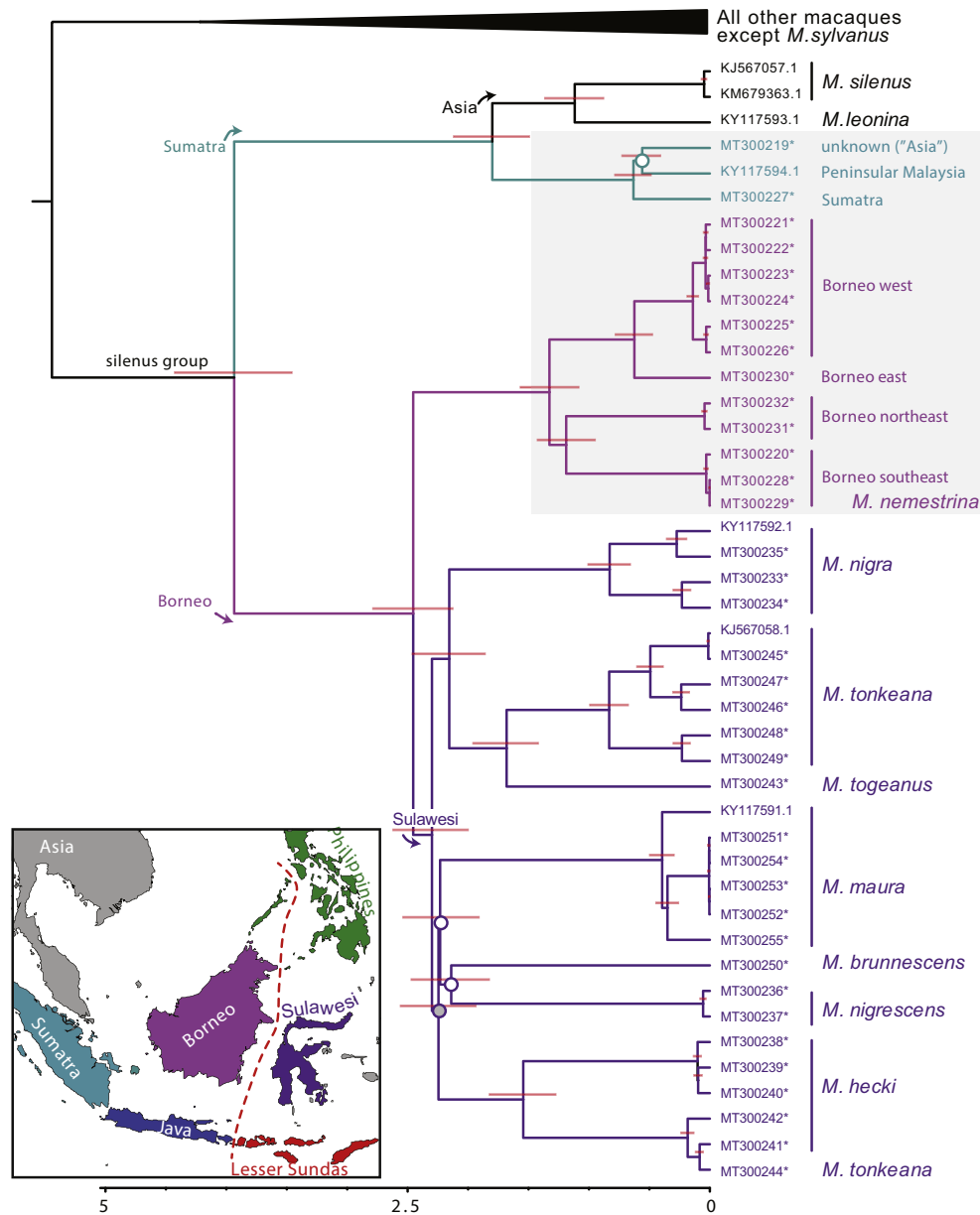


Figure 4. Inferred evolutionary relationships among mitochondrial genomes of *silenus* group samples support paraphyly of *M. nemestrina* but fail to resolve early relationships among the Sulawesi macaques. Labeling follows Fig. 3, except that 95% confidence intervals for key divergence times are indicated with red bars above each node. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

hominins in Island Southeast Asia extends to ~1.5 Ma based on a juvenile fossil hominin cranium found at Mojokerto, Java, in 1936, which is typically attributed to *Homo erectus* (Morwood et al., 2003; Huffman et al., 2006). Other evidence of *H. erectus* in Java includes fossils and stone artifacts found at other sites in Java including Sangiran (~1.3–0.9 Ma; Matsu'ura et al., 2020), Trinil (~0.5 Ma; Joordens et al., 2015), and Ngandong (~0.55–0.11 Ma; Rizal et al., 2020). East of Huxley's modification of Wallace's line, archaic hominins are known from the islands of Luzon in the Philippines and Flores in Indonesia (Morwood et al., 2004; Brown et al., 2004; Detroit et al., 2019). In addition, stone artifacts of unknown hominins dated to between ~200 and 100 ka have been recovered on Sulawesi at Talepu (van den Bergh et al., 2016b), well before the earliest skeletal evidence of modern humans in Island Southeast Asia (73–63 ka in Sumatra; Westaway et al., 2017). On Luzon, skeletal remains of *Homo luzonensis* are dated to minimum ages of

50 ka and 67 ka (Detroit et al., 2019), whereas stone artifacts and butchered rhinoceros bones derive from sediments dated to 777–631 ka (Ingicco et al., 2018). On Flores, skeletal and cultural remains of *Homo floresiensis*, an extinct human species that stood about 106 cm tall, weighed ~27.5 kg, and had a chimpanzee-sized brain (~420 cm³), are dated to ~190–50 ka at Liang Bua (Brown et al., 2004; Falk et al., 2009; Kubo et al., 2013; Grabowski et al., 2015; Sutikna et al., 2016). *Homo floresiensis*-like teeth and lower jaw fragments dated to ~700 ka are known from Mata Menge (Brumm et al., 2016; van den Bergh et al., 2016a), and stone artifacts from Wolo Sege are at least 1 Ma (Brumm et al., 2010). Thus, it is certainly plausible that *M. fascicularis* dispersed to the Lesser Sunda Islands around the same time as early hominins.

An alternative hypothesis is based on a lack of substantial morphological differentiation across the southern portion of Wallace's Line and a dearth of fossil evidence of macaques in the Lesser

Sunda Islands and posits that *M. fascicularis* dispersed to these islands more recently and possibly was introduced by modern humans (Musser, 1981; Heinsohn, 2001; Fooden, 2006; Sutikna et al., 2018). Numerous examples exist of modern human-mediated introduction of macaques in Southeast Asia, including *M. nigra* to the Bacan Islands in the Moluccas (Wallace, 1869; Rosenbaum et al., 1998), *M. togeanus* to Malenge Island in the Togian archipelago (Froehlich and Supriatna, 1996), and *M. fascicularis* to many locations, including Kabaena Island, Mauritius, Angaur Island, New Guinea, and elsewhere (Poirier and Smith, 1974; Froehlich et al., 2003; Long, 2003). Although modern humans have been present in this region for tens of thousands of years, it is possible that transport of macaques and other non-endemic animals to the Lesser Sunda Islands did not take place until recent migrations associated with Neolithic culture < 5 ka (Musser, 1981; Heinsohn, 2001; Fooden, 2006; Sutikna et al., 2018). Evidence of modern humans in the Lesser Sunda Islands extends back to ~46–40 ka (Morley et al., 2017; O'Connor et al., 2010, 2011; Sutikna et al., 2018) and in Australia extends back to as early as ~65 ka (Clarkson et al., 2017; but see O'Connell et al., 2018). Genetic analyses of living humans in the Lesser Sunda Islands and the surrounding regions show that they are the descendants of early Australo-Melanesian populations that admixed with Austronesian populations (Hudjashov et al., 2017). This relatively recent dispersal of Austronesian populations into Southeast Asia originated in Taiwan and reached the Lesser Sunda Islands, possibly by way of Luzon (in the Philippines) and Sulawesi (Hudjashov et al., 2017), and roughly coincides with the earliest evidence of Neolithic cultural practices on these Wallacean islands (Bellwood et al., 1995; Bellwood, 2005; Spriggs, 2012; Ko et al., 2014; O'Connor, 2015).

Direct evidence of Neolithic cultural practices, such as pottery, evidence of agriculture, and putatively introduced animals, appears in Southeast Asia with variable timing across this region, perhaps before ~3,000 years ago in portions of Borneo, the Philippines, the Lesser Sunda Islands, and the Moluccas (O'Connor, 2015). No evidence of macaques older than ~4 ka has been recovered on Timor (Glover, 1986; O'Connor, 2015) or Liang Bua (~190 ka to present; Sutikna et al., 2018) or at older sites on Flores (~1.0–0.7 Ma; Brumm et al., 2016). Similarly, on Sumba, which was not sampled in this study but is inhabited by *M. fascicularis* (Fig. 1), faunal remains have been recovered from Pleistocene deposits ~1 Ma and from Holocene deposits ~3.5–2.0 ka, but neither of these included macaques (Turvey et al., 2017). On Timor, macaque remains have only ever been recovered from archaeological contexts that are younger than ~4 ka and that also include pottery and other putatively introduced mammals such as pigs, but none are found in deposits older than ~5 ka (Glover, 1986). Macaques may have been intentionally introduced to the Lesser Sunda Islands by modern humans as a potential source of food to be hunted (Rosenbaum et al., 1998). Macaques are also kept as pets in Indonesia and elsewhere (Jones-Engel et al., 2005; Klegarth et al., 2017; Nijman et al., 2017), and this may also have provided a rationale for transport of macaques between islands in the past. Escaped or released animals could thus contribute to the distribution and genetic variation of modern macaque populations.

Our results have implications for discerning between these two hypotheses, but fail to conclusively reject either one. We found that the most recent divergences within three *M. fascicularis* mitochondrial clades that span the southern portion of Wallace's Line have 95% CIs of 40–20, 90–40, and 20–0 ka for clades B1-a1, B2-a, and B3-a, respectively (SOM Table S3). The phylogenetic placement of clades B2 and B3 points to recent (< 90 ka) eastward dispersal from Bali or Penida across Wallace's Line to Lombok, and these dispersal events may have occurred separately or concurrently with a polymorphic population. However, there are multiple interpretations of clade B1, summarized in Fig. 5, that are conceivably

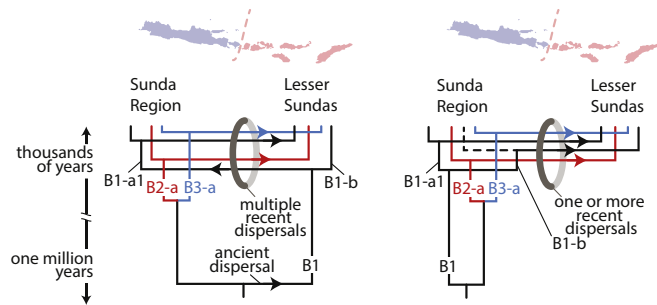


Figure 5. Two alternative dispersal scenarios for the ancestors of *M. fascicularis* in the Lesser Sunda Islands, under the assumption that well-supported relationships among mitochondrial genomes estimated in this study are accurate. The phylogenies on the left and right are a simplified subset of relationships from Figures 2 and 3. On the left is a scenario involving an early dispersal (~1 Mya) followed by more recent ones (thousands of years ago). On the right is a scenario with one or more recent dispersals and no early one; a dashed lineage represents an unsampled or extinct lineage from the Sunda region.

consistent either with ancient (~1 Ma) or more recent dispersal. One possibility is that *M. fascicularis* dispersed to the Lesser Sunda Islands ~1 Ma, which initiated the divergence of clade B1 from the other lineages in clade B, and then more recently, ~80 ka, descendants of these early migrants dispersed back to Java from Lombok, initiating divergence of clade B1 into B1-a1 and B1-b. The latter divergence would then have been followed by a recent dispersal from Java back to Lombok, which then initiated divergence within subclade B1-a1 ~30 ka (Fig. 5; left). This scenario, however, is at odds with the archaeological/fossil record from the Lesser Sunda Islands and with the shallow divergence between lineages on separate islands of the Lesser Sunda Islands (i.e., divergence ~70 ka lineages within clade B1-b from Timor, Sumbawa, Flores), unless the effective population size of clade B1 mitochondrial genomes from the Lesser Sunda Islands was very small.

A second interpretation of clade B1, which is also not consistent with the archaeological/fossil record, is that diversification of this clade began when macaques dispersed more recently (< 80 ka) from Java/Bali/Penida across Wallace's Line to the Lesser Sunda Islands, bringing with them lineages within clade B1 (Fig. 5; right). This dispersal could have involved a polymorphic population (or multiple separate ones) carrying subclades B1-a and B1-b (and perhaps clades B2 or B3 as well). In other words, in addition to subclades B3-a and B2-a, early diversification of clade B1 also could have occurred on Java or elsewhere in the Sunda region, with Javan *M. fascicularis* belonging to subclade B1-b either being extinct or not yet sampled. Overall, a recent dispersal of *M. fascicularis* to the Lesser Sunda Islands tens of thousands, rather than several hundreds of thousands, of years ago is more parsimonious in the sense that a recent dispersal minimally involves four lineages dispersing across Wallace's Line, whereas an ancient dispersal (~1 Ma) involves at least five lineages (Fig. 5). If correct, a < 80-ka-old dispersal of *M. fascicularis* to the Lesser Sunda Islands may have been mediated either intentionally or unintentionally by early modern human movements through Wallacea.

Some perspective on divergence times can be gleaned from the ancient samples from Liang Bua on Flores. Three of the four fragments of *Macaca* sp. from Liang Bua sampled for this study were positively identified as *M. fascicularis* based on their mitochondrial genomes, and all three are nested within subclade B1-b (Fig. 3). These specimens yielded calibrated ¹⁴C ages of ~1.5 ka (two samples) and ~0.2 ka (one sample; Table 1). DNA from the fourth specimen was identified as Cercopithecidae but was not preserved enough for sequencing, although it did yield a calibrated ¹⁴C age of

~2.5 ka. After excluding missing data, the available sequences from the three genomes from Liang Bua (15,619 base pairs) are identical, despite their different ages, which limits their utility for calibration purposes. Nonetheless, this provides a coarse expectation (based on a small sample size) that one substitution typically takes more than ~1400 years to occur. Within clade B1-a, there are ~10 differences between *M. fascicularis* mitochondrial genomes from Sumbawa + Flores compared with those from Timor. It follows that these lineages diverged more than 14,000 years ago, which is consistent with the phylogenetically estimated divergence time for this node of 30 ka (40–20 ka). Overall, these divergence estimates seem consistent with macaque residency in the Lesser Sunda Islands before ~4 ka, but perhaps not reaching back to ~1 Ma. With caveats discussed in the following paragraphs, most 95% CIs for divergence times suggest that our sample is not substantially affected by very recent dispersals or recently escaped or released captive animals, or if it possibly was (e.g., clade B3-a; SOM Table S3), the geographic scope of this effect is limited.

Our tests for recombination suggest that these data do not appear to be substantially affected by recombination in mitochondrial DNA, which increases branch lengths and leads to exaggerated divergence times. However, there are several cautionary notes with respect to the divergence estimates in this study. Perhaps most importantly (and as illustrated by this study compared to previous ones), unsampled mitochondrial diversity from extant and prehistoric macaque populations on these islands could identify new variation that influences phylogeographic interpretations.

Another concern is that the rate of evolution at the tips of a phylogeny is generally higher than the long-term rate of substitution at deeper portions of a phylogeny because some variants at tips are polymorphisms that will be removed by natural selection before they contribute to divergence (Ho et al., 2011). We did not observe a dramatic difference in the rate estimates at the tips of the tree compared with the older branches (SOM), but this likely is an artifact of our calibration points, which are both old. To explore this possibility further, we estimated the rate ratio of nonsynonymous and synonymous substitutions per site (dN/dS) on internal and terminal branches (SOM). This rate ratio (ω) was significantly higher on terminal branches (0.1232) than on internal branches (0.0928; $p = 0.01$). This finding is consistent with the proposals that (i) the rate of evolution is higher on terminal than on interior branches and (ii) our divergence estimates for terminal nodes (SOM Table S3) may be overestimates.

Thus, the disparity between the archaeological and paleontological evidence and the divergence estimates reported here can be partially explained by overestimation of divergence times at the tips of the phylogeny (Ho et al., 2011). Another factor that could cause this disparity is that divergence of mitochondrial lineages often can be older than the divergence time of descendant populations or species, and the disparity between gene divergence and species divergence increases with the population size, as does the probability of incomplete lineage sorting (Edwards and Beerli, 2000). Female philopatry and a female-skewed adult sex ratio in macaques also have the potential to cause divergence times in mitochondrial DNA to exceed the divergence times of populations (Melnick and Hoelzer, 1992; Hoelzer, 1997; Hoelzer et al., 1998). For these reasons, and in light of the archaeological and paleontological record, we do not consider our findings strong enough to reject the hypothesis that macaques were introduced in association with the Neolithic cultural diaspora in this region (i.e., < 5 ka; Glover, 1986; Sutikna et al., 2018), although we do interpret the data to favor a scenario of more recent (thousands of years ago) rather than ancient (several hundreds of thousands of years ago) dispersal to the Lesser Sunda Islands. Future work could further explore these

scenarios by searching for evidence of macaques on the Lesser Sunda Islands that are more than a few thousand years old. In tandem, collection and analysis of more macaque genetic data, including better geographical sampling of mitochondrial DNA and multilocus analysis of nuclear DNA, would permit more precise estimates of key parameters associated with this dispersal, such as ancestral population size, dispersal time, and the presence or absence of migration after the initial dispersal to the Lesser Sunda Islands. This is because far higher rates of recombination in nuclear DNA allow one to sample independent coalescent histories of molecular variation to a greater extent than in mitochondrial DNA.

4.3. Rapid dispersal on Sulawesi, fragmentation, and hybridization

New data and analyses reported here support previous inferences based on partial mitochondrial genomes including mitochondrial paraphyly of *M. nemestrina* with respect to Sulawesi macaques, but they also fail to resolve phylogenetic relationships within Sulawesi (Figs. 2 and 4; Evans et al., 1999; Evans et al., 2010). Although our conclusions are similar to previous studies, the present study pushes forward our understanding of macaque evolution in this region by increasing the size and likely the quality of mitochondrial sequences being considered. For example, Sanger sequences of partial mitochondrial genomes by Evans et al. (1999, 2010) comprised < 14% of the genome and, as mentioned previously, are more likely to be influenced by NUMTs than the current data.

Similar to *M. fascicularis*, the deepest diversification within *silenus* group macaques is found in the Sunda region. Because we did not sequence a mitochondrial genome of a *M. nemestrina* specimen from south Sumatra, we were unable to determine whether this early bifurcation occurs in mitochondrial DNA of *M. nemestrina* from northern and southern Sumatra, which is the case with *M. fascicularis* (Figs. 2 and 3; Yao et al., 2017), or from Sumatra and Borneo. On Sulawesi, mitochondrial diversity generally clusters by species, but with poorly resolved relationships among some of these clusters. This phylogenetic pattern with short internodes and long terminal branches matches expectations for an initially expanding population (Nordborg, 2001). Monophyly of mitochondrial genomes (outside of hybrid zones) is consistent with the proposal that evolution of much of Sulawesi's terrestrial fauna was influenced by habitat fragmentation (Evans et al., 2003b).

Mitochondrial paraphyly in *M. mulatta*, *M. fascicularis*, and *M. nemestrina* (Fig. 2) has been reported previously (Evans et al., 1999, 2010; Matsudaira et al., 2018; Tosi et al., 2002, 2003) and is possibly a consequence of sex-specific dispersal and other demographic characteristics of macaques (Melnick and Hoelzer, 1992). Incomplete lineage sorting could account for several inferred relationships among mitochondrial genomes of Sulawesi macaques (Figs. 2 and 4) that are discordant with those among nuclear genomes, although with equivocal statistical support between the Bayesian and maximum likelihood analyses of the mitochondrial genomes.

We identified one female *M. tonkeana* specimen (PF511) that carried a mitochondrial genome that is almost identical to that carried by *M. hecki* specimens. PF511 was previously incorrectly classified as *M. hecki* based on partial mitochondrial DNA sequences (Evans et al., 2003a), but a reassessment of photos and consideration of whole-genome sequences indicates that the genetic ancestry of this individual is mostly *M. tonkeana* (data not shown). In this case, incomplete lineage sorting is an unlikely explanation for this observation because pairwise divergence between other *M. tonkeana* and *M. hecki* mitochondrial genomes is ~20 times higher (Fig. 2). A more plausible explanation is that this specimen has recent mixed ancestry stemming from hybridization between

these two species, followed by several generations of backcrossing with *M. tonkeana* individuals.

5. Conclusions

New mitochondrial genome sequences presented here provide new insights into macaque evolution in Southeast Asia. Many of these insights are possible because DNA was accessible from museum specimens and three archaeological specimens from Flores. Analysis of new samples and geographic localities of *M. fascicularis* yielded substantially more recent divergence estimates across the southern portion of Wallace's Line that suggest that the period of *M. fascicularis* dispersal to the Lesser Sunda Islands probably occurred within the past 100,000 years. Divergence times across the southern portion of Wallace's Line generally antedate the Neolithic. However, molecular evolution and population genetic considerations and the possibility of unsampled variation bring these observations in closer alignment with the archaeological/paleontological record and with the hypothesis that macaques dispersed to the Lesser Sunda Islands fairly recently and possibly with the arrival of Neolithic culture. On Sulawesi, new data presented here offer glimpses into a dynamic evolutionary history including early arrival and rapid colonization (short internodes, possible incomplete lineage sorting), fragmentation (monophyly of species-specific lineages), and at least one instance of recent mitochondrial gene flow (at the hybrid zone between *M. tonkeana* and *M. hecki*). Overall, this study offers unique insights into the dynamic evolution of macaques and implications of human cultural diasporas on biodiversity.

Data accessibility

The 60 new mitochondrial genome sequences have been deposited in GenBank accessions MT300196–MT300255.

Conflict of interest

There is no competing interest.

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Supplementary Online Material

Supplementary online material to this article can be found online at <https://doi.org/10.1016/j.jhevol.2020.102852>.

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