

## PHYLOGEOGRAPHY OF THE ASIAN ELEPHANT (*ELEPHAS MAXIMUS*) BASED ON MITOCHONDRIAL DNA

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**Abstract.**—Populations of the Asian elephant (*Elephas maximus*) have been reduced in size and become highly fragmented during the past 3000 to 4000 years. Historical records reveal elephant dispersal by humans via trade and war. How have these anthropogenic impacts affected genetic variation and structure of Asian elephant populations? We sequenced mitochondrial DNA (mtDNA) to assay genetic variation and phylogeography across much of the Asian elephant's range. Initially we compare cytochrome *b* sequences (cyt *b*) between nine Asian and five African elephants and use the fossil-based age of their separation (~5 million years ago) to obtain a rate of about 0.013 (95% CI = 0.011–0.018) corrected sequence divergence per million years. We also assess variation in part of the mtDNA control region (CR) and adjacent tRNA genes in 57 Asian elephants from seven countries (Sri Lanka, India, Nepal, Myanmar, Thailand, Malaysia, and Indonesia). Asian elephants have typical levels of mtDNA variation, and coalescence analyses suggest their populations were growing in the late Pleistocene. Reconstructed phylogenies reveal two major clades (A and B) differing on average by HKY85/Γ-corrected distances of 0.020 for cyt *b* and 0.050 for the CR segment (corresponding to a coalescence time based on our cyt *b* rate of ~1.2 million years). Individuals of both major clades exist in all locations but Indonesia and Malaysia. Most elephants from Malaysia and all from Indonesia are in well-supported, basal clades within clade A, thus supporting their status as evolutionarily significant units (ESUs). The proportion of clade A individuals decreases to the north, which could result from retention and subsequent loss of ancient lineages in long-term stable populations or, perhaps more likely, via recent mixing of two expanding populations that were isolated in the mid-Pleistocene. The distribution of clade A individuals appears to have been impacted by human trade in elephants among Myanmar, Sri Lanka, and India, and the subspecies and ESU statuses of Sri Lankan elephants are not supported by molecular data.

**Key words.**—Asian elephant, *Elephas maximus*, evolutionarily significant units, *Loxodonta africana*, mitochondrial DNA, phylogeography, rate calibration.

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Molecular assessments of phylogeographic structure, in concert with paleontological and geological information, are highly valuable for the reconstruction of evolutionary scenarios. Molecular analyses of phylogeography can reveal evidence for both ancient and recent demographic events such as population size changes or dispersal (Slatkin and Maddison 1989; Tajima 1989; Avise 1994; Harpending 1994). Determination of phylogeographic structure can also be important for the purposes of conservation management (e.g., Dizon et al. 1992; Moritz 1994; Vogler and DeSalle 1994). In the case of Asian elephants (*Elephas maximus*), humans have been responsible both for fragmentation of populations through habitat change and local extirpation and for potentially high levels of gene flow through partial domestication and transport of elephants for trade and war (Sukumar and Santiapillai 1996). In this paper we assess mitochondrial DNA (mtDNA) variation in Asian elephants from throughout their remnant range. We combine the analysis with information about geological, paleontological, and human history to address alternative evolutionary scenarios that may explain the phylogeographic patterns we find. Our results suggest that most Asian elephant populations we studied are derived from relatively recent mixing of two formerly allopatric lineages

(perhaps even forms described as different fossil species) because of natural and human-assisted dispersal. Not all study populations show evidence of this mixing, and we consider the genetic evidence for subspecies and evolutionarily significant units (ESUs) and discuss the conservation implications of our findings.

The Asian elephant is the most endangered extant proboscidean, with fewer than 55,000 individuals remaining compared to more than 500,000 African elephants (*Loxodonta africana*). Their wild populations were greatly reduced and fragmented in the late Holocene by the actions of humans (Sukumar and Santiapillai 1996). The number of wild Asian elephants was estimated at more than 200,000 as recently as 1900, but current estimates (Sukumar and Santiapillai 1996) place the wild population at 37,000 to 55,000. An additional 15,000 are in captivity in a semidomesticated status, most of which do not breed. Because of their long generation time (minimum of 14–15 years and usually 18–20 years to female breeding age; Shoshani and Eisenberg 1982; Sukumar 1989) and low reproductive output (a calf every 2.5 to 8 years; Shoshani and Eisenberg 1982), population recovery in the wild will be a slow process (assuming that elephants and their habitats are suitably protected).

Although subspecies taxonomy of Asian elephants has varied among authors, the most recent treatment (Shoshani and

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Eisenberg 1982) recognizes three subspecies: *E. m. indicus* on the mainland, *E. m. maximus* in Sri Lanka; and *E. m. sumatranus* from Sumatra of Indonesia. These subspecies designations are based primarily on body size and slight differences in coloration and the fact that *E. m. sumatranus* has relatively larger ears and one extra pair of ribs (Shoshani and Eisenberg 1982). The Sri Lankan subspecies designation is weakly supported by analysis of allozyme loci (Nozawa and Shotake 1990), but not by analysis of mtDNA sequences (Hartl et al. 1996; Fernando et al. 2000). No assessment of the Sumatran subspecies was made in previous molecular studies.

There is a remarkably complete and detailed fossil record of the Elephantidae since the late Miocene (Maglio 1973; Coppens et al. 1978; Todd and Roth 1996). Fossil remains of the primitive elephantine *Primelephas gomphotheroides* have been found from the late Miocene to early Pliocene of Africa (Maglio 1973). This species likely gave rise to *Loxodonta* and *Elephas* in the early Pliocene (Maglio 1973; Tassy and Shoshani 1988), although alternative evolutionary scenarios exist (see Todd and Roth 1996). The lineage of mammoths (genus *Mammuthus*) evolved shortly after, probably from the *Loxodonta* lineage (Noro et al. 1998; but see Yang et al. 1996; Ozawa et al. 1997). K-Ar dating of strata associated with these fossils reveal that none date prior to about 4.5 million years ago, and Maglio (1973: fig. 15) estimates the split of *Loxodonta* and *Elephas* occurred about 5 million years ago. The *Elephas* lineage itself split into one lineage that remained in Africa (*E. recki* and derivatives) and at least one lineage that migrated into Asia about 3 million years ago (*E. planifrons* and *E. hysudricus* and derivatives; Maglio 1973). *Elephas maximus* likely evolved directly from *E. hysudricus*, perhaps during the last 0.2 million years (Maglio 1973). A Javan form, *E. hysudrindicus*, probably split from the *E. hysudricus* lineage 0.8–1.0 million years ago.

Asian vegetation and biogeography were greatly impacted by glaciation during the Pleistocene, and these changes likely affected the distribution of *Elephas*. At glacial maxima much of northern and western India was desert, and because of lower sea levels, the Sunda peninsula was large and included Sumatra, Java, and Borneo (Fig. 1). *Elephas* may have been separated into refugia in southern India and the Sunda peninsula during glacial maxima.

Humans apparently began domestication of Asian elephants about 4000–5000 years ago in the earliest settlements of the Indus Valley (Tennant 1861; Carrington 1958; Lahiri-Choudhury 1991). Until the early 19th century, large numbers of captive animals were moved across national borders, primarily as beasts for war or burden or, more recently, for timber extraction. The geographic extent and scale of this trade will never be completely known, but there are records of transport of literally thousands of elephants, over thousands of kilometers, especially during periods of war (Sukumar 1989). For example, one historical trade route existed between Sri Lanka and India by about 300 B.C., and later, in medieval times, another developed between Pegu in southern Myanmar to Sri Lanka and Bengal, and then to the Sultanate of Northern India (where wild elephants had been largely extirpated by this time; Digby 1971). Because escapes of domestic elephants were and continue to be relatively com-

mon occurrences (Ferrier 1947; Stracey 1963), translocations of elephants are likely to contribute genes to wild populations. Thus, escapes from captivity may have had major impacts on genetic structure. Natural dispersal occurs via movement of males among matrilineal groups (Sukumar 1989; Fernando and Lande 2000).

Here we report analyses of mtDNA sequences from Asian elephant populations across most of their native range. We calculate a divergence rate for the cytochrome *b* (*cyt b*) gene based on an African and Asian elephant comparison and their estimated date of ancestry (i.e., 5 million years ago; Maglio 1973; Coppens et al. 1978). We then apply this local rate calibration to estimate how long ago the Asian elephant mtDNA lineage split into two divergent clades (this study; Hartl et al. 1996). We also assess variability within and among Asian elephant populations and phylogeography for a much larger sample of individuals by analysing a segment of mtDNA that includes about half of the control region along with a small part of *cyt b*, and two tRNA genes. Last, we interpret the patterns in light of paleontological, biogeographical, and human history and address the implications of our results for the conservation of Asian elephants.

## MATERIALS AND METHODS

### Samples

All elephants sampled were captives (Table 1), but for nearly all individuals we know from where in the wild they originated. Asian elephant samples were collected from elephant work camps or orphanages in rural regions of Asia (Nepal, Thailand, Indonesia, Sri Lanka), or from zoos, either in Asia (Myanmar, Malaysia, Thailand) or North America (i.e., Indian samples; Table 1, Fig. 1). The records for most samples indicate that individuals are derived from local populations. These records showed that (1) Indonesian samples originated in three localities from across the island of Sumatra (i.e., Aceh in the far north, Riau in the center, and Lampung in the far south); (2) Malaysian samples came from five localities located throughout peninsular Malaysia (i.e., Perak, Selangor, Johor, Pahang, and Taman Negara); (3) three Myanmar samples came from Taungoo in Pegu; (4) all Nepal samples came from Chitwan National Park; and (5) one Indian sample came from Assam in far northeastern India (Fig. 1). We do not have exact capture localities for samples from Thailand or Sri Lanka, but based on the knowledge of local experts these are also derived from local wild captures.

All but three of our original sequences are derived from blood samples; two India-zoo sequences are from DNA isolated from toenail clippings, and one African and some Genbank sequences originated from salvaged hard tissues. Blood was taken in syringes, either in the presence of heparin or EDTA to prevent coagulation. White blood cells were separated by centrifugation. Samples were frozen and remained so during transfer to the laboratory. In total, using both control region sequences (57 individuals) and *cyt b* sequences (three different individuals) we determined the mitochondrial clade membership for 60 individual Asian elephants from seven countries (Table 1). We also obtained new *cyt b* sequences for two African elephants (Jacksonville Zoo and San Diego Zoo).

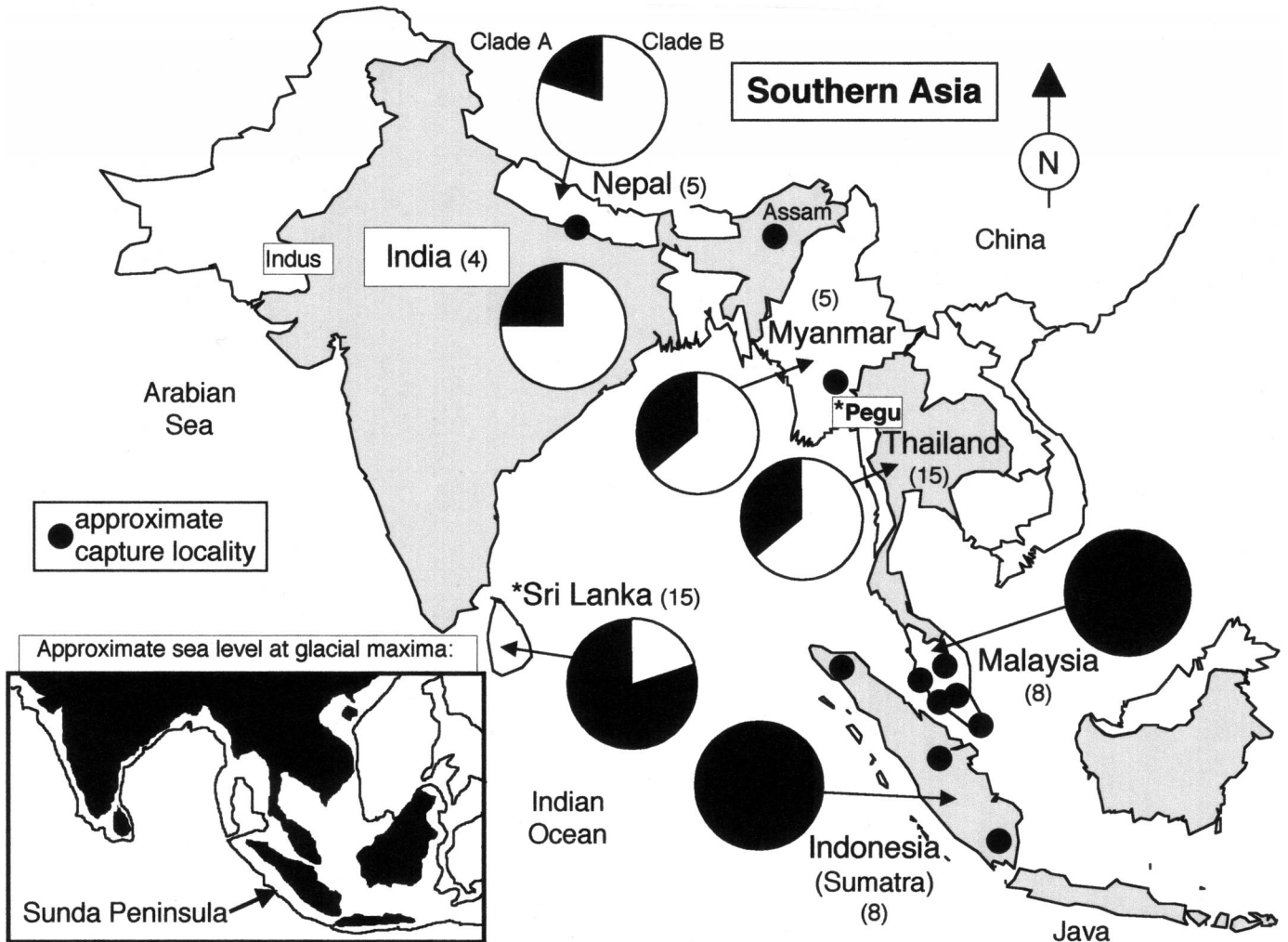


FIG. 1. Map of southern Asia showing the countries from which Asian elephant samples used in this study were collected. Sample sizes of genotyped elephants are in parentheses. Dots indicate sampling localities of elephants, when known (see text). Pie diagrams depict the relative frequencies of clade A (black) and clade B (white) in each sample (see text and Figs. 2 and 5 for explanation). Sample sizes include all elephants designated by clade. This includes three individuals typed with cytochrome *b* only, for a total of 60. Pegu and the Indus Valley are noted. Inset shows the approximate area of southern Asia at present (black) and at periods of glacial maximum (outline).

#### Molecular Protocols

DNA was isolated from blood samples and hard tissues by digestion in saline buffer-SDS with proteinase k followed by phenol-chloroform extractions and ethanol-Na-acetate precipitation. DNA was hooked from solution and placed in Tris-EDTA buffer for storage and subsequent use.

We amplified two segments of mtDNA from elephant genomic DNA via the polymerase chain reaction (PCR). The first segment, part of the *cyt b* gene, was amplified and sequenced from a subset of individuals, primarily to determine the sequence divergence between African and Asian elephants to calibrate a substitution rate. The second region, which we here label as "control region" (CR), was amplified from a much larger sample of Asian elephants (but could not be amplified cleanly from our African elephant samples). CR contains a small piece of the 3' end of the *cyt b* gene, *tRNA<sup>thr</sup>*, and *tRNA<sup>pro</sup>*, and the 5' end of the control region. The *cyt b* product totaled 1001 bp and was amplified with primers

*cyt b1* (3' base: L-14,608 of bovine mtDNA sequence, Anderson et al. 1982; same primer as L-14,841 of human; from Kocher et al. 1989) and *cyt b-EE* (H-15,800 of bovine mtDNA, Anderson et al. 1982; 5'-GTATAGAATT-GAGGCTATTG-3'). The CR product totaled 721 bp and was amplified with primers L-15,775 (bases 17–38 from the sequence in table 1 of Irwin et al. 1991; L-15,540 of bovine mtDNA, Anderson et al. 1982) and EW470 (H-16,265 of bovine mtDNA, Anderson et al. 1982; 5'-CCTGAAGTAG-TAGGAACCAGATG-3').

Standard PCR components (at 1.5 mM  $MgCl_2$ ) and approximately 100 ng of template DNA (estimated by comparison of EtBr fluorescence to a known amount of size marker on a minigel) were combined in a total volume of 50  $\mu$ l. Thermal cycling began with a hot start at 94°C for 3 min, and was followed by 25 cycles of 94°C for 50 sec, 55° for 50 sec, and 72° for 1 min. This was followed by a period of 3 min at 72°C for product extension. PCR products were

TABLE 1. Sample sizes and sampling localities of elephants sequenced in this study (additional sequences for cytochrome *b* genes were obtained from Genbank; see text).

Species/Region	<i>N</i>	Sampling Institutions
African elephant	2	San Diego Zoo (1), Jacksonville Zoo (1)
Asian elephant (60)		
India	4	Birmingham Zoo (1), El Paso Zoo (1), Los Angeles Zoo (1), Malaka Zoo (1)
Indonesia	8	Sumatra: Taman Safari (from three locations in the wild)
Malaysia	8	Malaka Zoo (4), Singapore Zoo (2), Zoo Negara (2)
Myanmar	5	Rangoon Zoo
Nepal	5	Royal Chitwan National Park
Sri Lanka	15	Malaka Zoo (2), Pinnewala Elephant Orphanage (13)
Thailand	15	Khao Kheow Zoo (3), Dusit Zoo (4), Siam Park (7), Jacksonville Zoo (1)

checked and quantified on an agarose gel and approximately 100 ng of Qiagen purified product was used for cycle sequencing reactions according to the manufacturer's protocol (Applied Biosystems, Inc., Foster City, CA). These reactions were run on an ABI 373 automated sequencer. Sequence chromatograms for light and heavy strands were aligned and checked for correct base calls in SeqEd (ver. 1.0.3, Applied Biosystems, Inc.) and/or Sequencher (ver. 3.0, GeneCodes Corp., Ann Arbor, MI). For *cyt b*, sequence was generated using the *cyt b1* and *cyt b-EE* end primers along with *cyt b2*

(Kocher et al. 1989) and *cyt b-DD* (L-15,076 of bovine mtDNA sequence, Anderson et al. 1982; 5'-CATTTCATCCTTCCATTAC-3') internal primers. For CR we used the PCR primers (above) for sequencing.

For *cyt b* we sequenced five Asian elephants (Indonesia<sub>2</sub>, Malaysia<sub>2</sub>, Myanmar<sub>3</sub>, Thailand<sub>2</sub>, and studbook number 130 from the Jacksonville Zoo, but originally from Thailand; Fig. 2) and one African elephant (*L. africana*) from the Jacksonville Zoo (studbook number 394). This African sequence differed substantially from that of Irwin et al. (1991; Genbank accession number X56285). Therefore we obtained tissue from O. Ryder (San Diego Zoo) and resequenced *cyt b* from the individual originally sequenced by Irwin et al. (1991). Four additional Asian elephant (numbers D83048, Ozawa et al. 1997; D50844, D50845, and AB002412, Noro et al. 1998; collection localities unknown), and three African elephant *cyt b* sequences were obtained from Genbank (numbers D84150–D84152, Noro et al. 1998). Thus, we have five African elephant and nine Asian elephant *cyt b* sequences for the comparison and rate calibration. A dugong *cyt b* sequence (*Dugong dugong*; U07564) was included to root the tree for a likelihood ratio test of molecular rate homogeneity (see below). Last, we obtained sequences from 57 Asian elephants for the CR segment. When combined with the three individuals for which we had *cyt b* sequences only, we could determine clade membership for 60 individuals.

#### Analysis of Mitochondrial DNA Sequence Data

We initially aligned the elephant sequences to each other and the published bovine one (Anderson et al. 1982) using Sequencher. We estimated the number of haplotypes and segregating sites, and the gene diversity (*h*) from both the aligned *cyt b* and CR sequences. Nucleotide diversity ( $\pi$ , or the mean of pairwise sequence difference) and its standard error were estimated uncorrected as in Nei (1987) using the program NucDiversity (C. E. McIntosh, unpubl.).  $\pi$  was calculated for the entire sample for each species, for the two primary clades (see below), and for each country sample of Asian elephants.  $\pi$  estimates  $\theta$  ( $=2N_e\mu$  for haploid mtDNA) under the assumptions of neutrality of substitutions and mutation drift equilibrium. An alternative estimate of  $\theta$ ,  $\theta_p$ , can be calculated from the proportion of segregating sites ( $p_N$  of Nei 1987) and this can be compared via Tajima's test (Tajima 1989) to  $\theta_\pi$  to assess whether impacts of selection or population change can be detected. Tajima's test was executed

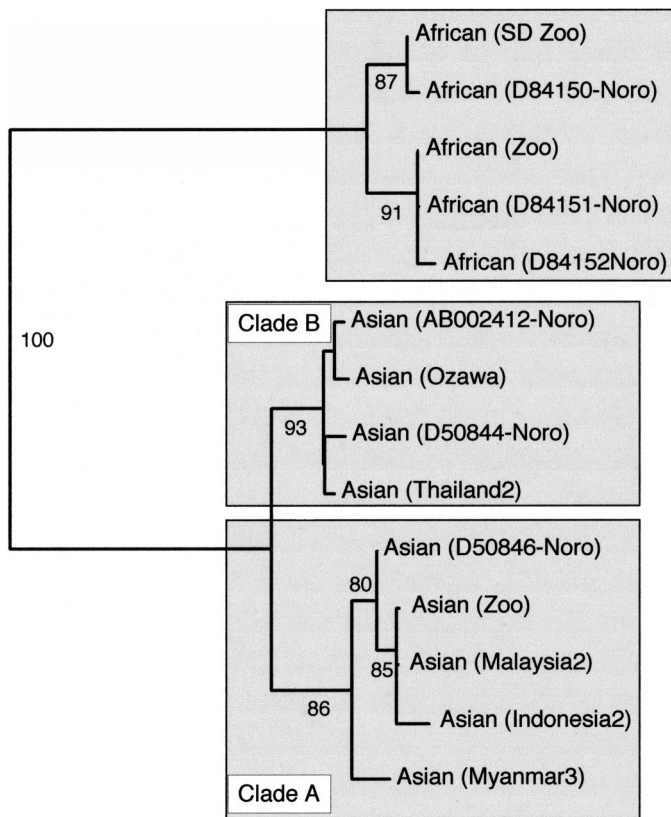


FIG. 2. Phylogeny of Asian and African elephant cytochrome *b* sequences. This midpoint-rooted tree is the shortest ( $D = 0.107$ ) derived via heuristic searches using a minimum-evolution criterion. The model of sequence change is the HKY85 model with  $\Gamma$ -correction ( $\alpha = 1.45$ ). A cladistic parsimony criterion with a branch-and-bound search results in four maximum-parsimony trees with an almost identical consensus topology.

in Arlequin (ver. 2.000, Schneider et al. 1999). Haplotype diversity ( $h$ ) corrected for sampling error was calculated following Nei (1987).

To further assess demographic change indicated by the mtDNA data, we constructed a mismatch distribution and computed the raggedness index of Harpending (1994) for the CR data using Arlequin (ver. 2.000, Schneider et al. 1999). We compared the mismatch distribution to Poisson expectation (Slatkin and Hudson 1991), and the raggedness index to generalizable simulation results of Harpending (1994). Last, we used the graphical coalescent approach of Nee et al. (1995) to determine whether the pattern of increase in the number of lineages over time (i.e., genetic distance) in a phylogram matches the pattern expected for stable, growing or decreasing populations. We used branch lengths (HKY85 and  $\Gamma$ -corrected; Sullivan et al. 1995; Swofford et al. 1997) from a UPGMA (i.e., ultrametric) tree to estimate coalescence time to a node. Both the mismatch and graphical approaches were conducted using both the entire set of CR sequences and each major clade separately.

Genetic distances among sequences of Asian and African elephants were calculated in PAUP\* (ver. 4.0b, Swofford 1997). To calculate unbiased 95% confidence limits of mean distances we used the method of Steel et al. (1996). This method requires a Jukes-Cantor correction (Swofford et al. 1997) and was conducted in CIPROGRAM (ver. 1.01b, C. E. McIntosh, unpubl.; available via <http://www.si.edu/organization/museums/zoo/zooview/research/genetics/mgl.htm>). For comparison to the Jukes-Cantor correction (Swofford et al. 1997), we also used PAUP\* to correct sequence divergence using a more complex, but perhaps more realistic, HKY85 model with a  $\Gamma$ -correction for rate variation among sites (Sullivan et al. 1995). All mean divergences were also corrected for potential lineage sorting by subtracting the weighted mean of within-taxon distances from mean intertaxon distances and their 95% confidence limits (Wilson et al. 1985; Nei 1987). The corrected mean and 95% confidence limits of the *Loxodonta-Elephas* separation were divided by 5 million years, the estimated date of cladogenesis from the fossil record, to provide a local maximum rate of overall *cyt b* sequence divergence.

We analyzed population structure by performing analysis of molecular variance (AMOVA; Arlequin ver. 2.000, Excoffier et al. 1992; Schneider et al. 1999) and constructing phylogenetic trees for the CR dataset. For the AMOVA, we make (perhaps unrealistic) assumptions that samples from each country represent populations and that the populations are in drift-migration equilibrium (see below). We calculate  $F_{ST}$ -statistics that incorporate Tamura-Nei corrected sequence divergences among haplotypes as well as haplotype frequencies. We use a nonparametric permutation procedure to test whether  $F_{ST}$ -statistics are significantly different from zero (Arlequin ver. 2.000, Excoffier et al. 1992; Schneider et al. 1999).

We also assessed population structure by estimating the genealogy of haplotypes via phylogenetic reconstruction. We built trees (in PAUP\*, Swofford 1997; Swofford et al. 1997) and a minimum spanning network (in Arlequin ver. 2.0b2, Schneider et al. 1999). Corrected distance matrices (HKY85 and  $\Gamma$ ) were analysed using a parsimony criterion (minimum

evolution) in PAUP\*: Starting trees obtained by the neighbor-joining method were subjected to branch swapping in heuristic searches until minimal length trees were obtained. If more than one tree was obtained a 50% majority rule consensus tree was calculated. Similar heuristic searches were conducted using unweighted, character-based (cladistic) parsimony (*cyt b* and CR). Distance and cladistic searches also included 500 repetition bootstraps to estimate support for particular nodes. Maximum-likelihood analysis was conducted on the *cyt b* data using a F84 model of sequence evolution with iterative or direct estimates of base frequencies, transition to transversion ratio, and rate variation among sites (PAUP\*, Swofford 1997). Log-likelihoods were calculated with and without a molecular clock from the *cyt b* tree and compared using a likelihood-ratio test with (number of terminal nodes - 2) as the degrees of freedom (Felsenstein 1981).

## RESULTS

### *Cytochrome b Sequences and Calibration*

We sequenced between 896 and 941 bp from the 1001-bp *cyt b* product. To this we added up to 110 bp of *cyt b* from the CR segment to make a total *cyt b* sequence of up to 1020 bp (base 14,631 to base 15,650 of the bovine sequence; Anderson et al. 1982). We aligned our sequences to the African elephant *cyt b* sequence from Irwin et al. (1991). The Irwin et al. African sequence has an inserted amino acid in residue 326 that is absent in all other elephantid sequences (Ozawa et al. 1997; Noro et al. 1998) and sequences of other mammal species (Irwin et al. 1991). When we aligned our resequence of the Irwin et al. sample, we found the bases for residue 326 missing and 14 additional substitutions (seven transitions and seven transversions). We believe that the Irwin et al. (1991) sequence may contain sequencing errors or perhaps represents a nuclear transposition of mtDNA sequence (e.g., Greenwood and Pääbo 1999). Irwin et al. (1991) found, as we did for both species, that African elephant *cyt b* has one less amino acid than other mammalian *cyt b* sequences. Asian elephant has a TAA termination codon, like African, rather than AGA as in most other mammals. No other unusual features were noted in our *cyt b* sequences.

The five African *cyt b* sequences contain four different haplotypes (our Jacksonville Zoo and one Noro et al. [1998] sequence were identical) and 11 variable sites; thus  $P = 0.012$  and  $\theta_p = 0.0056$  (SE = 0.0034). All 11 substitutions involved transitional changes (six A:G and five C:T); nine occurred in third positions and the remaining two substitutions occurred in second positions. The nucleotide diversity ( $\pi$  of Nei 1987) averaged 0.0064 (SE = 0.0016) substitutions per site.

In contrast, the nine Asian *cyt b* sequences contained 31 variable sites and eight haplotypes (our zoo and Malaysian2 sequences were identical). All 31 substitutions were transitions (six A:G and 25 C:T); 21 of the 31 substitutions occurred in third positions, and seven occurred in first and three in second positions. The  $\theta$  estimated from the mean number of segregating sites ( $p_N = 0.033$ ) was 0.0121 (SE = 0.0054; Nei 1987) and  $\pi$  was 0.0126 (SE = 0.0022).

The Asian sequences fall clearly into two differentiated clades (labeled "A" and "B" in Fig. 2) that differ at 16

sites. The topology from an heuristic, minimum evolution search is shown in Fig. 2 (HKY85 model with  $\Gamma$ -correction, shape parameter  $\alpha = 1.45$ , shortest tree score is 0.107). Tree topologies found by the cladistic parsimony and maximum-likelihood approaches only differ in resolution of some of the terminal branches. Support for the two clades is 100% based on 500 repetition bootstraps of both minimum evolution and cladistic trees. We expected this pattern of two divergent clades from three earlier studies, one using a 335-bp *cyt b* sequence (Hartl et al. 1996), one a restriction site analysis of PCR-amplified ND5 and ND6 (Georgiadis et al. 1994), and a third based on control region sequences from a limited geographical sampling (Fernando et al. 2000). We compared the region of overlap of our *cyt b* sequences with those of Hartl et al. (1996) and made the same clade designations (i.e., A or B).

The African and Asian *cyt b* sequences differ at 52 sites. The mean Jukes-Cantor-corrected divergence between the five African and the nine Asian sequences is 0.069. This distance occurs in the lower end of the range of intragenetic distances for mammals (Johns and Avise 1998). We evaluated the dugong-rooted tree with and without a molecular clock in PAUP\* and found a marginally significant difference in the log-likelihoods ( $G = 22.5$ ,  $df = 13$ ,  $P = 0.05$ ). Assessment of relative branch lengths suggests that the marginal rate heterogeneity is not caused by differences between the African and Asian clades, so we proceeded with the rate calibration. The HKY85 and  $\Gamma$ -corrected distance is 0.078, or 1.14 times the Jukes-Cantor distance. The unbiased 95% confidence limits (Steel et al. 1996) for the overall Jukes-Cantor mean (0.069) are estimated to be 0.056–0.088. We made an approximate HKY85 correction by multiplying the 95% confidence limit extremes by 1.14 (i.e., 0.064–0.100). After subtracting the intraspecific distance (0.011; Wilson et al. 1985; Nei 1987), we divided the three values by 5 million years (the estimated *Loxodonta-Elephas*) to obtain a rate of 0.013 *cyt b* sequence divergence per million years (95% CI = 0.011–0.018 per million years). The accuracy of the calibration relies on *Loxodonta* and *Elephas* sharing a common ancestor about 5 million years ago. However, because the two lineages are diagnosable in the fossil record for the past 4.5 million years (Maglio 1973), they must coalesce before then, and thus the rate we have calibrated is likely a maximum one.

We used the *cyt b* rate to estimate a date for the separation of the two major Asian elephant clades. The mean Jukes-Cantor corrected *cyt b* divergence between clades A and B is 0.019 (95% CI = 0.011–0.027) and the weighted mean within-clade distance is 0.004. The mean HKY85 and  $\Gamma$ -corrected distance is, as expected, only slightly larger, at 0.020. Applying the mean corrected rate for *cyt b* from above (0.013 per million years) yields a mean coalescence time for clades A and B of 1.2 million years (95% CI = 0.5–1.7 million years). We also calculated a rate of sequence divergence based on the mean African/Asian distance from the Georgiadis et al. (1994) ND5/ND6 RFLP dataset and the same split of 5 million years as 0.015 per million years. The distance between their two Asian clades is 0.016 (less within-clade distance) and the estimated coalescence time is thus 1.1 million years, very close to our *independent cyt b* estimate

of 1.2 million years. These results suggest the two mitochondrial lineages arose in the mid-Pleistocene, or perhaps even earlier, given that we have calibrated maximum rates.

#### Control Region Variation

The 675 bp of the Asian elephant CR segment includes 110 bp of the 3' end of *cyt b*, 67 bp of each of tRNA<sup>thr</sup> and tRNA<sup>pro</sup>, and up to 431 bp of the 5' end of the control region (including 354 bp of the left and 77 bp of the central domains). The 110 bp of *cyt b* aligns well to other elephant (Irwin et al. 1991; Ozawa et al. 1997; Noro et al. 1998) and bovine (Anderson et al. 1982) sequences. Both tRNA genes contain expected anticodon sequences and conserved stem sequence complementarity in the stem leading to the anticodon loop (Anderson et al. 1982). The CR sequence does not align to bovine sequence until 351 bp from its 5' start, presumably at the beginning of the conserved central domain. There is no evidence that our sequences represent transpositions of mtDNA sequences to the nuclear genome as found by Greenwood and Pääbo (1999). Our sequences differ by a minimum of 18 substitutions (15 transitions and three transversions) from the 281 bp of nuclear (Numt) CR sequence they amplified from elephant hair. However, one of our sequences (NE1) differs by only a single base from their mtDNA sequence amplified from DNA isolated from blood.

We found 47 variable sites and 36 haplotypes among the 57 CR sequences of up to 675 bp. This results in an overall  $\theta_p$  of 0.018 (SE = 0.003). Variable sites are not randomly distributed among the different genes: *cyt b* has 10 variable sites (0.091), tRNA<sup>thr</sup> has two (0.030), tRNA<sup>pro</sup> has three (0.045), the left domain of the CR has 29 (0.082), and the central domain piece has three (0.039). Only three variable sites contain more than two character states. Thus of 50 possible substitutions, only six involve transversions (a transition/transversion ratio of 7.33:1). The  $\pi$  over all 57 sequences (0.019  $\pm$  0.001) is not significantly different from  $\theta_p$  by Tajima's (1989) test ( $D = 1.40$ ,  $P = 0.090$ ). Positive values of  $D$  can indicate evidence for balancing selection or possibly admixture (Tajima 1989). Haplotype diversity was high overall ( $H = 0.972$ ).

#### Genetic Structure and Phylogeography

As found for the *cyt b* data (this study; Hartl et al. 1996) and for the CR data of Fernando et al. (2000), the CR phylogram (Fig. 3) and minimum spanning network (not shown) was split into two divergent clades, with 22 individuals in clade B and 35 in clade A. The two clades differ by a Jukes-Cantor corrected mean of 0.029 (95% CI = 0.018–0.041; Steel et al. 1996). The mean HKY85 and  $\Gamma$ -corrected distance (shape parameter  $\alpha = 0.063$ ; method of Sullivan et al. 1995) is much higher, at 0.050, indicating that there is greater rate variation among sites for CR than for *cyt b* (as expected; e.g., Wakeley 1993). This could account in part for the very low rate ( $\sim$ 0.01 per million years) of elephant CR sequence evolution estimated by Fernando et al. (2000), because they used a Jukes-Cantor correction rather than an HKY85/ $\Gamma$ -correction and may have underestimated divergence. We used the *cyt b*-based interclade coalescence time (i.e., 1.2 million years) to obtain a rough estimate of the rate of corrected

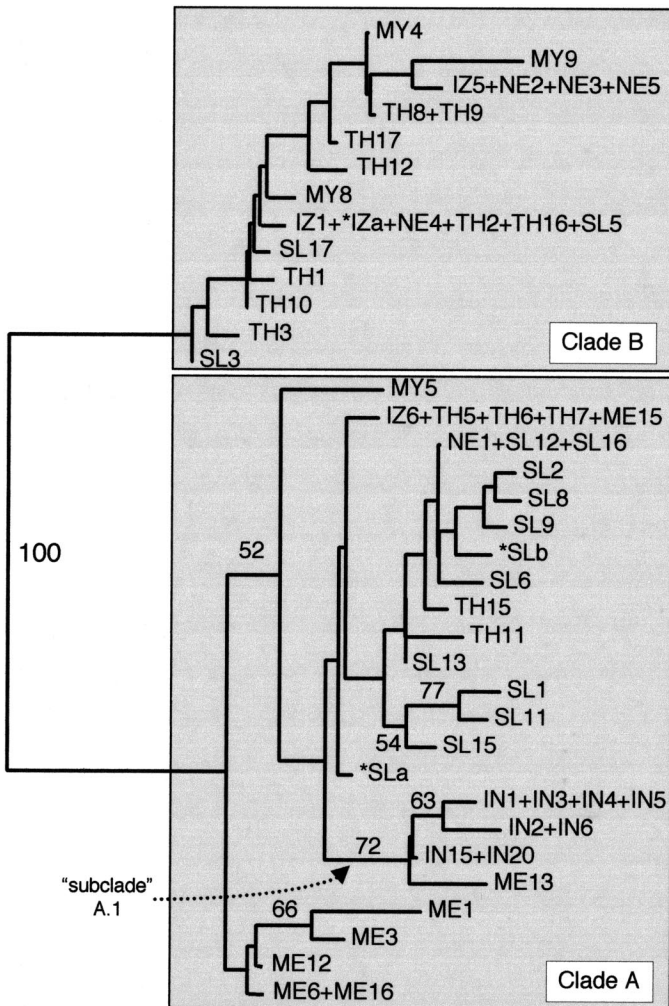


FIG. 3. Phylogeny of Asian elephant control region sequences. The tree was derived by criteria and methods as in Figure 2 (with  $\alpha = 0.063$ ). Note the deep split into two major clades (labeled A and B). The subclade A.1 contains all of our Indonesian (Sumatran) sample, plus one Malaysian individual. IN, Indonesia; IZ, India (zoo); ME, Malaysia; MY, Myanmar; NE, Nepal; SL, Sri Lanka; TH, Thailand.

sequence divergence for the CR segment as 0.035 per million years.

Nucleotide diversity is significantly smaller in clade B than clade A ( $\pi = 0.003 \pm 0.001$  for B and  $\pi = 0.012 \pm 0.001$  for A; Fig. 4), and also for the Sumatran (Indonesian) versus other populations (Fig. 4). In Sumatra and Malaysia we found only sequences of clade A (Figs. 1, 3). All eight Indonesian sequences and most Malaysian occur in well-supported clades basal to the other sequences in clade A (Fig. 3, Indonesian subclade A.1). This subclade is supported by unique bases at three sites and a bootstrap of 72% and shows a Jukes-Cantor distance (corrected by within-clade variation) from the other members of clade A of 0.012 (95% CI = 0.004–0.020). Applying the very roughly-estimated CR rate (0.035 per million years) suggests that this Indonesian clade may have begun diverging from the other clade A elephants about 0.34 (0.12–0.57) million years ago.

No significant differences exist between nucleotide diver-

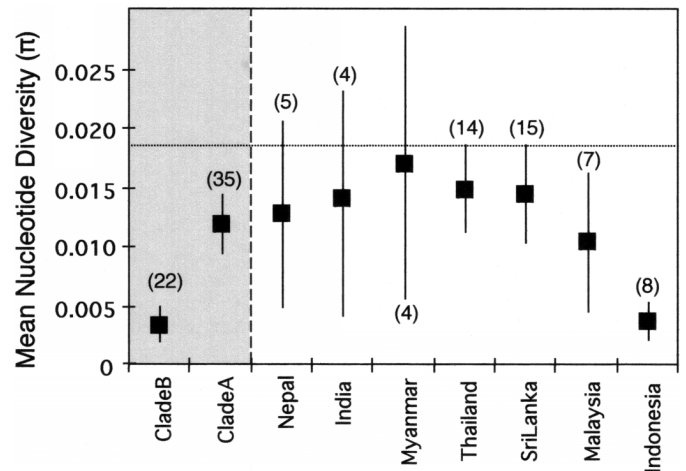


FIG. 4. Plot of mean nucleotide diversity ( $\pi$ ) of control region sequences within each sample and the two divergent clades (A and B). Means (squares) and 95% confidence limits (vertical bars) are calculated following equations from Nei (1987).

sity ( $\pi$ ) and  $\theta_p$  in the total sample (noted above), nor for each clade analyzed separately: In clade A,  $\pi = 0.004 \pm 0.001$  and  $\theta_p = 0.006 \pm 0.002$  (Tajima's  $D = -0.65$ ,  $n = 22$ ,  $P = 0.28$ ); in clade B,  $\pi = 0.012 \pm 0.001$  and  $\theta_p = 0.016 \pm 0.004$  (Tajima's  $D = -0.20$ ,  $n = 35$ ,  $P = 0.44$ ). Haplotype diversity is 0.905 in clade A and 0.963 in clade B.

The mismatch distribution for all 57 sequences is significantly different from Poisson expectation (Kolmogorov-Smirnoff test,  $P = 0.016$ ) and has a flattened shape usually suggestive of a stationary rather than expanding population (Fig. 5a; Slatkin and Hudson 1991; Harpending 1994). However, the mismatch distributions for clades A and B analyzed separately show no significant differences from Poisson expectation ( $P > 0.90$ ; Figs. 5b, 5c) and have shapes usually associated with expanding populations. Such unimodal patterns could, however, be generated by variation in substitution rates among sites (Aris-Brozou and Excoffier 1996), and CR and tRNA sequences are especially prone to such rate variation (e.g., Tamura and Nei 1993). Thus, other analyses are needed to test whether the match to a Poisson distribution is due to population expansion.

The raggedness index was low for analysis of all 57 sequences ( $r = 0.022$ ) and for each clade analyzed separately (clade A:  $r = 0.082$ ; clade B:  $r = 0.014$ ) and within the range indicating a smooth distribution and thus a population expansion (Harpending 1994). A plot of the log of the cumulative number of lineages against their HKY85 distances was made for each clade separately and combined. Analysis of both clades results in a line concave up that, according to the theoretical construct of Nee et al. (1995), should indicate a long-term stable or declining population. Analysis of each clade separately, however, reveals linear or slightly concave down plots, which indicate a growing population for the period during which each clade was evolving. Given the rate at which coalescent events occur for mtDNA, it is not likely that we can detect recent, human-caused population changes.

AMOVAs were conducted using Tamura-Nei corrections in Arlequin (ver. 2.000, Tamura and Nei 1993; Schneider et

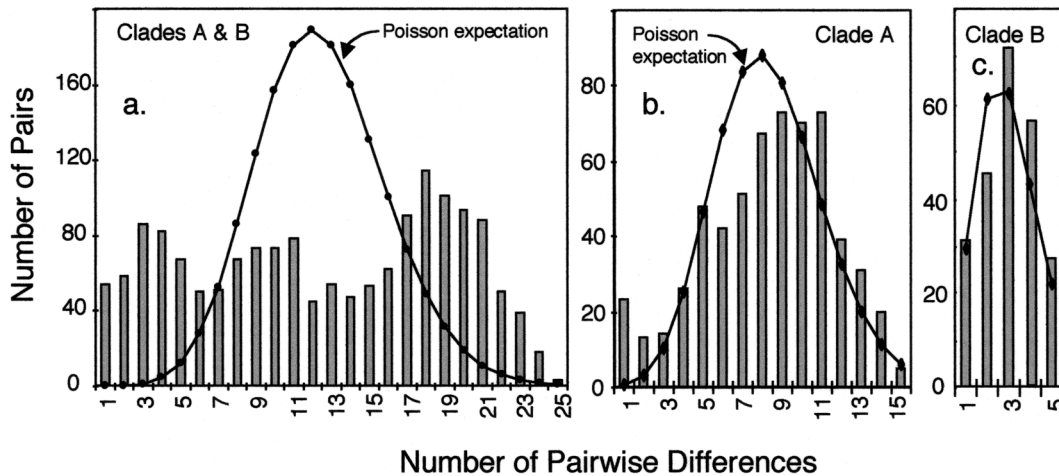


FIG. 5. Mismatch distribution (Harpending 1994): histogram of pairwise differences for control region sequences. (a) Both clades; (b) clade A only; (c) clade B only. The solid line in each graph is the Poisson expected given means of 11.5 (both clades), 7.35 (clade A), and 2.05 (clade B) differences among sequences. The expected is significantly different by Kolmogorov-Smirnov test only in (a) ( $P = 0.016$ ).

al. 1999) to generate  $F_{ST}$ -values of the CR sequence variation. Two AMOVAs were conducted: one across the seven country samples and one across the three subspecies. A hierarchical analysis was not possible because permutation tests require more than one population per group. Both analyses revealed significant genetic structuring. Among the seven populations,  $F_{ST} = 0.30$ , and the permutation test revealed this to be significantly different from zero ( $P < 0.0001$ ). Using equation 13.25 of Nei (1987), we calculate an average long-term  $Nm$  (migration rate) of about 1.2 migrants per generation among populations.  $F_{ST}$  for an AMOVA comparing the three subspecies (mainland, Sri Lankan, and Indonesian) was also high ( $F_{ST} = 0.29$ ,  $P < 0.0001$ ). However, analyzing  $F_{ST}$  pairwise revealed a lower level of divergence for Sri Lanka versus the mainland subspecies ( $F_{ST} = 0.14$ ,  $P = 0.009$ ). The divergence was considerably higher ( $F_{ST} = 0.42$ ,  $P < 0.0001$ ) for the mainland and Sri Lankan subspecies when compared pairwise with the Indonesian one.

The two major clades (A and B) evident in the phylogenetic analyses show an unexpected correlation with geography (Fig. 1): The more southerly of our samples have a higher frequency of individuals of clade A than the more northerly ones. This relationship is borne out by a highly significant ( $r = -0.78$ ,  $n = 16$ ,  $P = 0.0004$ ) regression between the proportion (arcsine-transformed) of individuals from clade A and the mean latitude of the sample. This regression includes clade designations from the present study (based on 57 CR and three *cyt b* sequences) as well as those of Hartl et al. (1996; 53 *cyt b* sequences from six localities) and Fernando et al. (2000; 118 CR sequences from three localities).

## DISCUSSION

### *Phylogeography and History*

Our results indicate that Asian elephants have typical levels of within population mtDNA variation. Thus, there is no evidence to suggest that historically recent range fragmentation and population decreases have impacted within-pop-

ulation genetic variability to any great extent. In addition, we found significant structuring of genetic variability among localities, in spite of a potentially very high, human-assisted dispersal level. Also, haplotypes are consistently separated into two highly differentiated clades that differ in their representation across localities and, to some extent, subspecies. These two clades can also be identified in three earlier studies of mtDNA in Asian elephants (Georgiadis et al. 1994; Hartl et al. 1996; Fernando et al. 2000), however, clinal distributions of clade proportions (Fig. 1) were not noted in these studies. The Georgiadis et al. (1994) study used North American zoo animals and their origins in Asia were not assessed, and the Hartl et al. (1996) and Fernando et al. (2000) studies dealt primarily with samples collected from central latitudes. Their Sri Lankan and southern Indian samples were similar in clade A frequencies to our Sri Lankan sample.

A split into two highly differentiated clades has been noted for the African elephant (Georgiadis et al. 1994). In this case, as with a number of other studies of natural populations (e.g., Wayne et al. 1990; Quinn 1992; Taberlet et al. 1992; Hoelzer et al. 1994; Arctander et al. 1996; Thomaz et al. 1996; Wooding and Ward 1997), highly divergent clades are found within single populations. In some cases, the clades show a broader geographic pattern suggesting that prior allopatry generated them (e.g., Wayne et al. 1990; Quinn 1992; Arctander et al. 1996; Wooding and Ward 1997). Georgiadis et al. (1994) favor the hypothesis that the two highly divergent clades in African elephant populations result from long-term retention of two ancestral, nonrecombining lineages rather than hybridization between two differentiated allopatric populations or species (e.g., *Loxodonta africana* and *L. atlantica*).

In support of the lineage retention hypothesis, Georgiadis et al. (1994) calculated the expected time to coalescence ( $T_c$ ) for haplotypes in a population as  $N_e[1 - (1/n)]$  generations. With our estimate of 1.18 million years for  $T_c$  for the A and B clades and a 20-year generation time, we used this equation to estimate a long-term  $N_e$  sufficient to maintain the two lineages to be approximately 29,000 females. This is only

slightly larger than current population estimates of 18,500–27,500 females (assuming at least half the population is female) and certainly smaller than the population of the last century. Thus, unlike Fernando et al. (2000), we feel that we cannot rule out from this analysis that our two clades could have been maintained in a single large population purely by stochastic lineage retention over the long term.

We also cannot rule out an alternative explanation for the existence of the two divergent clades: that the clades evolved in geographically separate populations, and after a period of isolation, some barrier to dispersal was removed and hybridization occurred. The cline in the proportion of clade A individuals from Indonesia to Nepal somewhat supports this allopatry hypothesis. However, the cline, and lack of clade B in our Malaysian and Indonesian samples, could also have been caused by loss of B through drift, perhaps caused by the decreasing land mass of the Sunda in the Holocene. The Tajima tests and the mismatch and other analyses on clades A and B suggest that the populations of each clade have been stable or expanding over time (but not bottlenecked). These changes may have occurred at different rates given the significant difference in  $\pi$  between the two clades. Different histories of each clade seems incongruous with a single-population, lineage sorting hypothesis.

If clades A and B do represent formerly allopatric populations that have recently merged, then their initial separation may have occurred more than 1 million years ago. Maglio (1973) notes that fossil *E. maximus*, although rare overall, is not found until the late Pleistocene (0.2 million years ago to present). The Javanese endemic, *E. hysudrindicus*, considered a sister taxon to *E. maximus*, appears to have separated from the *E. hysudrindicus/E. maximus* lineage 0.8–1.0 million years ago (Maglio 1973; Van den Bergh et al. 1996). It should be noted, however, that comparisons of morphological variability in extant elephants have not yet been made, and “the variability observed in the modern forms thus may raise questions about the validity of some of the nominal species of fossils” (Todd and Roth 1996, p. 201). In addition, the basal positions of the Malaysian and Indonesian individuals in clade A (Fig. 3), suggest that clade A most likely arose on the southern Sunda peninsula. Oddly, *E. maximus* does not show up in the fossil record of this region until the last interglacial, perhaps 0.05 million years ago, and only after *E. hysudrindicus* has disappeared (Maglio 1973; Van den Bergh et al. 1996). The estimated divergence time of about 0.34 million years between clade A.1 and other haplotypes in clade A (Fig. 3) suggests that clade A was in situ on the Sunda peninsula long before the putative arrival of *E. maximus* 0.05 million years ago. It is possible that the explosive eruption of Toba 0.071 million years ago and the volcanic winter that followed (Rose and Chesner 1990; Ambrose 1998) may have initiated the major dispersal events that led to mixing of the taxa.

One point possibly counter to the allopatry hypothesis is the high proportion of clade A individuals found in Sri Lanka and southern India. However, Sri Lanka (Ceylon) is unusual in that it was heavily impacted by the elephant trade, having been a major elephant import/export depot for more than 1000 years (Sukumar 1989). Strachan (in Tennant 1861) gives accounts of Arab traders transporting Ceylonese elephants to

India in the 1600s and Jayewardene (1994, p. 60) calculates that “over 3,253 were exported in the second half of the 19th century.” Sukumar (1989, p. 5) points out that “elephants from certain regions, such as Sri Lanka, were imported because they were considered especially suited for war.” War between regions resulted in major movements and escapes of elephants: of the Delhi Sultanate’s 3000 war elephants, “most came as captures from enemies in South India, as tribute from subordinate rulers, or as imports from various regions, including East Bengal, Sri Lanka and Pegu in lower Burma” (Sukumar 1989, p. 5). Sri Lanka also imported large numbers of elephants for breeding and training. The Pegu region (Fig. 1) of southern Myanmar, in particular, was a major source of elephants for Sri Lanka (Digby 1971).

If trade from Pegu brought clade A elephants to Sri Lanka and southern India then the haplotypes of these elephants should be like ones from southern Myanmar and adjacent Thailand. Most Sri Lankan and Indian elephants in clade A do have haplotypes more like ones from Thailand than like ones from Malaysia and Indonesia (Fig. 3). Likewise, in the study by Hartl et al. (1996), the most common clade A haplotype (MAX V) in Sri Lanka (36%) and southern India (55%) is also the only clade A haplotype found by them in southern Myanmar. A recent study by Fernando et al. (2000) revealed that wild elephant populations in Sri Lanka had a similar frequency of haplotypes in clade A (only 65.3% of 81 elephants) to the captive ones from our and the Hartl et al. (1996) studies. However, they found only 24% of these clade A haplotypes in Bhutan or Laos. These results suggest that trade among non-Sunda Asian populations may have erased some preexisting genetic structure in these areas (but apparently did not on the lower Sunda). Haplotypes from Sri Lanka deserve further study.

We speculate that clade A haplotypes could be descended from haplotypes of the Sunda species *E. hysudrindicus*, whereas clade B haplotypes are descended from those of the northern *E. maximus* (formerly *E. hysudrindicus*). The geographic pattern we see today would thus be a result of hybridization between the two forms, perhaps caused in the late Pleistocene by a combination of habitat changes, the impacts of Toba’s eruption, and, in later periods, by the elephant trade. The timing based on our molecular clock estimates and the geographic pattern we found do not support the alternative suggestion that the two haplotypes occur because of hybridization between a much earlier *Elephas* form (i.e., *E. namadicus*) and the lineage leading to *E. maximus* (Fernando et al. 2000). Because of higher female philopatry (Fernando and Lande 2000) and maternal inheritance of mtDNA, and perhaps little or no trade involving Indonesia and Malaysia, we still see genetically divergent, relictual populations in these regions, whereas other regions reflect very high admixture.

#### *Implications for Conservation*

Our results have implications for future in situ and ex situ management of Asian elephants. Genealogical analysis of mtDNA markers enables us to apply definitions of evolutionarily significant units (e.g., Ryder 1986; Moritz 1994; Vogler and DeSalle 1994). We confirmed earlier results of Hartl et al. (1996) and Fernando et al. (2000) that the Sri

Lankan subspecies is not supported as an ESU by current patterns of mtDNA variation. Significant haplotype frequency divergence of Sri Lankan elephants from mainland ones (also in Fernando et al. 2000) does suggest that Sri Lankan elephants may be a management unit (Moritz 1994). However, if we ignore the single Malaysian individual in clade A.1 (Fig. 3), the Sumatran subspecies, *E. m. sumatranus*, is monophyletic and diagnosable. Thus, under both the phylogeographic definition of Moritz (1994) and the single diagnostic character definition of Vogler and DeSalle (1994) we could define this taxon as an ESU and thus suggest that Sumatran elephants be managed separately from other elephants.

Before making this recommendation, however, we note some caveats to the rationale for ESUs (Fleischer 1998). First, no nuclear markers have been analyzed, and their analysis may lead to different conclusions given the difference in dispersal between the sexes (Fernando and Lande 2000). Second, we found major genetic divergence between haplotypes in clades A and B (more than four times that between Indonesian clade A.1 and related sequences within clade A). In most populations that we (and others) assessed, both haplotypes occur at intermediate frequencies. If the high divergence between clades A and B does not cause problems for reproduction or survival, should the smaller divergences between Sumatran, Malaysian, and the other clade A haplotypes matter for fitness in the event of crosses? We suspect outbreeding depression is unlikely, but the success of crosses in captive populations needs to be assessed directly. For example, what would happen to the extra rib in hybrids?

Finally, beyond outbreeding depression is a philosophical argument for retaining genetic structure, that is, to allow evolution to proceed on its natural or historical path. However, given the long and complicated historical interaction between elephants and humans, it may be difficult to determine what was the prehistoric situation for most populations, let alone to reconstruct it. In addition, if certain populations do become so small that reduced genetic variation and inbreeding compromise fitness within them (an unfortunately realistic possibility with the Sumatran population given recent trends in the loss of Indonesian lowland forest; Jepson et al. 2001; Whitten et al. 2001), then we may not have the luxury of maintaining the ESU. At that point, as originally suggested for ESUs by Ryder (1986), the question of crossing should be reevaluated.

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