



Short Note

High levels of mitochondrial genetic diversity in Asian elephants (*Elephas maximus*) from Myanmar

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Abstract

We analysed mtDNA control region sequences and 11 microsatellites in 78 Asian working elephants (*Elephas maximus*) from two camps in Myanmar (ca. 60 km apart), which holds the second largest elephant population in Asia. We found limited heterozygosity (overall H_o and H_e of 0.55 and 0.59) but high mtDNA diversity (overall haplotype and nucleotide diversities of 0.89 and 0.011, respectively) due to the presence of both mitochondrial lineages (α and β) known for Asian elephants. The fact that 13 of the 23 haplotypes found in this study were novel emphasises the importance of Myanmar for the conservation of this endangered species. Both markers support the occurrence of a single panmictic population in the region. Demographic tests produced some indication of a recent bottleneck in the microsatellite dataset, but the mtDNA sequences did not show either a signature of past expansion or bottlenecks.

The Asian elephant (*Elephas maximus*) is the largest terrestrial mammal of the Asian continent and of high ecological importance. Due to anthropogenic impacts, however, it has vanished from approx. 95% of its former distribution range, with present total population size estimates of ca. 40000–50000 (<http://www.iucnredlist.org/details/7140/0>). Population numbers are declining, and the species is listed as Endangered by the IUCN (<http://www.iucnredlist.org/details/7140/0>). Although Myanmar is a stronghold of the species (4000–5000 animals), very little is known about the genetic diversity of its elephants. To our knowledge, there are only very few studies that included Myanmar elephants (Hartl et al. 1996; Vidya et al. 2009). None of these studies analysed nuclear DNA (only mitochondrial loci), and none of them had a sample size larger than 24.

Two distinct mtDNA haplogroups are found in Asian elephants, α and β (or B and A). Unfortunately, the group called α by some authors is called B by others, and accordingly β is the equivalent of A (Hartl et al., 1996; Fernando et al., 2000, 2003; Fleischer et al., 2001). In the present study, we follow the α and β terminology. The two lineages are believed to have diverged between 1.6 and 2.1 Mya (Vidya et al., 2009). They co-occur in large parts of the range, and Myanmar is believed to be an ancestral area of the α -clade (Vidya et al., 2009). More detailed knowledge on the genetic make-up of the Myanmar elephants is important and timely. Our study aims at filling this gap by analysing elephants from two camps in Myanmar with respect to sequences of the mtDNA and for the first time, nuclear markers.

A total of 78 Asian elephants (38 males and 40 females, from the age of several months to 57 years) were sampled in two working camps

in Myanmar: Myaing Hay Wun (MHW) ($n=40$) and Taikkyi ($n=38$). Camp elephants are kept in semi-captivity; they are taken to work on logging sites 4 to 6 hrs per day 5 days per week. At night they are released into the forest for unsupervised foraging where also wild elephants occur. It is supposed that most of the calves born in captivity are sired by wild bulls. Both camps are located in the southeast of the country, to the northwest of Yangon and geographically close to each other (≈ 60 km). Additional information on Myanmar elephants can be found on Appendix A (Supplemental S1).

Blood samples were taken in March and April 1996 and stored in DMSO buffer. Specific ethical requirements beyond those pertaining to veterinary examinations did not exist. DNA was extracted using the GenElute™ Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich). A portion of the mitochondrial control region was amplified using the primers LoxProL and HiCtrIIH (following Fickel et al., 2007), sequencing both strands on an automated sequencer (3730xl DNA Analyzer, Applied Biosystems). Individual genotypes were established through amplification of 11 nuclear microsatellite loci (Nyakaana and Arctander, 1988; Comstock et al., 2000; Eggert et al., 2000; Fernando et al., 2001; Appendix B, Supplemental S2). PCR runs consisted of 5 min at 95 °C followed by 35 amplification cycles (30 s at 95 °C, 30 s at the locus-specific annealing temperature and 30 s at 72 °C) and a final extension step of 10 min at 72 °C. Annealing temperature was 60 °C except for the loci LafMSO2 and LafMSO3 (55 °C) and FH102 (52 °C).

Sequences were aligned using BioEdit 7.2.3 (Hall, 1999). Number of haplotypes, number of polymorphic sites (S), haplotype (H_d) and nucleotide diversities (π) were calculated with DnaSP 5.10.1 (Librado and Rozas, 2009). Pairwise Φ_{ST} , Chakraborty's amalgamation test, neutrality/demographic tests (Tajima's D and Fu's F_S , based on 10000 permutations) and mismatch distribution analyses were calculated using

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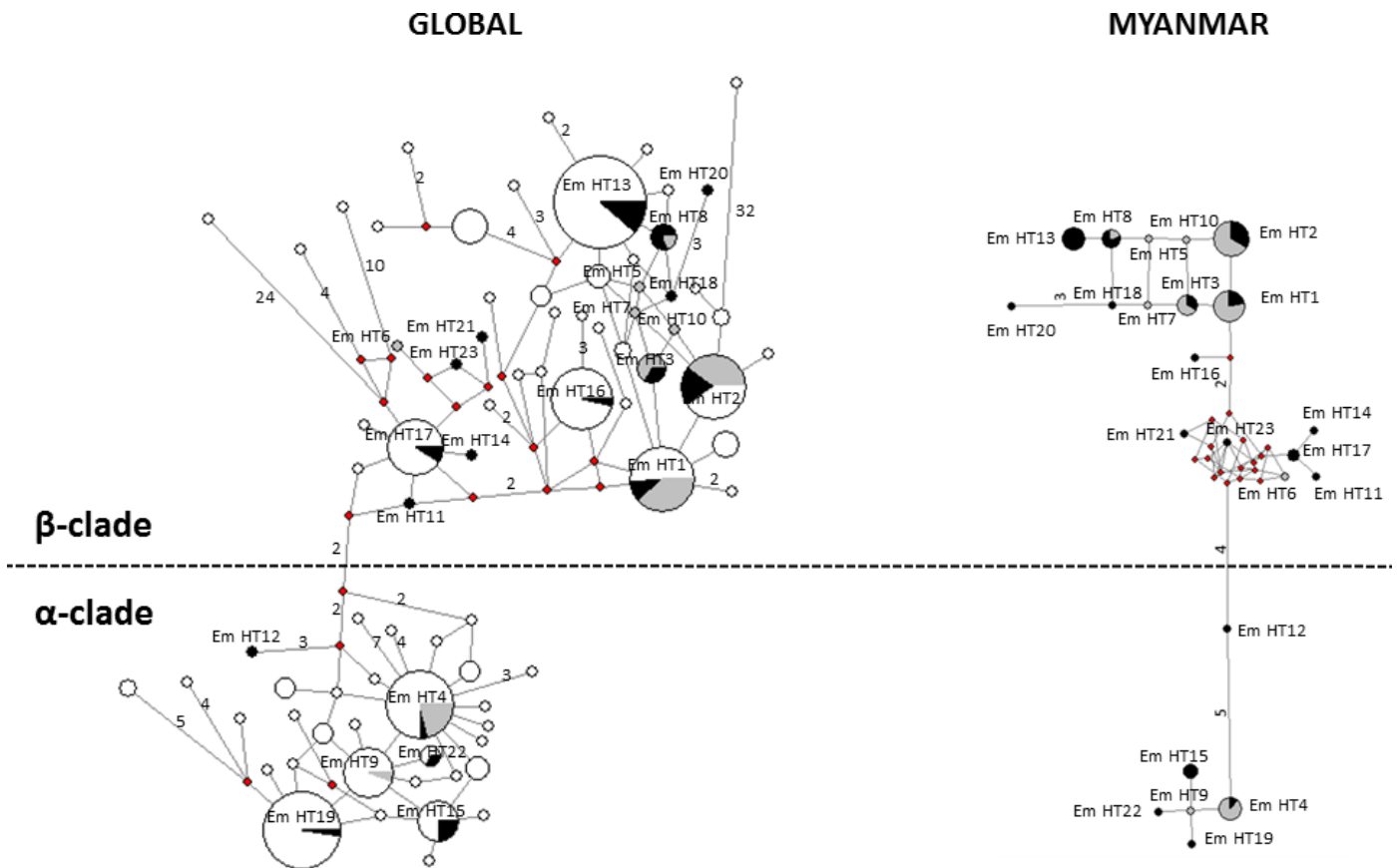


Figure 1 – Median-joining networks of the 23 haplotypes found in Myanmar in the present study (right, based on 563 bp) and of an enlarged dataset including 309 Asian elephant sequences from GenBank (left, based on 336 bp). Circle sizes are proportional to haplotype frequencies. Numbers denote mutational steps, colours refer to our sampling sites — grey: MHW, black: Taikkyi; GenBank sequences are white. The dashed line separates the two known mtDNA clades α and β . Red dots represent median vectors.

ARLEQUIN 3.5.2 (Excoffier and Lischer, 2010). NETWORK 5.0.0.1 (Bandelt et al., 1999) was used to construct two median-joining networks using default parameters: (1) based on the haplotypes of the present study (563 bp); (2) including 309 previously published (Appendix C, Supplemental S3) Asian elephant sequences from GenBank (336 bp).

Occurrence of microsatellite null alleles was tested for with MICROCHECKER 2.2.3 (Van Oosterhout et al., 2004). Number of alleles per locus, observed (H_o) and expected (H_e) heterozygosities and deviations from Hardy-Weinberg equilibrium (HWE) were calculated with ARLEQUIN 3.5.2 which was also used to calculate the pairwise F_{st} value between the two camps. GENEPOP 3.4 (Raymond and Rousset, 1995) was used to test for pairwise linkage between loci. A factorial correspondence analysis (FCA) and a Structure cluster analysis (Appendix D) were carried out for the total dataset. Effective population size (N_e) was estimated using the bias-corrected linkage disequilibrium approach (LDNe) as implemented in NeESTIMATOR 2.01 (Do et al., 2014) with three different frequency cut-off levels (0.05, 0.02, 0.01) below which alleles were not considered. Recent reduction in effective population size was tested for with BOTTLENECK 1.2.0.2 (Cornuet and Luikart, 1996), using a Wilcoxon test under a two-phase mutation model of microsatellite evolution with 1000 iterations and 70% step-wise mutations.

Our 78 control region sequences (563 bp) yielded 23 different haplotypes (Em HT1-HT23; GenBank accession numbers: KX882107–KX882129) based on 19 variable sites. In accordance with Vidya et al. (2009), we found both α (6) and β (17) haplotypes in Myanmar (Fig. 1), and the respective frequencies were also similar: the 24 Myanmar elephants in Vidya et al. (2009) yielded 25% α and 75% β , while our study yielded 15 α (19.2%) and 63 β (80.8%) haplotypes. This is in clear contrast to the results from the neighbouring population in Thailand, where Fickel et al. (2007) found 50% of each clade in their sample of 78 elephants from throughout the country based on 411

bp. Thirteen of our 23 haplotypes were novel. Only five haplotypes were shared by both populations, while 15 haplotypes were private to one of them, but differentiation between the camps was non-significant ($\Phi_{ST}=0.23$, $p=0.127$, Chakraborty's amalgamation test: $p=0.09$). Haplotype (H_d) and nucleotide diversities (π) were high in both camps and overall (Tab. 1). One-hundred-and-two Lao elephants (Ahlering et al., 2011) only yielded six different control region haplotypes resulting in a lower haplotype diversity of 0.74, while nucleotide diversity was identical to ours (0.011). In Thai elephants haplotype and nucleotide diversities were very similar ($H_d=0.90$ vs. our 0.89) or slightly higher (π of 0.016 vs. 0.011) than in our Myanmar elephants. The comparatively high nucleotide diversity in Thailand (Fickel et al., 2007), Sri Lanka and Myanmar (Vidya et al., 2009) is due to the presence of substantial numbers of animals from both clades. The mismatch distribution for our total population was bimodal, indicating a population without past population expansion or bottleneck. This was supported by statistically insignificant values of Tajima's D and Fu's FS ($D=1.617$, $p=0.96$; $FS=-3.531$, $p=0.15$). The median-joining network confirmed the lack of conspicuous geographic structuring in that both camps harboured haplotypes from both previously identified mtDNA haplogroups. All major haplotypes in the global network were present in Myanmar, which, together with the private haplotypes found in this study, means that Myanmar elephants harbour a large portion of the species' overall mtDNA diversity. Our network also showed that there are elephants from two β -subclades in Myanmar, which Vidya et al. (2009) interpreted as evidence of independent colonisation of elephants from the southwest (India/Sri Lanka) and the southeast (Sunda region). Myanmar seems to be a phylogeographic crossroads for both major clades (α and β) and two β -subclades that originated in allopatry.

Microsatellite genotyping was successful at six or more loci in 61 elephants (percentage of missing data: 11.47%), 35 from MHW and 26 from Taikkyi. All loci were in HWE in both camps and in the total population, and no linkage disequilibrium between any two loci or null

Table 1 – Sample size and genetic diversity indices for the mtDNA sequences and nuclear genotypes found in Myanmar elephants. Standard deviations are in parentheses.

Camp	n	Number of haplotypes	Number of polymorphic sites	Singleton haplotypes	Haplotype diversity	Nucleotide diversity	Mean observed heterozygosity	Mean expected heterozygosity
MHW	40	10	17	5	0.810 (0.035)	0.009 (0.002)	0.550 (0.169)	0.580 (0.198)
Taikkyi	38	18	19	10	0.929 (0.022)	0.012 (0.001)	0.541 (0.224)	0.591 (0.201)
Total	78	23	19	15	0.893 (0.019)	0.011 (0.001)	0.547 (0.186)	0.586 (0.191)

alleles were detected. No differentiation between the two camps was found as revealed by pairwise F_{st} (0.001, $p=0.41$), FCA and Structure analysis (Appendix D, Supplemental S4), suggesting a single genetically homogeneous population. Overall mean allelic diversity was 4.55, and observed and expected heterozygosities averaged over all loci for the total population were 0.55 and 0.59, respectively (Appendix B, Supplemental S2). These values are within the range of previously reported genetic variability for Asian elephants in Borneo, Sri Lanka, Vietnam, Myanmar, Malaysia, Cambodia, Lao PDR, Thailand, Bhutan and India (Fernando et al., 2000, 2001, 2003; Vidya et al., 2005, 2009; Fickel et al., 2007; Thitaram et al., 2010), although expected heterozygosity was found to be considerably higher in elephants from the Lao PDR (0.75 vs our 0.59). For the total Myanmar sample of our study N_e was estimated at between 40.1 (CI 22.7–91.0) and 53.6 (31.8–117.0), depending on the cut-off allele frequency. Thitaram et al. (2010) analysed diversity and effective population sizes of 156 working elephants from Thailand based on 14 microsatellite loci (eight of which were also included in our study). Heterozygosity values were similar to ours (between 0.58 and 0.60). Their estimate of overall effective population size based on the LDNe approach was about 200 and thus four to five times higher than ours, but the geographic origin of their elephants spanned the whole of Thailand, which precludes a direct comparison. The bottleneck test yielded significant p values for our total dataset (<0.001), suggesting a relative excess of heterozygosity and a recent bottleneck.

Taken together, rather low levels of heterozygosity, but high levels of mtDNA diversity and private haplotypes were found in Myanmar elephants. No evidence for deviation from panmixia was found in either marker set for our total sample. The semicaptive status of our camp elephants allows for sufficient gene flow in the region through mating with wild bulls to consider the two camps being part of a single genetic population. In contrast to Thai elephants, where nuclear differentiation between animals carrying α and β haplotypes was found (Fickel et al., 2007), no such separation exists in our Myanmar elephants (Appendix D, Supplemental S4). Although our study is the most comprehensive to date on Myanmar elephants, we only covered a small part of their range in the country, and generalisations of our demographic results should not be made until other regions have been studied. What even our limited dataset shows, however, is that Myanmar not only harbours a significant number of the remaining Asian elephants but also a large part of the species' genetic diversity. ☞

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Supplemental information

Additional Supplemental Information may be found in the online version of this article:

- Supplemental S1** Appendix A. Additional information on Myanmar elephants.
- Supplemental S2** Appendix B. Observed number of alleles (N_a), observed (H_o) and expected (H_e) heterozygosities and p values for deviation from HWE in the two camps and the total population.
- Supplemental S3** Appendix C. Accession numbers and information of the Asian elephant sequences downloaded from GenBank.
- Supplemental S4** Appendix D. Test of genetic differentiation at 11 autosomal microsatellites.