

# The Elephants of Gash-Barka, Eritrea: Nuclear and Mitochondrial Genetic Patterns

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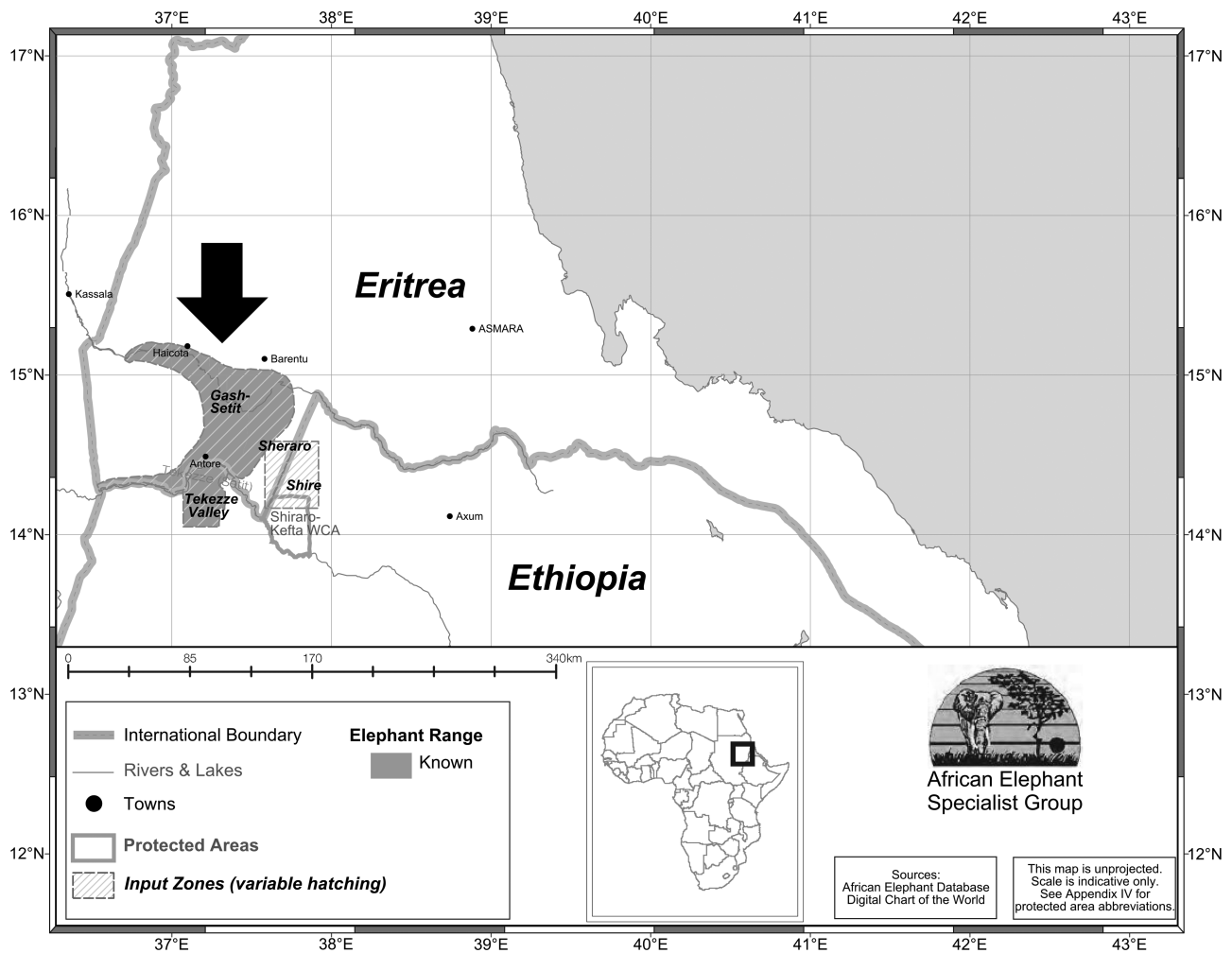
## Abstract

Eritrea has one of the northernmost populations of African elephants. Only about 100 elephants persist in the Gash-Barka administrative zone. Elephants in Eritrea have become completely isolated, with no gene flow from other elephant populations. The conservation of Eritrean elephants would benefit from an understanding of their genetic affinities to elephants elsewhere on the continent and the degree to which genetic variation persists in the population. Using dung samples from Eritrean elephants, we examined 18 species-diagnostic single nucleotide polymorphisms in 3 nuclear genes, sequences of mitochondrial *HVR1* and *ND5*, and genotyped 11 microsatellite loci. The sampled Eritrean elephants carried nuclear and mitochondrial DNA markers establishing them as savanna elephants, with closer genetic affinity to Eastern than to North Central savanna elephant populations, and contrary to speculation by some scholars that forest elephants were found in Eritrea. Mitochondrial DNA diversity was relatively low, with 2 haplotypes unique to Eritrea predominating. Microsatellite genotypes could only be determined for a small number of elephants but suggested that the population suffers from low genetic diversity. Conservation efforts should aim to protect Eritrean elephants and their habitat in the short run, with restoration of habitat connectivity and genetic diversity as long-term goals.

**Keywords:** fecal DNA, *Loxodonta*, microsatellites, mitochondrial DNA, single nucleotide polymorphism, war elephants

Eritrea marks the northeast boundary of current African elephant distribution, with elephants persisting in a small fragment of their formerly extensive range. African elephants were once found throughout Eritrea, but by the early 20th century, they were believed to have been extirpated (Gowers 1948) although a small population was found to have persisted at low population densities in the southwest (Hagos et al. 2003; Yalden, Largen and Kock 1986). The current distribution of elephants lies within the Zoba Gash-Barka, one of the 6 administrative zones, located in the southern part of western Eritrea (Figure 1) (Blanc et al. 2007). Gash-Barka is a dry region with habitat consisting mostly of doum palm, ziziphus bush, acacia woodland, and open grassland savanna

(Hagos et al. 2003). Most surveys report sightings of only a few individuals (Barnes et al. 1999; Litoroh 1997). However, one estimate suggested that 100–200 elephants persisted in the 1950s (Largen and Yalden 1987; Leuenberger 1955), whereas an aerial survey conducted in Gash-Barka between 2001 and 2003 estimated that around 100 African elephants persisted in Eritrea (Hagos et al. 2003; Shoshani et al. 2004). In 2012, the government of Eritrea indicated that the numbers and range of elephants appear to be increasing and that around 120 elephants persist (Anonymous 2013). Protecting elephant habitat is considered by the government to be a priority for biodiversity conservation (Weddeyohannes and Siratu 2010).



**Figure 1.** Map of Eritrea showing current African elephant distribution. The shaded region pointed to by the arrow indicates the current range of elephants in Eritrea and bordering Ethiopia (Litoroh 1997; Shoshani et al. 2004). This map is from the IUCN Elephant Specialist Group—African Elephant Status Report (Blanc et al. 2007), which permits reproduction for educational purposes.

Within Eritrea, the geographic range of elephants is approximately 5293 km<sup>2</sup> which includes narrow corridors connecting the northern and southern extents of their range (Blanc et al. 2007; Yacob et al. 2004). During the wet season, some Eritrean elephants migrate into northern Ethiopia (Shoshani, Hagos and Yacob 2000; Shoshani et al. 2004), utilizing the additional range within the Tkeze Valley Wildlife Reserve, which is 1130 km<sup>2</sup> (Blanc et al. 2007). The study of these elephants is made difficult by their migration between Eritrea and Ethiopia. During a 27-month survey, the mortality rate was estimated to be 4.9% per year, which is comparable to an annual average of 4.71% for the Samburu savanna elephant population of Kenya (Witemyer, Daballen and Douglas-Hamilton 2013) and less than the mortality rate (17.1% of juveniles or 10.5% of adults) that has been estimated as necessary to prevent population growth in savanna elephants (Woolley et al. 2008). The Eritrean herds observed include a substantial proportion of infant and subadult individuals (Hagos et al. 2003; Shoshani et al. 2004). Although some elephant deaths in Eritrea are attributable to the human

presence in the area, ivory poaching has not been of major concern (Yacob et al. 2004).

The Eritrean elephant population is isolated, with the nearest other elephant population more than 400 km away (Blanc et al. 2007). Eritrean elephants are thus vulnerable to a decrease in fitness due to inbreeding and loss of genetic variation (Reed and Frankham 2003). Understanding the genetic diversity and affinities of this population, and determining the effects of limited gene flow, can contribute to scientifically sound conservation practices to ensure their long-term persistence. We therefore examined genetic markers previously characterized in elephants across Africa to examine the genetic diversity and affinities of Eritrean elephants.

## Material and Methods

### Samples

This study was conducted in compliance with the University of Illinois Institutional Animal Care and Use Committee

(IACUC) approved protocol number 09036. Samples were obtained in full compliance with required permits. Thirty-three dung samples were collected from elephants in the Gash-Barka region of Eritrea between 2001 and 2003 and stored in “blue” alcohol (ethanol with methanol additive). Dung was collected as part of the elephant population census reported by Shoshani et al. (2004). The dung was collected in various geographic locations during this time period (Table S1, Figure S1). To minimize the possibility that different samples may have come from the same individual, exclusion criteria were used consisting of 1) ability of elephants to travel distances given differences in time of sample collection; 2) similarity of herd or group composition; and 3) similarity of markings including ear and body marks, tusk characters, and soleprints (Hagos et al. 2003; Shoshani et al. 2004). A total of 83 distinct elephants were counted during the census, though an additional 45 sightings of elephants were excluded from the census total using these conservative exclusion criteria. None of the samples that were successfully amplified (see below) were from the potential duplicates; thus all sequences and genotypes were from distinct individuals.

### Mitochondrial and Nuclear DNA Amplification and Sequencing

DNA was extracted using the QIAamp DNA Stool Kit (Qiagen Inc., Valencia, CA) following the recommended protocol. Several DNA markers were unable to be amplified despite repeated attempts and utilization of techniques that typically increase PCR success rate—thus limiting the analyses possible for some individuals. Two regions of the mitochondrial genome were amplified and sequenced. A 319-bp region of the mitochondrial *NADH dehydrogenase 5* (*ND5*) was amplified as previously described (Roca et al. 2005). A 314-bp region of the hypervariable region 1 (*HVR1*) was amplified in 2 overlapping segments using a combination of 4 primers developed for low quality DNA, *CR-F1* (TGGTCTTGTAAGCCATAAATGAAA) with *CR-R1* (GCTTTAATGTGCTATGTAAGACTATG) and *CR-F2* (TCGTGCATCACATTATTACCC) with *CR-R2* (TGGTCCTGAAGAAAGAACAG). PCR was run with an initial step of 95 °C for 9 min 45 s; with cycles of 20 s at 94 °C; followed by 30 s at 60 °C (first 3 cycles), 58 °C (next 5 cycles), 56 °C (5 cycles), 54 °C (5 cycles), 52 °C (5 cycles), or 50 °C (final 22 cycles); followed by 30-s extension at 72 °C; with a final extension after the last cycle of 7 min at 72 °C. Short species-diagnostic regions of nuclear DNA sequences for genes *Biglycan* (*BGN*), *Phosphorylase kinase alpha subunit 2* (*PHKA2*) and *Proteolipid protein 1* (*PLP*) were amplified following methods previously described (Ishida et al. 2011a). All products were enzyme purified (Hanke and Wink 1994) then sequenced using the BigDye Terminator system (ABI), purified, and resolved at the University of Illinois at Urbana-Champaign Core Sequencing Facility. The software Sequencher (Gene Codes Corporation, Ann Arbor, MI) was used to edit and concatenate sequences. There were no indications of nuclear DNA sequences of mitochondrial origin (numts) among the results (Brandt et al.

2012; Roca et al. 2007). Sequences of 4 mitochondrial DNA (mtDNA) haplotypes (2 novel) were submitted to GenBank (KC608163-KC608166).

### Haplotype Analyses

Mitochondrial DNA sequences were aligned using CLUSTALW 2.0 (Larkin et al. 2007) with default parameters, in European Bioinformatics Institute Web Services (McWilliam et al. 2009); alignment output was visually inspected. Haplotype diversity indices were calculated with ARLEQUIN version 3.5 (Excoffier and Lischer 2010). *HVR1* sequences were combined with a larger dataset (Ishida et al. 2013) and weighted maximum likelihood distances were used to generate a median-joining network using the software NETWORK version 4.6.1 (Bandelt, Forster and Rohl 1999).

### Single Nucleotide Polymorphisms Analyses

The identities of nuclear DNA sequences were established using NCBI BLAST (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>) and were compared with published DNA sequences from savanna elephant, forest elephant, and Asian elephant (Roca et al. 2005). Species-specific diagnostic nucleotide sites for each gene (*BGN*, *PHK* and *PLP*) were examined as described previously (Ishida et al. 2011a). Because the sex of the individuals was not known, nuclear sequences were conservatively estimated as originating from 1 rather than 2 X-chromosomes.

### Microsatellites

Eight microsatellite loci developed in savanna elephants, LAF10, LAF11, LAF12, LAF13, LAF29, LAF37, LaT05, and LaT06 (Archie, Moss and Alberts 2003; Ishida et al. 2011b), and 3 loci developed in Asian elephants, EMX3, EMX4, and EMX5 (Fernando, Vidya and Melnick 2001), were amplified in the Eritrean samples by PCR. Primers were tagged for fluorescence detection (Boutin-Ganache et al. 2001) and amplification followed a touchdown thermocycle profile as previously described (Ishida et al. 2011b; Menotti-Raymond et al. 2005). Samples were genotyped on an ABI 3100 Genetic Analyzer and scored using GeneScan 3.7 and Genotyper 2.5 software (Applied Biosystems); alleles were subsequently binned using Allelogram (Morin et al. 2009). To verify genotypes and to check for allelic dropout or false alleles, samples were genotyped at least 3 additional times; in no cases were allelic dropouts detected. PCR mixes included 10–25 µg bovine serum albumin. Positive and negative controls were run. In fulfillment of data-archiving guidelines (Baker 2013), we have deposited the primary data underlying these analyses with Dryad.

The results for Eritrea are reported here for the first time. One amplification for the Eritrean elephants was generated concurrent with and alongside an additional 555 African elephants from 23 locales that included both savanna elephants (*Loxodonta africana*) and forest elephants (*L. cyclotis*) (Ishida et al. 2011b), thus ensuring consistency in platforms and in allelic size comparisons. The previously genotyped elephants were from the following locations: SL-Sierra

Leone; LO-Lope, in Gabon; OD-Odzala, in Republic of the Congo; DS-Dzanga Sangha, in Central African Republic; BF-Bili, GR-Garamba, in the Democratic Republic of the Congo; BE-Benoue, WA-Waza in Cameroon; KE-Central Kenya/Laikipia, MK-Mount Kenya, AB-Aberdares, AM-Amboseli, in Kenya; SE-Serengeti, NG-Ngorongoro, TA-Tarangire, in Tanzania; SW-Sengwa, ZZ-Zambezi, HW-Hwange, in Zimbabwe; CH-Chobe, SA-Savuti, MA-Mashatu, in Botswana; KR-Kruger, in South Africa; NA-Northern Namibia/Etoshia.

Diversity indices for microsatellites were calculated using ARLEQUIN version 3.5 (Excoffier and Lischer 2010) and population structure was examined using the software STRUCTURE 2.3.3 (Hubisz et al. 2009). Four models (Pritchard, Stephens and Donnelly 2000) were used to examine the effects of various combinations of assumptions of individual genetic ancestry and genetic relatedness among populations: 1) admixture with correlated allele frequencies; 2) admixture with independent allele frequencies; 3) no admixture with correlated allele frequencies; and 4) no admixture with independent allele frequencies. Each model was run 3 times using values of K (possible number of populations) between 1 and 24, which is the maximum number of putative populations assigned a priori. Each analysis was run for a minimum of 1 million Markov chain Monte Carlo steps following a burn-in of at least 100 000 steps. The uppermost hierarchical level of population structure was examined using the ad hoc statistic delta K based on the rate of change in  $\ln P(D)$  between successive K values (Evanno, Regnaut and Goudet 2005), implemented in Structure Harvester (Earl and vonHoldt 2012).

To identify the genetic affinity of Eritrean elephants, short tandem repeat (STR) data from savanna elephants from North Central and East Africa were combined with data from Eritrean elephants, and parameters of the STRUCTURE software were modified to allow for “learning samples.” Default parameters were used for migrant priors, allele frequencies were only updated from north central and eastern savanna elephant populations, and an admixture model with correlated allele frequencies and  $K = 2$  clusters was assumed.

## Results

A total of 33 elephant dung samples were collected in the Gash-Barka zone of Eritrea. DNA extraction was attempted at least twice on all samples. The DNA proved to be of low quality, because for 10 samples amplification was never successful for any locus. Sequences of mtDNA were obtained for 21 samples and short nuclear fragments were sequenced for 9 samples. However, only 3 individuals successfully amplified for at least 7 of the STR loci. Results were confirmed by repeated genotyping or sequencing.

### Mitochondrial Haplotypes

We successfully sequenced mtDNA *ND5* in 20 samples. We identified a single *ND5* haplotype (GenBank accession number KC608166) for all; this haplotype also occurs in savanna

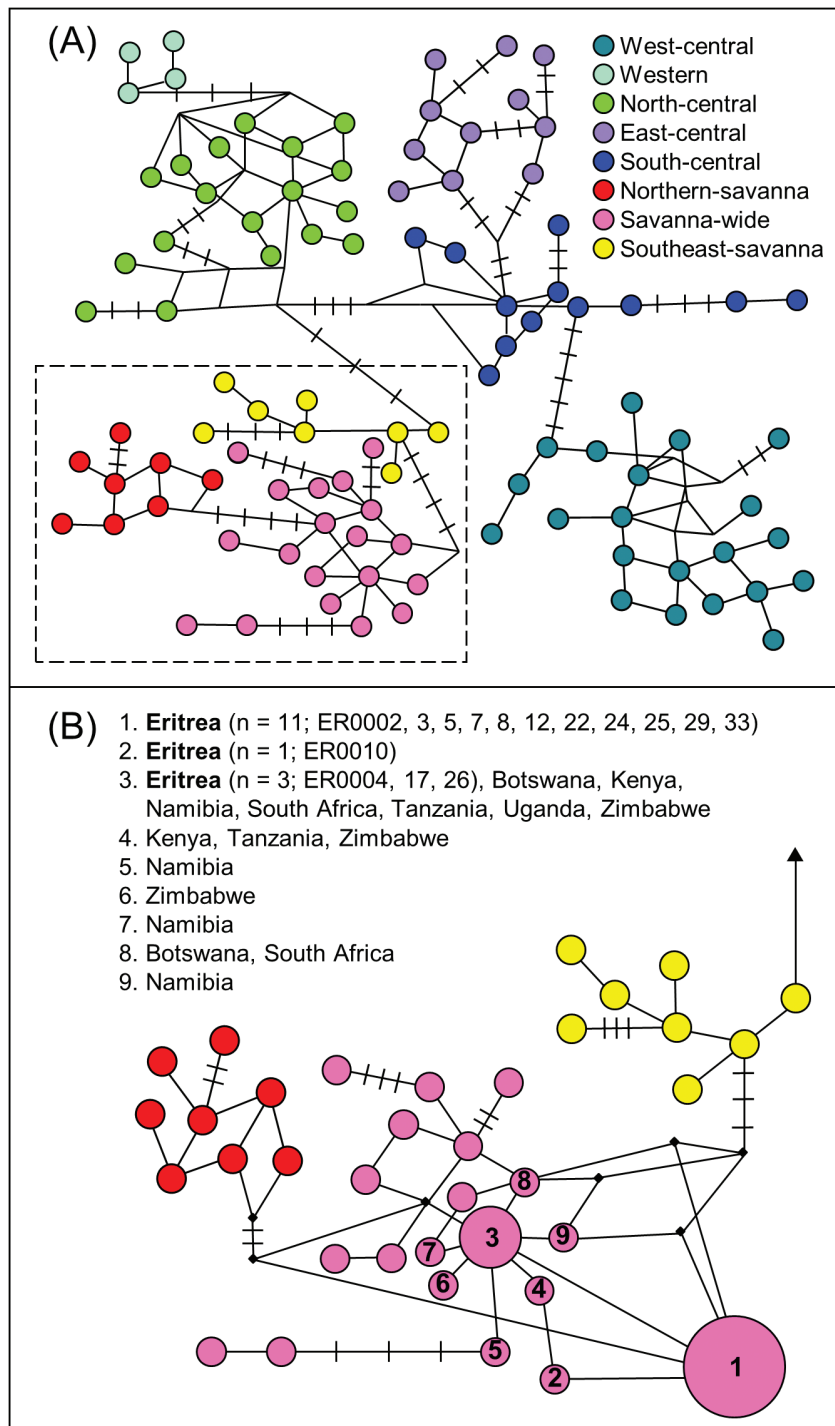
elephants throughout Eastern and Southern Africa (Ishida et al. 2013; Roca et al. 2005). Among 15 Eritrean elephant samples that were successfully amplified and sequenced for *HVR1*, 3 unique haplotypes were detected, distinguished by only 2 polymorphic nucleotide sites (Figure 2). Thus, at *HVR1* the Eritrean elephants had low nucleotide diversity ( $\pi = 0.0015$ ;  $SD \pm 0.0016$ ) and haplotype diversity of 0.4476 ( $SD \pm 0.1345$ ). The 3 unique *HVR1* Eritrean haplotypes were aligned with those previously published for African elephants and used to generate a median joining network (Figure 2A). Across African elephants, 8 mtDNA groups had previously been identified (Ishida et al. 2013); all 3 Eritrean haplotypes formed part of the “savanna wide” mtDNA group (Figure 2). One haplotype (found in 3 Eritrean samples) was identical to a previously reported haplotype (Figure 2; GenBank accession number AY741325) (Nyakaana, Arctander and Siegmund 2002), which occurs in elephants across Eastern and Southern Africa (Debruyne 2005; Eggert, Rasner and Woodruff 2002; Johnson et al. 2007; Nyakaana et al. 2002). The other 2 haplotypes (found in 1 and 11 samples) were novel and confined to Eritrean elephants; they did not exactly match sequences reported by any previous study (Figure 2).

### Nuclear SNPs

Three X-linked nuclear genes (*BGN*, *PHK*, *PLP*) have nucleotide sites with character state differences that distinguish forest from savanna elephants (Roca et al. 2005). Primers for PCR amplification of very short fragments containing one or more of these diagnostic sites have previously been developed for use with degraded DNA (Ishida et al. 2011a). Using these primers, we obtained at least one of the genic sequences for 9 of the Eritrean samples (Table 1), generating a total of 21 unlinked sequences with diagnostic sites that distinguish forest from savanna elephants. At every one of the diagnostic sites, savanna elephant-specific nucleotide character states were present (Table 1); sequences with sites that matched a character state typical of forest elephants were never found (Table 1). There were no significant differences in nucleotide character states between Eritrean elephants and other savanna elephants (Fisher’s exact tests,  $P \approx 1.00$ , Table S2) (Ishida et al. 2011a). By contrast there were highly significant differences between nucleotide character states found in Eritrean elephants and those in previously examined forest elephants (Fisher’s exact tests,  $P < 10^{-4}$ , Table S2) (Ishida et al. 2011a).

### Microsatellites

For microsatellites, only 3 elephants from Eritrea were successfully genotyped: 2 at 8 loci and 1 at 7 loci. The low success rate may be attributable to degraded DNA, perhaps due to field or storage conditions. Allele scores were confirmed by repeated PCR and genotyping (at least 3 replicates), no allelic dropouts were detected. Within Eritrea, 5 of the loci were polymorphic and 3 were monomorphic, with an average number of alleles per locus of 1.46. Observed and expected heterozygosity were 0.36 and 0.29, respectively;  $F_{IS}$  was  $-0.37$



**Figure 2.** Median-joining network of 314bp mitochondrial *HVR1* haplotypes in Eritrean and other African elephants. (A) The 8 mitochondrial groups reported by [Ishida et al. \(2013\)](#) are color coded. Haplotypes in the groups within the box are carried only by savanna and not forest elephants. (B) Close-up of 3 groups, showing the haplotypes carried by Eritrean elephants (numbered 1, 2, and 3), for which circle size is proportional to the number of individuals from Eritrea carrying each haplotype. Haplotypes 1 and 2 (Genbank accessions KC608163 and KC608165, respectively) were detected only in Eritrea; haplotype 3 was found in Eritrea and elsewhere. Haplotypes 4–9 differ by a single nucleotide from those present in Eritrea. Haplotypes 3 through 9 have Genbank accessions AY741325, AY742801, AF106236, AF106226, AF106239, AY741074, and AF106235 ([Debruyne 2005](#); [Nyakaana et al. 2002](#)).

**Table 1** Species-diagnostic nucleotide sites present in 3 nuclear genes examined in Eritrean elephants

	BGN												PHKA2					PLP			
	N	287	<b>304</b>	308	472	<b>485</b>	499	508	513	515	516	<b>570</b>	N	<b>39</b>	71	<b>871</b>	872	N	319	345	<b>361</b>
<i>E. maximus</i>	12	G	C	G	A	C	T	G	A	GΔ	Δ	T	12	C	G	C	G	12	G	C	G
<i>L. cyclotis</i>	116	GA	C	GC	AG	C	Δ	T	G	Δ	Δ	T	71	C	A	C	GA	118	GA	T	G
<i>L. africana</i>	806	G	<b>T</b>	G	A	<b>T</b>	ΔT	<b>G</b>	G	GΔ	ΔG	<b>C</b>	721	<b>T</b>	A	<b>T</b>	G	661	G	T	<b>A</b>
ER0001		G	<b>T</b>	G	A	<b>T</b>	T	<b>G</b>	G	G	G	<b>C</b>	—	—	—	—	—	—	—	—	—
ER0002		G	<b>T</b>	G	A	<b>T</b>	T	<b>G</b>	G	G	G	<b>C</b>	<b>T</b>	A	<b>T</b>	G		G	T	<b>A</b>	
ER0003		G	<b>T</b>	G	A	<b>T</b>	T	<b>G</b>	G	G	G	<b>C</b>	<b>T</b>	A	<b>T</b>	G		G	T	<b>A</b>	
ER0004		G	<b>T</b>	G	—	—	—	—	—	—	—	—	<b>T</b>	A	<b>T</b>	G		G	T	<b>A</b>	
ER0007		G	<b>T</b>	G	A	<b>T</b>	T	<b>G</b>	G	G	G	<b>C</b>	<b>T</b>	A	<b>T</b>	G		G	T	<b>A</b>	
ER0008		G	<b>T</b>	G	—	—	—	—	—	—	—	—	<b>T</b>	A	<b>T</b>	G		G	T	<b>A</b>	
ER0022		G	<b>T</b>	G	A	<b>T</b>	T	<b>G</b>	G	G	G	<b>C</b>	<b>T</b>	A	<b>T</b>	G		G	T	<b>A</b>	
ER0024		—	—	—	—	—	—	—	—	—	—	—	<b>T</b>	A	—	—		—	—	—	
ER0025		—	—	—	—	—	—	—	—	—	—	—	<b>T</b>	A	—	—		—	—	—	

Species-diagnostic nucleotide sites in 3 genes that distinguish savanna (*L. africana*) from forest (*L. cyclotis*) elephants (Ishida et al. 2011a) are indicated in boldface; at variable sites both bases are shown.

N is the number of chromosomes sequenced by previous transnational studies for each of 3 species of elephant (Ishida et al. 2011a; Roca et al. 2005); results are also shown for individual Eritrean elephants (code ER); nucleotide site positions are from Roca et al. (2005).

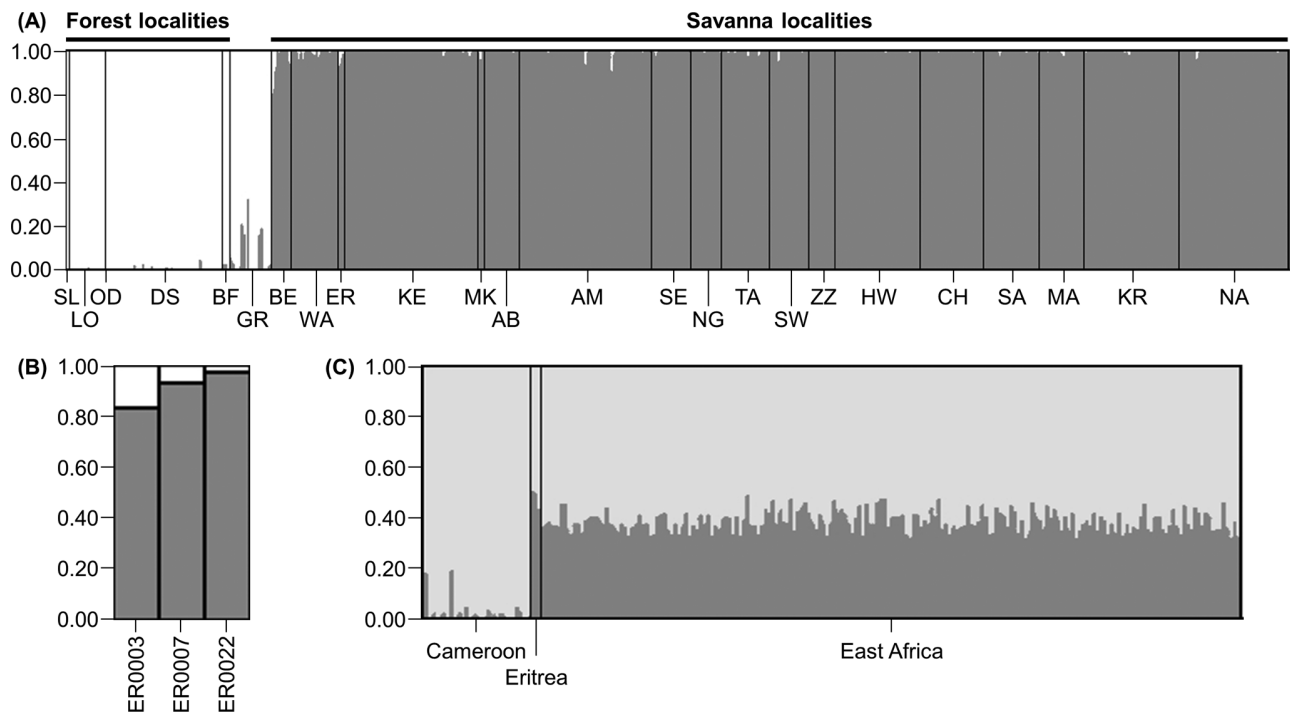
Dash indicates that sequence was not generated for a sample; delta indicates a deletion; *E. maximus* is the Asian elephant.

and all polymorphic loci were in Hardy–Weinberg equilibrium. For these same loci, among savanna elephants from across the African continent genotyped by a previous study (Ishida et al. 2011b), the average number of alleles per locus had been 9.88 whereas observed and expected heterozygosity had been 0.57 and 0.58, respectively. To account for the small sample size from Eritrea, 3 individuals from each non-Eritrean African savanna elephant population were randomly chosen for analysis. In this analysis, elephants from the rest of Africa were still more diverse than Eritrean elephants: the average number of alleles per locus per population (for sample size  $n = 3$ ) was 1.97 (standard deviation of 0.21) whereas observed and expected heterozygosity were 0.54 and 0.59, respectively.

Bayesian clustering analysis was performed using STRUCTURE (Pritchard et al. 2000) for 2 data sets that combined the Eritrean individuals with genotypes from a larger group of elephants that had been previously reported (Ishida et al. 2011b). The analysis included 555 forest and savanna elephants from outside Eritrea. This supported splitting Africa's elephants into 2 clusters ( $K = 2$ ; Figure 3A, Figure S2 and Table S3), one corresponding to African forest elephants, the other to African savanna elephants (Ishida et al. 2011b). Partitioning of the 3 Eritrean elephants identified them as savanna elephants (Figure 3B). The overall proportion of the Eritrean elephants assigned to the forest elephant partition was 0.08. This partitioning appeared to reflect local differences in savanna elephant allele frequencies rather than admixture from forest elephants. We examined the data closely, finding 3 alleles present in Eritrea that were more common in forest than savanna elephants (one allele at each of the loci LAF37, LaT06 and EMX4). These alleles occurred at high frequencies or were fixed in Eritrean elephants. Even so, these 3 alleles were also present in other savanna elephants, and no allele at any locus

in Eritrean elephants fell outside the size range expected of savanna elephants. No allele in Eritrea had a size that was typical of only forest and not savanna elephants in cases where the allelic size ranges vary between the species (Ishida et al. 2011b). With the caveat that DNA from only 3 individuals amplified, the close examination of STR allele sizes in Eritrea failed to find evidence for this population having any alleles that would be indicative of admixture from forest elephants.

A second STRUCTURE analysis included the 3 Eritrean elephants along with previously published genotypes of only savanna elephants from North Central Africa (Cameroon) and from East Africa (Kenya, Tanzania) in order to examine whether Eritrean elephants genetically had a closer affinity to elephants from one region or the other. The parameters of the STRUCTURE software were modified to allow for “learning samples” in which north central and eastern savanna elephants were a priori assigned to their known region of origin. The Eritrean elephants were not defined a priori as belonging to a population or region. Despite this modification, partitioning between elephants in the 2 regions was not complete, presumably due to limited differentiation between north central and eastern savanna elephants. Different patterns between the north central and the eastern savanna elephants were evident (Figure 3C). The patterns observed among Eritrean elephants more closely resembled those of eastern than those of north central savanna elephants (Figure 3C). This suggests that Eritrean elephants have a greater nuclear genetic affinity with East African than with North Central African savanna elephants, which is consistent with the finding that some Eritrean elephants share control region haplotypes with populations in Eastern Africa but are not known to share mtDNA haplotypes with Cameroon elephants (Figure 2).



**Figure 3.** Genetic subdivisions among African elephants. Multilocus genotype data were used to estimate population subdivision using the program STRUCTURE (Pritchard et al. 2000). (A) Two partitions that distinguish African forest elephants (white partition) from African savanna elephants (shaded partition) were successfully reconstructed (Ishida et al. 2011b). Three Eritrean (ER) elephants were assigned largely but not completely to the savanna elephant partition. (B) A close-up of STRUCTURE partitioning for the Eritrean elephants. Average assignment of Eritrean elephants to the partition corresponding to forest elephants was around 0.08, although this appears to result from drift and not from any admixture by forest elephants. (C) The genetic affinity of Eritrean elephants to other African savanna elephants was examined in STRUCTURE, with non-Eritrean individuals assigned to predefined populations as “learning samples.” Savanna elephant from Cameroon (BE, WA) were defined as belonging to a north central population whereas savanna elephants from Kenya and Tanzania (KE, MK, AB, AM, SE, NG, TA) were defined as belonging to an eastern population. However, the 2 partitions (lightly and darkly shaded) did not completely distinguish geographic regions (Cameroon and East Africa). The Eritrean elephants displayed patterns more closely resembling those of East African than of Cameroon elephants, indicating that Eritrean elephants were genetically more similar to the former. Abbreviations for elephant localities are listed in Materials and Methods.

## Discussion

Some scholars have speculated that African elephants used in warfare in the 3rd century BCE, which had likely been captured in what is now Eritrea, were forest rather than savanna elephants (Gowers 1948). This was based on a written account of the battle of Raphia in 217 BCE, fought between the armies of Ptolemy IV and Antiochus III during the Syrian Wars, and in which African and Asian elephants met in combat. The Asian elephants used by Antiochus are described as superior in size and strength over Ptolemy’s African elephants (Polybius 1923). Because African savanna elephants are larger than Asian elephants, some writers were led to conclude that the elephants used by Ptolemy could have been African forest elephants (Gowers 1948), which are smaller than savanna elephants (Grubb et al. 2000).

Sequences containing species-diagnostic nucleotide sites revealed only savanna elephant, and not

forest elephant, nuclear genotypes among the elephants of Eritrea (Figure 3, Table 1). Also, in Eritrea we detected only S clade mtDNA, which is carried only by savanna elephants (Ishida et al. 2011b). The mtDNA results may be especially telling, because savanna elephants in Eastern, Southern, and North Central Africa often carry F clade mitochondrial haplotypes that are geographically persistent and may record the ancient presence of forest elephants in a locality (Roca et al. 2005). The forest elephant mtDNA is geographically persistent because female African elephants are nondispersing (Ishida et al. 2011b). Although our results cannot completely rule out the possibility that forest elephants may have existed somewhere in Eritrea in the past, our data provide no support for this speculation. Eritrean elephants comprise a savanna elephant population in which even the forest-derived F clade mtDNA carried by many other savanna elephant populations was not detected. Although not consistent with previous speculation about the taxonomic affinity of Eritrean war

elephants, our results are consistent with the view that 2, and only 2, species of elephant occur in Africa and that currently hybrids between them are confined to a relatively narrow contact zone between forest and savanna habitats (Ishida et al. 2011b). Likewise, our results should dispense with rumors that Asian elephants brought to Eritrea in 1868 had admixed with African elephants in the region (Hagos et al. 2003; Shoshani et al. 2004).

Both nuclear and mitochondrial data support a closer relationship of Eritrean elephants to savanna elephants in East Africa than to savanna elephants of the North Central Sudanian/Sahelian region (Figures 2 and 3). Of 3 *HVR1* mtDNA haplotypes carried by elephants in Eritrea, one is widespread, occurring throughout Eastern and Southern Africa. The remaining 2 haplotypes are restricted to Eritrea but differ by a single nucleotide from haplotypes found in eastern but not north central Africa (Figure 2). The single *ND5* haplotype present in Eritrea has also been detected across Eastern and Southern Africa but not North Central Africa (Ishida et al. 2013; Roca et al. 2005). Although nuclear microsatellite genotypes were only successful for 3 Eritrean elephants, each of these had a closer genetic affinity to eastern savanna elephants than to the elephants of Cameroon (Figure 3).

Mitochondrial haplotype and nucleotide diversity were both low compared with other savanna elephant populations. For elephants across Africa, average *HVR1* haplotype diversity has been reported as 0.985 (Johnson et al. 2007) or 0.85 (Nyakaana et al. 2002), which is about twice the 0.45 of Eritrea. Mitochondrial nucleotide diversity has been reported as 0.030 (Johnson et al. 2007) or 0.02 (Nyakaana et al. 2002), which is much higher than the 0.0015 observed in Eritrea. Variation among haplotypes in Eritrea was low, as the 3 unique *HVR1* haplotypes were defined by 2 variable nucleotide sites, and only a single *ND5* haplotype was detected. Nuclear diversity was also very low, a previous study reported observed heterozygosity among all African elephant for the same STR loci as 0.50 (Ishida et al. 2011b), which is higher than the 0.36 observed in Eritrea.

The population of elephants in Eritrea is small; human-wildlife conflicts and habitat loss are major concerns. Currently, elephant migration into Ethiopia occurs only during the wet season (Yacob et al. 2004). This emphasizes the importance of the habitat in Eritrea for sustaining this population. An increase in suitable and protected habitat may be helpful to the long-term survival of Eritrean elephants. The agriculture ministry of Eritrea is committed to preservation of the elephants while minimizing human-elephant conflict and has reported that the numbers and range of elephants appear to be increasing (Anonymous 2013). Because the elephant population of Eritrea is small and isolated, it may in the future require genetic management. In the absence of habitat corridors that enable gene flow, genetic restoration may eventually become necessary—in which case our results suggest that the Eritrean population would best be augmented using individuals from Eastern Africa.

## Supplementary Material

Supplementary material can be found at <http://www.jhered.oxfordjournals.org/>.

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