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

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36 **Abstract**

37 Eritrea marks the northern range boundary for African elephants. Only about 100
38 elephants persist in the Gash-Barka administrative zone. Elephants in Eritrea have
39 become completely isolated, with no gene flow from other elephant populations. The
40 conservation of Eritrean elephants would benefit from an understanding of their genetic
41 affinities to elephants elsewhere on the continent, and of the degree to which genetic
42 variation persists in the population. Using dung samples from Eritrean elephants, we
43 examined 18 species-diagnostic single nucleotide polymorphisms in 3 nuclear genes,
44 sequences of mitochondrial *HVRI* and *ND5*, and genotyped 11 microsatellite loci. All
45 sampled Eritrean elephants carried nuclear and mtDNA markers establishing them as
46 savanna elephants, with closer genetic affinity to Eastern than to North-Central savanna
47 elephant populations, and contrary to speculation by some scholars that forest elephants
48 were found in Eritrea. Mitochondrial DNA diversity was relatively low, while two
49 haplotypes unique to Eritrea predominated. STR genotypes could only be determined for
50 a small number of elephants, but suggested that the population suffers from low
51 diversity. Conservation efforts should aim to protect Eritrean elephants and their habitat
52 in the short run, with restoration of habitat connectivity and genetic diversity as long-term
53 goals.

54

55 **Introduction**

56 Eritrea marks the northern boundary of current African elephant distribution, with
57 elephants persisting in a small fragment of their formerly extensive range. African
58 elephants were once found throughout Eritrea, but by the early 20th century they were
59 believed to have been extirpated (Gowers, 1948), although a small population was found
60 to have persisted at low population densities in the southwest (Hagos, *et al.*, 2003;
61 Yalden, Largen and Kock, 1986). The current distribution of elephants is restricted to a
62 5,293 km² area of land in the Zoba Gash-Barka, one of the six administrative zones,
63 located in the southern part of western Eritrea (Figure 1) (Blanc, *et al.*, 2007). Gash-
64 Barka is a dry region with habitat consisting mostly of doum palm, ziziphus bush, acacia
65 woodland and open grassland savanna (Hagos, *et al.*, 2003). Most surveys report
66 sightings of only a few individuals (Barnes, *et al.*, 1999; Litoroh, 1997). However, one
67 estimate suggested that 100 to 200 elephants persisted in the 1950s (Largen and Yalden,
68 1987; Leuenberger, 1955), while a recent aerial survey conducted in Gash-Barka between
69 2001 and 2003 estimated that ca. 100 African elephants remain in Eritrea (Hagos, *et al.*,
70 2003; Shoshani, *et al.*, 2004). In 2012, the government of Eritrea indicated that the
71 numbers and range of elephants appear to be increasing, and that ca. 120 elephants persist
72 (Anonymous, 2013). Protecting elephant habitat is considered by the government to be a
73 priority for biodiversity conservation (Weldeyohannes and Siratu, 2010).

74 Within Eritrea, the geographic range of elephants is approximately 4,200 km²
75 which includes narrow corridors connecting the northern and southern extents of their
76 range (Yacob, *et al.*, 2004). During the wet season, some Eritrean elephants migrate into
77 northern Ethiopia (Shoshani, Hagos and Yacob, 2000; Shoshani, *et al.*, 2004), utilizing

78 the additional range within the Tkezze Valley Wildlife Reserve, which is 1,130 km²
79 (Blanc, *et al.*, 2007). The study of these elephants is made difficult by their migrations
80 between Eritrea and Ethiopia. During a 27 month study, the mortality rate was estimated
81 to be 4.9% per year, which is comparable to the 14 year average of 4.71% in the Samburu
82 elephant population in northern Kenya (Wittemyer, Daballen and Douglas-Hamilton,
83 2013) and less than the mortality rate (17.1 % of juveniles or 10.5% of adults) that has
84 been estimated as necessary to prevent population growth in savanna elephants (Woolley,
85 *et al.*, 2008). Many elephant deaths in Eritrea are attributable to the human presence in
86 the area, although ivory poaching has not been of major concern (Yacob, *et al.*, 2004).
87 The Eritrean groups observed included a substantial proportion of infant and sub-adult
88 individuals (Hagos, *et al.*, 2003; Shoshani, *et al.*, 2004).

89 Elephants in the region are believed to be isolated, with the nearest other elephant
90 population over 400 km away (Blanc, *et al.*, 2007). Eritrean elephants are thus vulnerable
91 to a decrease in fitness due to inbreeding and loss of genetic variation (Reed and
92 Frankham, 2003). Understanding the genetic diversity and affinities of this population,
93 and determining the effects of limited gene flow, can contribute to scientifically sound
94 conservation practices to ensure their long-term persistence. We therefore examined
95 genetic markers previously characterized in elephants across Africa to examine the
96 genetic diversity and affinities of Eritrean elephants.

97

98 **Material and Methods**

99 *Samples*

100 This study was conducted in compliance with the University of Illinois
101 Institutional Animal Care and Use Committed (IACUC) approved protocol number
102 09036. Samples were obtained in full compliance with required permits. Thirty-three
103 dung samples were collected from elephants in the Gash-Barka region of Eritrea between
104 2001 and 2003 and stored in “blue” alcohol (ethanol with methanol additive). Dung was
105 collected as part of the elephant population census reported by Shoshani and others
106 (2004). The dung was collected in various geographic locations during this time period
107 (Table S1, Figure S1). To minimize the possibility of duplicate collection of samples,
108 exclusion criteria were used consisting of (1) ability of elephants to travel distances given
109 differences in time of sample collection; (2) similarity of herd or group composition; and
110 (3) similarity of markings including ear and body marks, tusk characters, and soleprints
111 (Hagos, *et al.*, 2003; Shoshani, *et al.*, 2004). A total of 83 distinct elephants were counted
112 during the census, though an additional 45 sightings of elephants were excluded from the
113 census total using these conservative exclusion criteria. None of the samples that were
114 successfully amplified (below) were from the potential duplicates, thus all sequences and
115 genotypes were from distinct individuals.

116

117 *Mitochondrial and Nuclear DNA Amplification and Sequencing*

118 DNA was extracted using the QIAamp DNA Stool Kit (Qiagen Inc., Valencia,
119 CA) following the recommended protocol. Several DNA markers were unable to be
120 amplified despite repeated attempts and utilization of techniques that typically increase
121 PCR success rate; thus limiting the analyses possible for some individuals. Two regions
122 of the mitochondrial genome were amplified and sequenced. A 319 bp region of the

123 mitochondrial *NADH dehydrogenase 5 (ND5)* was amplified as previously described
124 (Roca, Georgiadis and O'Brien, 2005). A 314 bp region of the *hyper variable region 1*
125 (*HVR1*) was amplified in two overlapping segments using a combination of four primers
126 developed for low quality DNA, CR-F1 (TGGTCTTGTAAGCCATAAATGAAA) with
127 CR-R1 (GCTTTAATGTGCTATGTAAGACTATG), and CR-F2
128 (TCGTGCATCACATTATTTACCC) with CR-R2
129 (TGGTCCTGAAGAAAGAACCAG). PCR was run with an initial step of 95°C for 9:45
130 min; with cycles of 20 sec at 94°C; followed by 30 sec at 60°C (first 3 cycles), 58°C
131 (next 5 cycles), 56°C (5 cycles), 54°C (5 cycles), 52°C (5 cycles), or 50°C (final 22
132 cycles); followed by 30 second extension at 72°C; with a final extension after the last
133 cycle of 7 min at 72°C. Short species-diagnostic regions of nuclear DNA sequences for
134 genes *Biglycan (BGN)*, *Phosphorylase kinase alpha subunit 2 (PHKA2)* and *Proteolipid*
135 *protein 1 (PLP)* were amplified following methods previously described (Ishida, *et al.*,
136 2011a). All products were enzyme-purified (Hanke and Wink, 1994) then sequenced
137 using the BigDye Terminator system (ABI), purified, and resolved at the University of
138 Illinois at Urbana-Champaign Core Sequencing Facility. The software *Sequencher* (Gene
139 Codes Corporation, Ann Arbor, MI) was used to edit and concatenate sequences. There
140 were no indications of nuclear DNA sequences of mitochondrial origin (numts) among
141 the results (Brandt, *et al.*, 2012; Roca, Georgiadis and O'Brien, 2007). Sequences of four
142 novel mtDNA haplotypes were submitted to GenBank (KC608163-KC608166).

143

144 *Haplotype Analyses*

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

152

153 *SNP Analyses*

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

161

162 *Microsatellites*

163 Eight microsatellite loci developed in savanna elephants: LAF10, LAF11, LAF12,
164 LAF13, LAF29, LAF37, LaT05, and LaT06 (Archie, Moss and Alberts, 2003; Ishida, *et*
165 *al.*, 2011b) and 3 loci developed in Asian elephants: EMX3, EMX4, and EMX5
166 (Fernando, Vidya and Melnick, 2001), were amplified in the Eritrean samples by PCR.
167 Primers were tagged for fluorescence detection (Boutin-Ganache, *et al.*, 2001) and

168 amplification followed a touchdown thermocycle profile previously described (Ishida, *et*
169 *al.*, 2011b; Menotti-Raymond, *et al.*, 2005). Samples were genotyped on an ABI 3100
170 Genetic Analyzer and scored using GeneScan 3.7 and Genotyper 2.5 software (Applied
171 Biosystems); alleles were subsequently binned using Allelogram (Morin, *et al.*, 2009). To
172 verify genotypes and to check for allelic dropout or false alleles, samples were genotyped
173 at least 3 additional times; in no cases were allelic dropouts detected. PCR mixes
174 included 10-25 ug bovine serum albumin. Positive and negative controls were run. In
175 fulfillment of data archiving guidelines (Baker, 2013), we have deposited the primary data
176 underlying these analyses with Dryad.

177 The results for Eritrea are reported here for the first time. One amplification for
178 the Eritrean elephants was generated concurrent with and alongside an additional 555
179 African elephants from 23 locales that included both savanna elephants (*Loxodonta*
180 *africana*) and forest elephants (*L. cyclotis*), and followed procedures described therein
181 (Ishida, *et al.*, 2011b), thus ensuring consistency in platforms and in allelic size
182 comparisons. Diversity indices for microsatellites were calculated using ARLEQUIN
183 v.3.5 (Excoffier and Lischer, 2010) and population structure was examined using the
184 software STRUCTURE 2.3.3 (Hubisz, *et al.*, 2009). Four models (Pritchard, Stephens
185 and Donnelly, 2000) were used to examine the effects of various combinations of
186 assumptions of individual genetic ancestry and genetic relatedness among populations: 1)
187 admixture with correlated allele frequencies, 2) admixture with independent allele
188 frequencies, 3) no admixture with correlated allele frequencies and 4) no admixture with
189 independent allele frequencies. Each model was run 3 times using values of K (possible
190 number of populations) between 1 and 24 genetic clusters, which is the maximum

191 number of putative populations assigned *a priori*. Each analysis was run for a minimum
192 of 1 million Markov chain Monte Carlo steps following a burn-in of at least 100,000
193 steps. The uppermost hierarchical level of population structure was examined using the
194 *ad hoc* statistic delta K based on the rate of change in ln P (D) between successive K
195 values (Evanno, Regnaut and Goudet, 2005), implemented in Structure Harvester (Earl
196 and vonHoldt, 2012).

197 To identify the genetic affinity of Eritrean elephants, STR data from savanna
198 elephants from north central and east Africa were combined with data from Eritrean
199 elephants, and parameters of the STRUCTURE software were modified to allow for
200 “learning samples.” Default parameters were used for migrant priors, allele frequencies
201 were only updated from north central and eastern savanna elephant populations, and an
202 admixture model with correlated allele frequencies and K=2 clusters was assumed.

203

204 **Results**

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

211

212 *Mitochondrial Haplotypes*

213 We successfully sequenced mtDNA *ND5* in 20 samples. We identified a single
214 *ND5* haplotype (GenBank accession number KC608166) for all; an NCBI BLAST search
215 revealed that this haplotype occurred in savanna elephants throughout eastern and
216 southern Africa (Ishida, *et al.*, 2013; Roca, *et al.*, 2005). Among 15 Eritrean elephant
217 samples that were successfully amplified and sequenced for *HVR1*, three unique
218 haplotypes were detected. These haplotypes were distinguished by only 2 polymorphic
219 nucleotide sites, so that the Eritrean elephant *HVR1* haplotypes differed by 1 nucleotide
220 character state each (Figure 2). Thus at *HVR1* the Eritrean elephants had low nucleotide
221 diversity ($\pi = 0.0015$; S.D. +/- 0.0016), and low haplotype diversity ($h = 0.4476$; S.D. +/-
222 0.1345). The three unique *HVR1* Eritrean haplotypes were aligned with those previously
223 published for African elephants, and used to generate a median joining network (Figure
224 2A). Eight mtDNA groups had previously been identified by Ishida and colleagues
225 (Ishida, *et al.*, 2013); the 3 Eritrean haplotypes were all within the “savanna wide” group
226 (Figure 2). One haplotype (found in 3 Eritrean samples) was identical to a previously
227 reported haplotype (Figure 2; GenBank accession number AY741325) (Nyakaana,
228 Arctander and Siegismund, 2002), which occurs in elephants from across Eastern and
229 Southern Africa (Debruyne, 2005; Eggert, Rasner and Woodruff, 2002; Johnson, *et al.*,
230 2007; Nyakaana, *et al.*, 2002). The remaining 2 haplotypes (found in 1 and 11 samples)
231 were novel and confined to Eritrean elephants; they did not match sequences reported by
232 any previous study (Figure 2).

233

234 *Nuclear SNPs*

235 Three X-linked nuclear genes (*BGN*, *PHK*, *PLP*) have nucleotide sites with fixed
236 character states that distinguish forest from savanna elephants (Roca, *et al.*, 2005).
237 Primers for PCR amplification of very short fragments containing one or more of these
238 diagnostic sites have previously been developed for use with degraded DNA (Ishida, *et*
239 *al.*, 2011a). Using these primers, we obtained at least one of the genic sequences for 9 of
240 the Eritrean samples (Table 1), generating a total of 21 unlinked sequences with sites that
241 distinguish forest from savanna elephants. At every one of these segments, savanna
242 elephant-specific nucleotide character states were present (Table 1); sequences with sites
243 that matched a character state typical of forest elephants were never found (Table 1).
244 Fisher's exact tests comparing these sites established that there were no significant
245 differences ($p \approx 1.00$) in the proportions of character states between Eritrean elephants
246 and savanna elephants (Ishida, *et al.*, 2011a) (Table S2). By contrast, a Fisher's exact test
247 found highly significant differences ($p < 10^{-4}$) between the character states found in
248 Eritrean elephants and those in previously examined forest elephants (Ishida, *et al.*,
249 2011a).

250

251 *Microsatellites*

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

258 respectively; F_{IS} was -0.37 and all polymorphic loci were in Hardy-Weinberg
259 equilibrium. For these same loci, among savanna elephants from across the African
260 continent genotyped by a previous study (Ishida, *et al.*, 2011b), the average number of
261 alleles per locus had been 9.88, while observed and expected heterozygosity had been
262 0.57 and 0.58 respectively. To account for the small sample size from Eritrea, 3
263 individuals from each non-Eritrean African savanna elephant population were randomly
264 chosen for analysis. In this analysis, elephants from the rest of Africa were still more
265 diverse than Eritrean elephants: the average number of alleles per locus per population
266 (for sample size $n=3$) was 1.97 (standard deviation of 0.21) while observed and expected
267 heterozygosity were 0.54 and 0.59, respectively.

268 Bayesian clustering analysis was performed using STRUCTURE (Pritchard, *et*
269 *al.*, 2000) for two data sets that combined the Eritrean individuals with genotypes from a
270 larger group of elephants that had been previously reported (Ishida, *et al.*, 2011b). The
271 analysis included 555 forest and savanna elephants from outside Eritrea. This supported
272 splitting Africa's elephants into two clusters ($K = 2$; Figure 3A, Figure S2 and Table S3),
273 one corresponding to African forest elephants, the other to African savanna elephants
274 (Ishida, *et al.*, 2011b). Partitioning of the 3 Eritrean elephants identified them as savanna
275 elephants (Figure 3B). The overall proportion of the Eritrean elephants assigned to the
276 forest elephant partition was 0.08. This partitioning appeared to reflect local differences
277 in savanna elephant allele frequencies, rather than admixture from forest elephants. We
278 examined the data closely, finding three alleles present in Eritrea that were more common
279 in forest than savanna elephants (one allele at each of the loci LAF37, LaT06 and
280 EMX4). These alleles occurred at high frequencies or were fixed in Eritrean elephants.

281 Even so, these three alleles were also present in other savanna elephants, and no allele at
282 any locus in Eritrean elephants fell outside the size range expected of savanna elephants.
283 No allele in Eritrea had a size that was typical of only forest and not savanna elephants, in
284 cases where the allelic size ranges vary between the species (Ishida, *et al.*, 2011b). With
285 the caveat that DNA from only three individuals amplified, the close examination of STR
286 allele sizes in Eritrea failed to find evidence for this population having any alleles that
287 would be indicative of admixture from forest elephants.

288 A second STRUCTURE analysis included the three Eritrean elephants along with
289 previously published genotypes of only savanna elephants from north central Africa
290 (Cameroon) and from east Africa, in order to examine whether Eritrean elephants
291 genetically had a closer affinity to elephants from one region or the other. The parameters
292 of the STRUCTURE software were modified to allow for “learning samples” in which
293 north central and eastern savanna elephants were *a priori* assigned to their known region
294 of origin. The Eritrean elephants were not defined *a priori* as belonging to a population or
295 region. Despite this modification, partitioning between elephants in the two regions was
296 not complete, presumably due to limited differentiation between north central and eastern
297 savanna elephants. Different patterns between the north central and the eastern savanna
298 elephants were evident (Figure 3C). The patterns observed among Eritrean elephants
299 more closely resembled those of eastern than those of north-central savanna elephants
300 (Figure 3C). This suggests that Eritrean elephants have a greater nuclear genetic affinity
301 with East African than with North-Central African savanna elephants, consistent with the
302 finding that some Eritrean elephants share control region haplotypes with populations in

303 Eastern Africa, but are not known to share mtDNA haplotypes with Cameroon elephants
304 (Figure 2).

305

306 **Discussion**

307 Some scholars have speculated that war elephants used in the 3rd century BCE
308 that had been captured in Eritrea were forest rather than savanna elephants (Gowers,
309 1948). This was based on a written account of the battle of Raphia in 217 BCE, fought
310 between the armies of Ptolemy IV and Antiochus III during the Syrian Wars, and in
311 which African and Asian elephants met in combat. The Asian elephants used by
312 Antiochus are described as superior in size and strength over Ptolemy's African elephants
313 (Polybius, 1923). Since African savanna elephants are larger than Asian elephants, some
314 writers were led to conclude that the elephants used by Ptolemy could have been African
315 forest elephants (Gowers, 1948), which are smaller and have a more compact build than
316 savanna elephants (Grubb, *et al.*, 2000).

317 Sequencing of diagnostic single nucleotide polymorphisms found only savanna
318 elephant and not forest elephant nuclear genotypes among the elephants of Eritrea (Figure
319 3, Table 1). Also, in Eritrea we detected only S clade mitochondrial DNA, which is
320 carried only by savanna elephants (Ishida, *et al.*, 2011b). The mtDNA results may be
321 especially telling, because savanna elephants in eastern, southern, and north-central
322 Africa often carry F clade mitochondrial haplotypes that are geographically persistent and
323 may record the ancient presence of forest elephants in a locality (Roca *et al.*, 2005). The
324 forest elephant mtDNA may be geographically persistent since female African elephants
325 are non-dispersing (Ishida, *et al.*, 2011b). Although our results cannot completely rule out

326 the possibility that forest elephants may have existed somewhere in Eritrea in the past,
327 our data provide no support for this speculation. Eritrean elephants comprise a savanna
328 elephant population in which even the forest-derived F clade mtDNA carried by many
329 other savanna elephant populations was not detected. While not consistent with previous
330 speculation about the taxonomic affinity of Eritrean war elephants, our results are
331 consistent with the view that two and only two species of elephant occur in Africa, and
332 that currently hybrids between them are confined to a relatively narrow contact zone
333 between forest and savanna habitats (Ishida, *et al.*, 2011b). Likewise, our results should
334 dispense with rumors that Asian elephants brought to Eritrea in 1868 had admixed with
335 African elephants in the region (Hagos, *et al.*, 2003; Shoshani, *et al.*, 2004).

336 Both nuclear and mitochondrial data support a closer relationship of Eritrean
337 elephants to savanna elephants in East Africa than to savanna elephants of the north-
338 central Sudanian/Sahelian region (Figures 2, 3). Of three *HVRI* mitochondrial DNA
339 haplotypes carried by elephants in Eritrea, one is widespread, occurring throughout
340 eastern and southern Africa. The remaining 2 haplotypes are restricted to Eritrea, but
341 differ by a single nucleotide from haplotypes found in eastern but not north-central Africa
342 (Figure 2). The single *ND5* haplotype present in Eritrea has also been detected across
343 eastern and southern Africa but not north central Africa (Ishida, *et al.*, 2013; Roca, *et al.*,
344 2005). Although nuclear microsatellite genotypes were only successful for three Eritrean
345 elephants, each of these had a closer genetic affinity to eastern savanna elephants than to
346 the elephants of Cameroon (Figure 3).

347 Mitochondrial haplotype and nucleotide diversity were both low compared to
348 other savanna elephant populations. For elephants across Africa, average *HVRI*

349 haplotype diversity has been reported as 0.985 (Johnson, *et al.*, 2007) or 0.85 (Nyakaana,
350 *et al.*, 2002), about twice the 0.45 of Eritrea. Mitochondrial nucleotide diversity has been
351 reported as 0.030 (Johnson, *et al.*, 2007) or 0.02 (Nyakaana, *et al.*, 2002), also about
352 twice the 0.0015 observed in Eritrea. Variation among haplotypes in Eritrea was low, as
353 the three unique *HVR1* haplotypes were defined by two nucleotide variable sites, and
354 only a single *ND5* haplotype was detected. Nuclear diversity was also very low, a
355 previous study reported observed heterozygosity among all African elephant for the same
356 STR loci as 0.50 (Ishida, *et al.*, 2011b) , higher than the 0.36 observed in Eritrea.

357 The population of elephants in Eritrea is small; human-wildlife conflicts and
358 habitat loss are major concerns. Currently elephant migration into Ethiopia occurs only
359 during the wet season (Yacob, *et al.*, 2004). This emphasizes the importance of the
360 habitat in Eritrea for sustaining this population. An increase in suitable and protected
361 habitat may be helpful to the long-term survival of Eritrean elephants. The Agriculture
362 Ministry of Eritrea is committed to preservation of the elephants while minimizing
363 human-elephant conflict, and recently reported that the numbers and range of elephants
364 appear to be increasing (Anonymous, 2013). Since the elephant population of Eritrea is
365 small and isolated, it may in the future require genetic management. In the absence of
366 habitat corridors that enable gene flow, genetic management or restoration may
367 eventually become necessary, in which case our results suggest that the Eritrean
368 population would best be augmented using individuals from eastern Africa.

369

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378

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- 501

502 **Tables and Figures**

503

504 **Figure 1. Map of Eritrea showing current African elephant distribution.** The shaded
505 region pointed to by the arrow indicates the current range of elephants in Eritrea (Litoroh,
506 1997; Shoshani, *et al.*, 2004). This map is from the IUCN African Elephant Specialist
507 Group – African Elephant Status Report (Blanc, *et al.*, 2007), which permits reproduction
508 for educational purposes.

509

510 **Figure 2. Relationships of Eritrean *HVRI* haplotypes to those previously reported**
511 **for African elephants.** A median joining network was constructed from 314 bp
512 mitochondrial *hypervariable region 1 (HVRI)* sequences using elephants from across
513 Africa (Ishida, *et al.*, 2013). (A) Eight subclades reported by Ishida *et al.* (2013) have
514 been color-coded. Haplogroups within the box have only been detected among savanna
515 elephants. (B) The northern-savanna, savanna-wide and southeast-savanna subclades are
516 shown, including the haplotypes carried by Eritrean elephants, numbered 1, 2 and 3.
517 Sizes of the circles representing haplotypes present in Eritrea are proportional to the
518 number of individuals from Eritrea carrying that haplotype. Haplotypes numbered 1 and
519 2 (Genbank accession numbers KC608163 and KC608165, respectively) were detected
520 only in Eritrea, number 3 was found in Eritrea and elsewhere, while numbers 4-9 are
521 haplotypes that differ by a single nucleotide from those present in Eritrea. Haplotypes 3
522 through 9 have been sequenced by previous studies (GenBank accession numbers
523 AY741325, AY742801, AF106236, AF106226, AF106239, AY741074, and AF106235
524 (Debruyne, 2005; Nyakaana, *et al.*, 2002), respectively).

525

526 **Figure 3. Species assignment and inferred population ancestry among African**

527 **elephants.** Multi-locus genotype data were used to estimate population subdivision using
528 the program STRUCTURE, which applies a model-based clustering algorithm to identify
529 subgroups that have distinct allele frequencies (Pritchard, *et al.*, 2000). (A) Two

530 partitions that distinguish African forest elephants (white partition) from African savanna
531 elephants (shaded partition) were successfully reconstructed (Ishida, *et al.*, 2011b). Three

532 Eritrean elephants included in the dataset were assigned largely but not completely to the
533 savanna elephant partition. (B) An enlargement of STRUCTURE partitioning for the

534 Eritrean elephant individuals. Average assignment of Eritrean elephants to the partition
535 corresponding to forest elephants was ca. 0.08 likely due to different frequencies in

536 savanna elephant alleles for Eritrea, rather than admixture by forest elephants. (C)

537 Genetic affinity of Eritrean elephants was determined by modifying STRUCTURE

538 parameters to allow for “learning samples” where individuals can be assigned to pre-

539 defined populations. Savanna elephant from Cameroon (BE and WA) were defined as

540 belonging to a north central population while savanna elephants from Kenya and

541 Tanzania (KE, MK, AB, AM, SE, NG, TA) were defined as belonging to an eastern

542 population. The two partitions (lightly and darkly shaded) did not completely conform to

543 geographic regions (Cameroon and East Africa). The Eritrean elephants displayed

544 patterns more closely resembling those of East African than of Cameroon elephants,

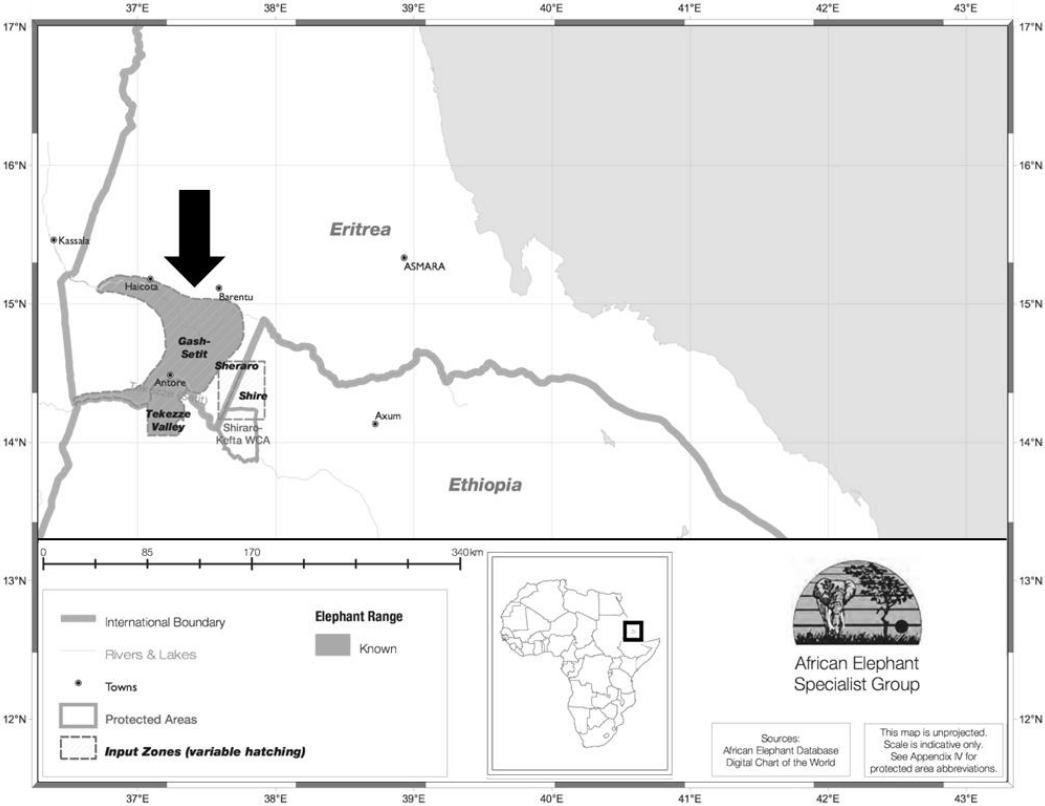
545 indicating genetic similarity to the former. Locality abbreviations are as follows: SL-

546 Sierra Leone; LO-Lope, in Gabon; OD-Odzala, in Republic of the Congo; DS-Dzanga

547 Sangha, in Central African Republic; BF-Bili, GR-Garamba, in the Democratic Republic

548 of the Congo; BE-Benoue, WA-Waza in Cameroon; ER-Eritrea; KE-Central
549 Kenya/Laikipia, MK-Mount Kenya, AB-Aberdares, AM-Amboseli, in Kenya; SE-
550 Serengeti, NG-Ngorongoro, TA-Tarangire, in Tanzania; SW-Sengwa, ZZ-Zambezi, HW-
551 Hwange, in Zimbabwe; CH-Chobe, SA-Savuti, MA-Mashatu, in Botswana; KR-Kruger,
552 in South Africa; NA-Northern Namibia/Etosha.
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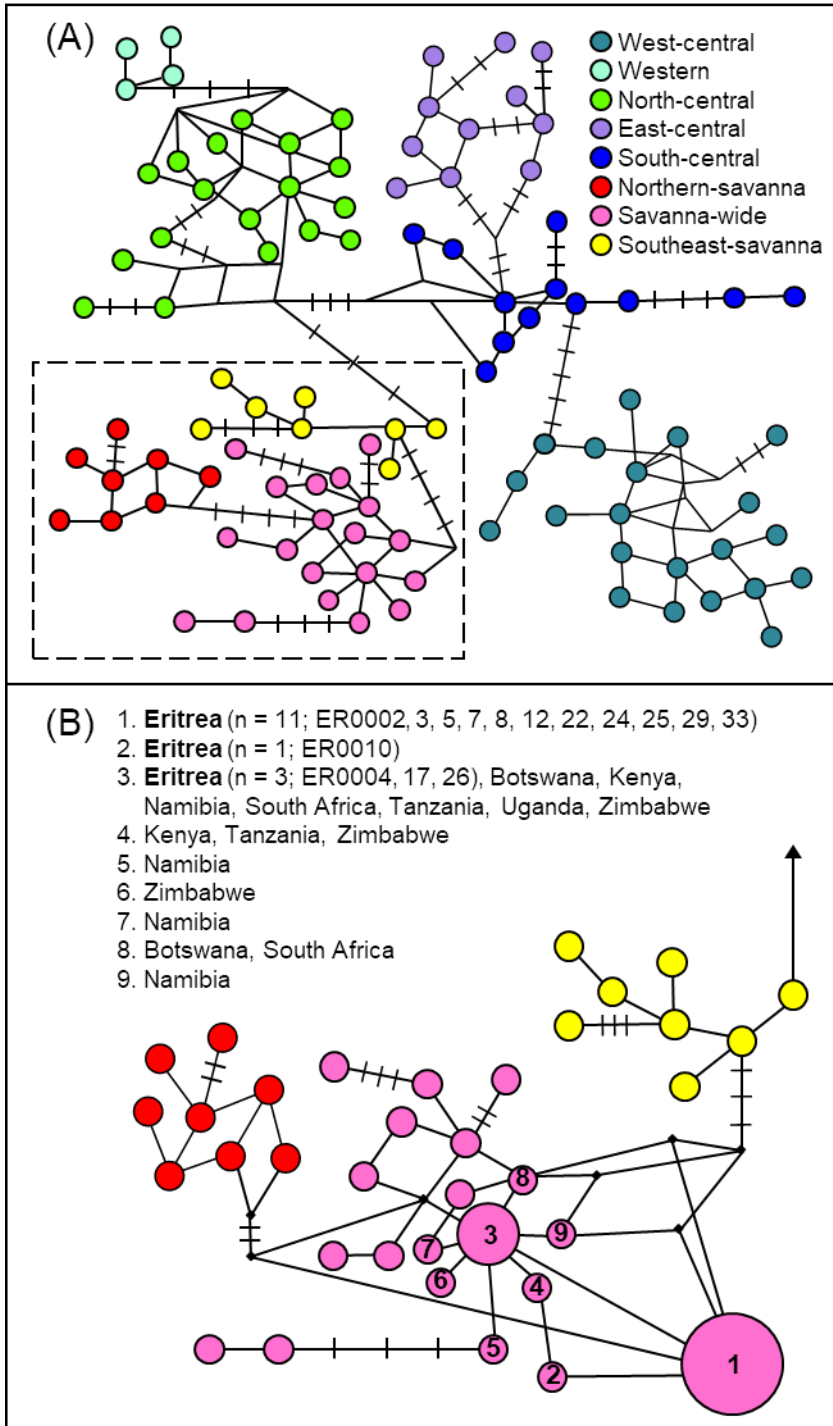
[Figure 1]



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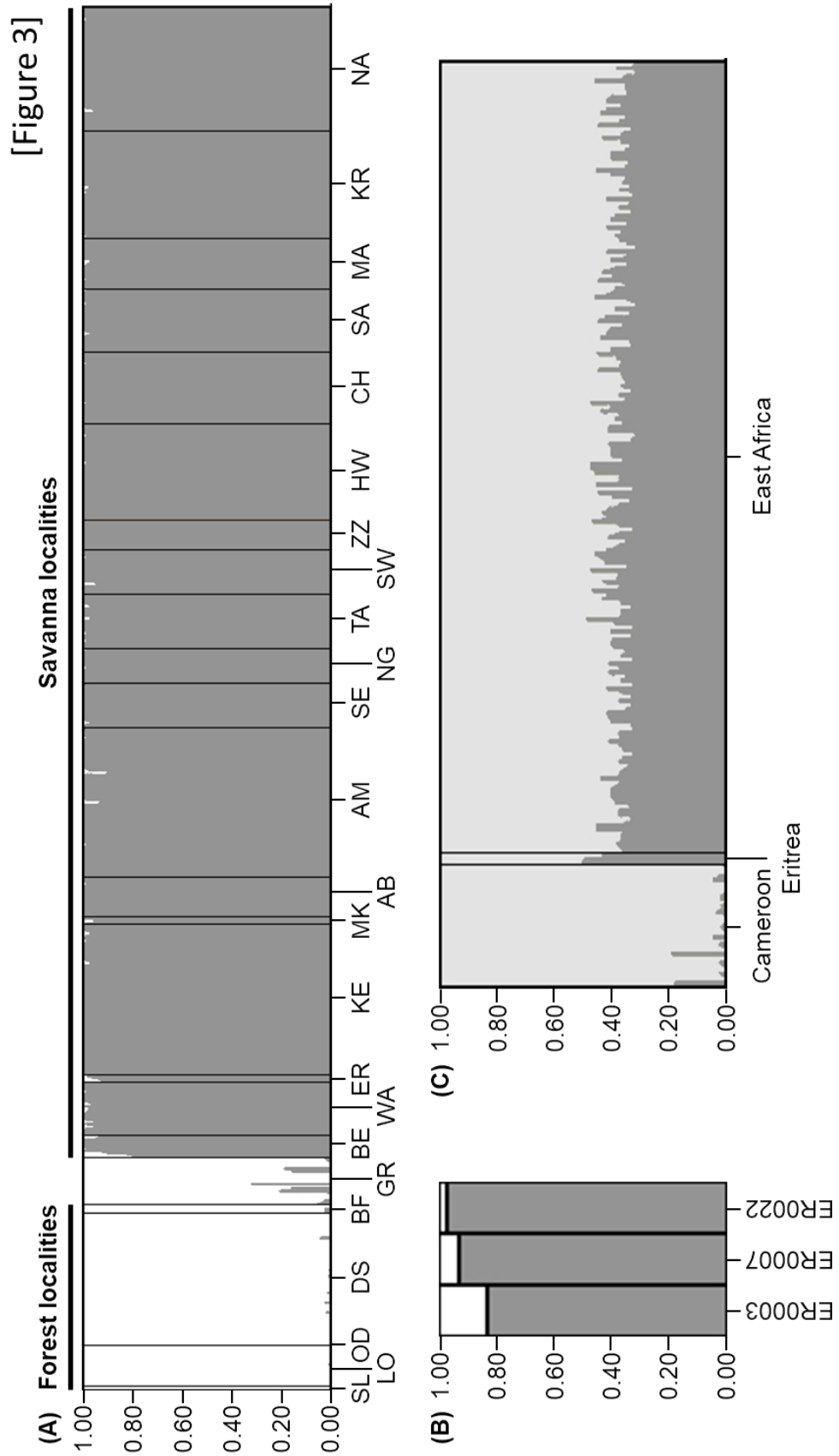
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[Figure 2]



556

557



558

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Table 1. Species-diagnostic nucleotide sites present in three nuclear genes examined in Eritrean elephants

	BGN															PHKA2					PLP				
	N=	287	304	308	472	485	499	508	513	515	516	570	N=	39	71	871	872	N=	319	345	361				
<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	T	G	A	T	ΔT	G	G	GΔ	ΔG	C	721	T	A	T	G	661	G	T	A				
ER0001		G	T	G	A	T	T	G	G	G	G	C		--	--	--	--		--	--	--				
ER0002		G	T	G	A	T	T	G	G	G	G	C		T	A	T	G		G	T	A				
ER0003		G	T	G	A	T	T	G	G	G	G	C		T	A	T	G		G	T	A				
ER0004		G	T	G	--	--	--	--	--	--	--	--		T	A	T	G		G	T	A				
ER0007		G	T	G	A	T	T	G	G	G	G	C		T	A	T	G		G	T	A				
ER0008		G	T	G	--	--	--	--	--	--	--	--		T	A	T	G		G	T	A				
ER0022		G	T	G	A	T	T	G	G	G	G	C		T	A	T	G		G	T	A				
ER0024		--	--	--	--	--	--	--	--	--	--	--		T	A	--	--		--	--	--				
ER0025		--	--	--	--	--	--	--	--	--	--	--		T	A	--	--		--	--	--				

Species diagnostic nucleotide sites in three 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 trans-national studies for each of three species of elephant (Ishida et al. 2011a, Lei et al. 2009, Roca et al. 2001 and 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; Δ indicates a deletion.

The elephants of Gash-Barka, Eritrea: Nuclear and mitochondrial DNA genetic patterns

Supplementary Material

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Figure S1. Map of Gash-Barka, Eritrea, showing sampling locations, which were approximated by the name of the nearest town or village (dots) (Shoshani, *et al.*, 2004).

Further details on sample collection can be found in Table S1.

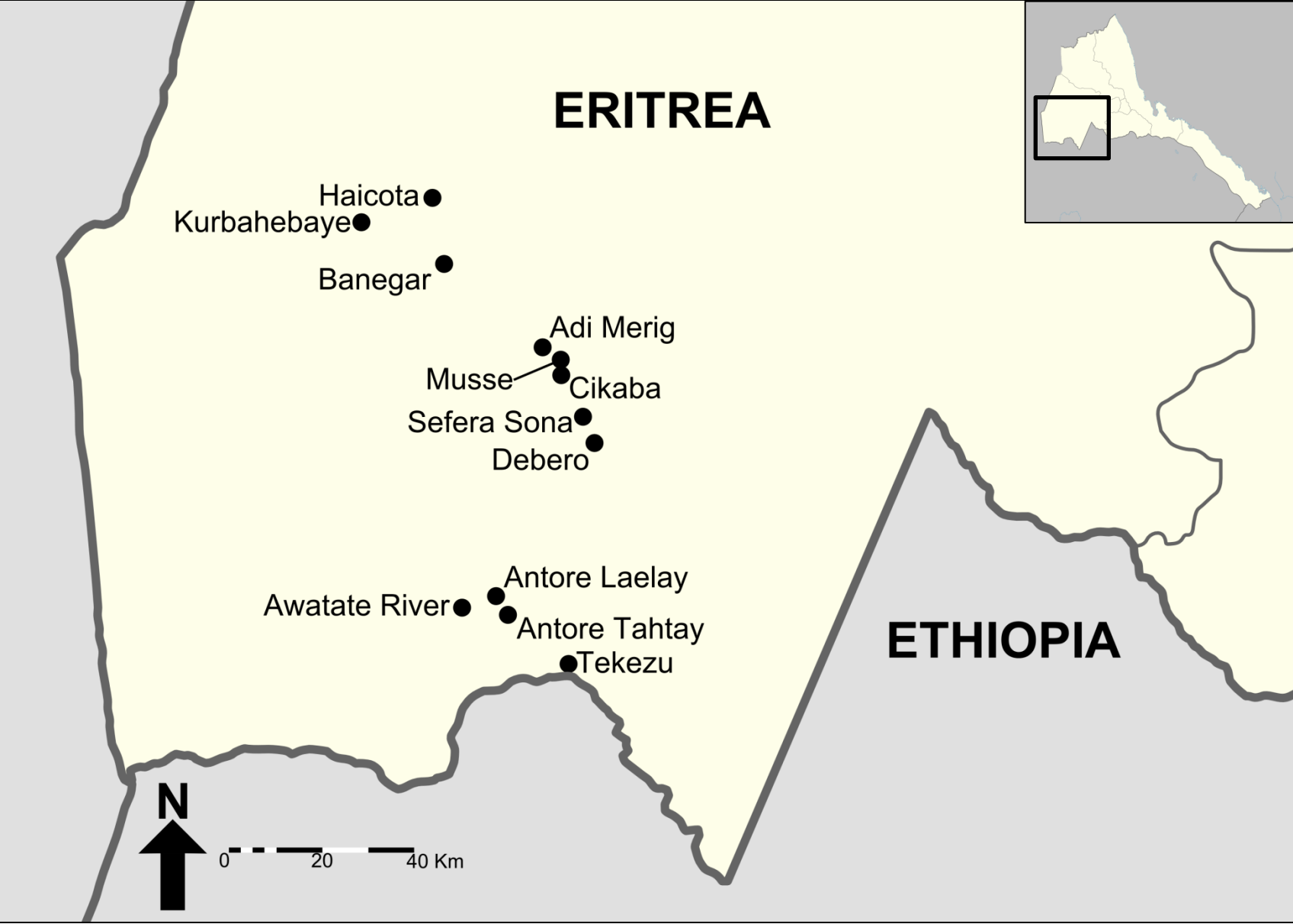
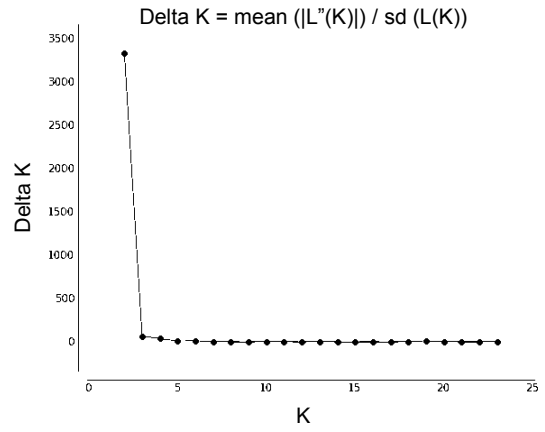
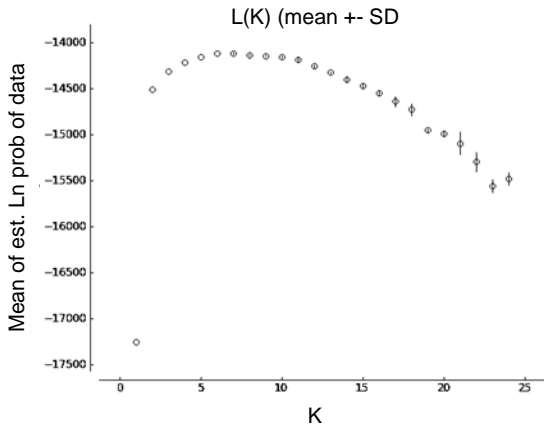
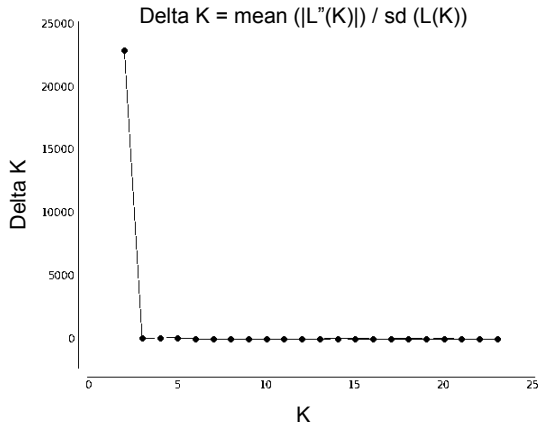
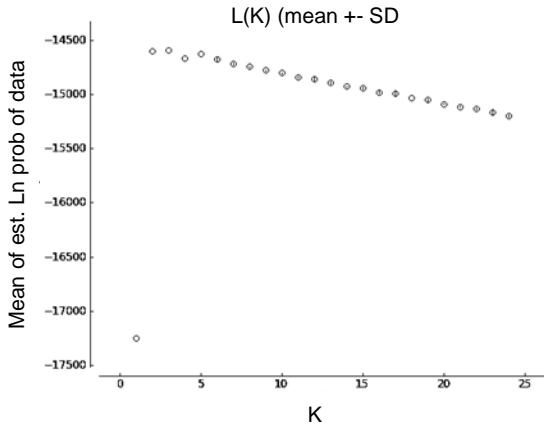


Figure S2. Graphical plots for examining the number of genetic subdivisions (K) in African elephants (genus *Loxodonta*). Results shown are based on the *ad hoc* method of Evanno (Evanno, Regnaut and Goudet, 2005) as implemented in Structure Harvester (Earl and vonHoldt, 2012). Combinations of assumptions of individual genetic ancestry and genetic relatedness among populations were tested: A) admixture with correlated allele frequencies, B) admixture with independent allele frequencies, C) no admixture with correlated allele frequencies and D) no admixture with independent allele frequencies. For the approach assuming no admixture and independent allele frequencies, the standard deviation of $\text{Ln}(K)$ was less than 10^{-7} and therefore K could not be estimated. For all other approaches, the Evanno method supported $K = 2$ clusters.

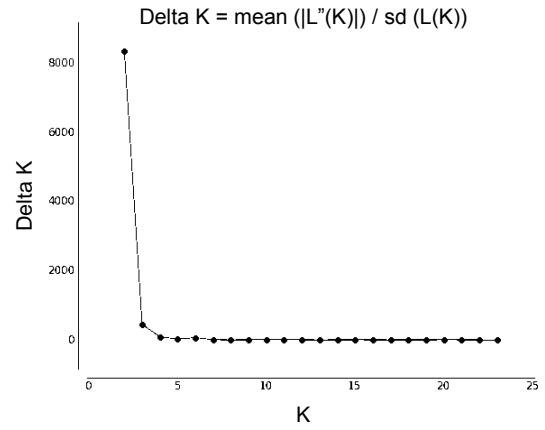
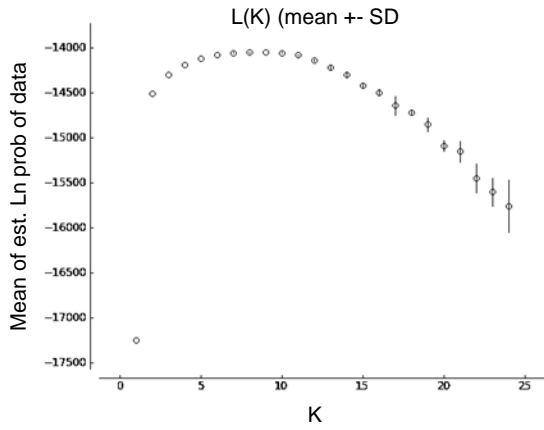
A) Admixture – Correlated



B) Admixture – Independent



C) Noadmixture – Correlated



D) Noadmixture – Independent

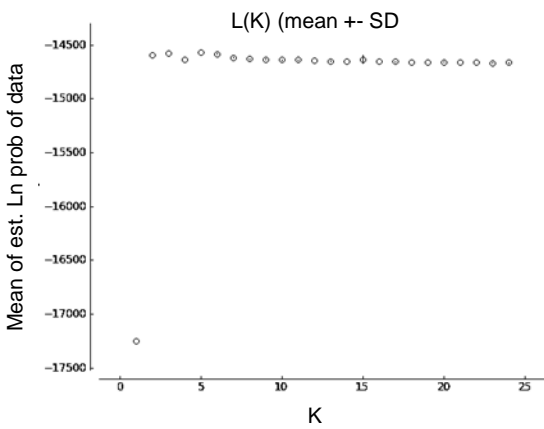


Table S1. Sampling data for Eritrean elephants

<i>Sample ID</i>	<i>Date Collected</i>	<i>Nearest Town / Village</i>
ER0001	2-Feb-02	Tekezu
ER0002	24-Dec-01	Banegar
ER0003	24-Dec-01	Banegar
ER0004	21-Apr-01	Debero
ER0005	21-Apr-01	Antore Laelay
ER0006	23-Jun-03	Tekezu
ER0007	22-Nov-02	Awtate River
ER0008	27-Dec-03	Haricota
ER0009	Unknown	Unknown
ER0010	2-Feb-03	Kurkahebaye
ER0011	9-May-03	Sefera Sona
ER0012	27-Jan-03	Adi Merig
ER0017	10-Feb-03	Antore Tahtay
ER0022	7-May-03	Musse
ER0024	12-Apr-03	Sefera Sona
ER0025	8-Nov-02	Tekezu
ER0026	22-Nov-02	Awtate River
ER0027	14-Mar-02	Debero
ER0029	13-Jun-02	Cikaba
ER0030	26-May-03	Unknown
ER0033	Unknown	Unknown

Sample locations are shown in Figure S1.

Table S2. Fisher's exact tests comparing Eritrean sequences to those of forest or savanna elephants

	<i>BGN</i>		<i>PHKA2</i>		<i>PLP</i>	
	<i>L. cyclotis</i> - typical sequences	<i>L. africana</i> - typical sequences	<i>L. cyclotis</i> - typical sequences	<i>L. africana</i> - typical sequences	<i>L. cyclotis</i> - typical sequences	<i>L. africana</i> - typical sequences
Eritrean elephants	0	7	0	8	0	6
Forest elephants	116	0	71	0	118	0
	<i>P</i> = 0.0000		<i>P</i> = 0.0000		<i>P</i> = 0.0000	
Eritrean elephants	0	7	0	8	0	6
Savanna elephants	0	806	0	721	2	661
	<i>P</i> = 1.0000		<i>P</i> = 1.0000		<i>P</i> = 0.9821	

Chromosome numbers are listed for each of three unlinked nuclear genes. Rows list the number of elephant chromosomes examined by the current study for elephant individuals from Eritrea vs. the number of chromosomes previously examined for either forest (*Loxodonta cyclotis*) or savanna (*L. africana*) elephants (Ishida et al. 2011a, Lei et al. 2009, Roca et al. 2001 and 2005). Columns show the number of chromosomal sequences that matched those previously shown to be typical for *L. cyclotis* or *L. africana*. Sex of the Eritrean elephants was unknown; therefore, nuclear amplicons were conservatively estimated as representative of 1 rather than 2 X-chromosomes. For the *PLP* gene, *Loxodonta africana* includes 2 putative hybrid elephants from Cameroon (Roca et al. 2005).

Table S3: Calculations to examine the number of elephant population subdivisions.

Admixture - Correlated

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	Ln''(K)	Delta K
1	3	-17251.6	0.458258	—	—	—
2	3	-14511.03333	0.763763	2740.566667	2546.9	3334.674868
3	3	-14317.36667	1.357694	193.666667	91.133333	67.123612
4	3	-14214.83333	1.154701	102.533333	43.5	37.672105
5	3	-14155.8	2.306513	59.033333	22.766667	9.870602
6	3	-14119.53333	2.83784	36.266667	35	12.333326
7	3	-14118.26667	6.017752	1.266667	19.4	3.223795
8	3	-14136.4	10.049378	-18.133333	8.766667	0.872359
9	3	-14145.76667	5.53022	-9.366667	0.966667	0.174797
10	3	-14154.16667	9.303942	-8.4	24.833333	2.66912
11	3	-14187.4	21.589118	-33.233333	39.3	1.820362
12	3	-14259.93333	16.519786	-72.533333	3.7	0.223974
13	3	-14328.76667	1.550269	-68.833333	5.533333	3.569274
14	3	-14403.13333	16.163642	-74.366667	7.733333	0.47844
15	3	-14469.76667	17.86962	-66.633333	8.4	0.470072
16	3	-14544.8	29.247051	-75.033333	21.666667	0.740815
17	3	-14641.5	57.161963	-96.7	10.533333	0.184272
18	3	-14727.66667	62.716532	-86.166667	137.433333	2.191341
19	3	-14951.26667	15.267067	-223.6	186.466667	12.213654
20	3	-14988.4	30.831153	-37.133333	67.866667	2.201237
21	3	-15093.4	119.902002	-105	95.8	0.798986
22	3	-15294.2	102.302346	-200.8	62.166667	0.607676
23	3	-15557.16667	71.396242	-262.966667	337.933333	4.733209
24	3	-15482.2	67.000224	74.966667	—	—

Admixture - Independent

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	Ln''(K)	Delta K
1	3	-17252.4	0.1	—	—	—
2	3	-14598.26667	0.11547	2654.133333	2645.5	22910.70206
3	3	-14589.63333	1.361372	8.633333	83.033333	60.992397
4	3	-14664.03333	1.569501	-74.4	112.8	71.869977
5	3	-14625.63333	1.096966	38.4	84.733333	77.243389
6	3	-14671.96667	14.365352	-46.333333	4.766667	0.331817
7	3	-14713.53333	9.282421	-41.566667	11.633333	1.253265
8	3	-14743.46667	4.62313	-29.933333	2.1	0.454238
9	3	-14775.5	6.657327	-32.033333	5.933333	0.891249
10	3	-14801.6	7.808329	-26.1	11.533333	1.477055
11	3	-14839.23333	8.894005	-37.633333	22.466667	2.526046
12	3	-14854.4	22.440811	-15.166667	20.9	0.931339
13	3	-14890.46667	10.96464	-36.066667	0.5	0.045601
14	3	-14926.03333	2.173323	-35.566667	21.866667	10.061397
15	3	-14939.73333	6.439203	-13.7	24.366667	3.784112
16	3	-14977.8	10.278132	-38.066667	23.266667	2.263706
17	3	-14992.6	16.284041	-14.8	23.333333	1.432896
18	3	-15030.73333	3.286842	-38.133333	20.466667	6.226847
19	3	-15048.4	13.383946	-17.666667	19.5	1.456969
20	3	-15085.56667	3.350124	-37.166667	13	3.880453
21	3	-15109.73333	3.652853	-24.166667	5.466667	1.496547
22	3	-15128.43333	6.296295	-18.7	15.133333	2.40353
23	3	-15162.26667	17.339358	-33.833333	2.633333	0.15187
24	3	-15198.73333	9.469072	-36.466667	—	—

Table S3: Continued.

<i>Noadmixture - Correlated</i>						
K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	Ln''(K)	Delta K
1	3	-17251.76667	0.152753	—	—	—
2	3	-14501.96667	0.305505	2749.8	2544.766667	8329.704197
3	3	-14296.93333	0.208167	205.033333	92.833333	445.956908
4	3	-14184.73333	0.51316	112.2	46	89.640633
5	3	-14118.53333	1.021437	66.2	20.3	19.873964
6	3	-14072.63333	0.416333	45.9	27.1	65.092095
7	3	-14053.83333	3.601851	18.8	14.266667	3.960926
8	3	-14049.3	4.340507	4.533333	0.933333	0.215029
9	3	-14045.7	3.315117	3.6	9.5	2.865661
10	3	-14051.6	3.835362	-5.9	22.833333	5.953371
11	3	-14080.33333	5.346338	-28.733333	26.466667	4.950429
12	3	-14135.53333	8.6031	-55.2	25	2.905929
13	3	-14215.73333	17.146525	-80.2	3.8	0.221619
14	3	-14299.73333	18.914104	-84	36.6	1.935064
15	3	-14420.33333	19.935981	-120.6	47.133333	2.364234
16	3	-14493.8	32.897264	-73.466667	72.766667	2.211937
17	3	-14640.03333	107.837115	-146.233333	65.866667	0.610798
18	3	-14720.4	23.84198	-80.366667	50.8	2.130696
19	3	-14851.56667	74.482638	-131.166667	102.833333	1.380635
20	3	-15085.56667	58.132636	-234	170.233333	2.928361
21	3	-15149.33333	114.366793	-63.766667	238.366667	2.08423
22	3	-15451.46667	161.061365	-302.133333	154.266667	0.957813
23	3	-15599.33333	154.876736	-147.866667	11.333333	0.073176
24	3	-15758.53333	294.328547	-159.2	—	—

The *ad hoc* method of Evanno et al. (2005) was used to examine the number of population subdivisions for elephants across Africa. The method was implemented in Structure Harvester (Earl et al. 2012). Calculations utilized the results from STRUCTURE software based on 1 million Markov chain Monte Carlo generations following a burn-in of 100,000 steps and 3 repetitions. Calculations for "Noadmixture - Independent" could not be performed since the standard deviation of the estimate of Ln Pr(Data) was less than 0.000001. Highlighted rows indicate the most likely value of K.

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