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Cytonuclear genomic dissociation in African elephant species

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African forest and savanna elephants are distinct species separated by a hybrid zone[1–4]. Because hybridization can affect the systematic and conservation status of populations, we examined gene flow between forest and savanna elephants at 21 African locations. We detected cytonuclear dissociation, indicative of different evolutionary histories for nuclear and mitochondrial genomes. Both paternally ($n = 205$ males) and biparentally ($n = 2{,}123$ X-chromosome segments) inherited gene sequences indicated that there was deep genetic separation between forest and savanna elephants. Yet in some savanna locales distant from present-day forest habitats, many individuals with savanna-specific nuclear genotypes carried maternally transmitted forest elephant mitochondrial DNA. This extreme cytonuclear dissociation implies that there were ancient episodes of hybridization between forest females and savanna males, which are larger and reproductively dominant to forest or hybrid males[1,2,5–7]. Recurrent backcrossing of female hybrids to savanna bulls replaced the forest nuclear genome. The persistence of residual forest elephant mitochondria in savanna elephant herds renders evolutionary interpretations based on mitochondrial DNA alone misleading and preserves a genomic record of ancient habitat changes.

There is a deep and almost complete separation between African forest elephants (*Loxodonta cyclotis*) and African savanna elephants (*Loxodonta africana*), as shown by morphological and nuclear genetic studies[1–4]. Skull measurements from 295 elephants of known provenance established that forest and savanna elephants fall into two morphologically distinct groups[1,2]. Nuclear DNA analyses using both slowly evolving nuclear gene sequences[3] and more rapidly evolving microsatellites[4] confirmed a deep evolutionary split between forest and savanna elephants occurring 2.6 million years ago (Mya)[3,8]. Only a few morphological intermediates[1] and genetic hybrids[3,4] have been detected in the zone of mixed forest-savanna habitat that surrounds the tropical forests of Africa. In contrast, analyses of mitochondrial DNA (mtDNA) identified high genetic diversity in savanna elephants[9–11] with patterns that sometimes appeared incongruent[11,12] with those of nuclear DNA studies, although mtDNA sequence phylogenies[10,11] were derived from individuals or populations different from those used to sequence nuclear DNA[3]. To investigate the apparent disparity across studies, we sequenced DNA from tissue samples of wild elephants to determine haplotypes with respect to three biparentally inherited X-linked gene introns ($n = 2{,}123$ X-chromosome segments), with respect to maternally transmitted mtDNA from 281 elephants, and with respect to a paternally inherited Y-chromosome gene intron in 205 males, using individuals from the same 21 African locations (**Fig. 1**) for each marker.

We sequenced three X-linked genes: *BGN* (647 bp)[3], *PHKA2* (1,002 bp)[13] and *PLP* (479 bp)[13]. Among African elephants, *BGN* had 16 variable sites defining 10 haplotypes, *PHKA2* had 25 variable sites defining 25 haplotypes and *PLP* had 8 variable sites defining 7 haplotypes. We determined *BGN* sequences for 733 *Loxodonta* chromosomes, including 112 from forest elephant populations, 598 from savanna elephant populations and 23 for elephants in Garamba National Park (**Fig. 1**), which includes both forest and savanna habitats[14]. *BGN* haplotypes were completely distinct between forest and savanna populations (**Fig. 2a**), with four fixed nucleotide differences between forest and savanna elephants (**Supplementary Figs. 1** and **2** online). Likewise, haplotypes of *PHKA2* were completely forest- or savanna-specific (**Fig. 2b**), with three fixed nucleotide differences between forest ($n = 67$) and savanna ($n = 511$) elephant gene segments (**Supplementary Figs. 1** and **2** online). The *PLP* haplotypes showed similar forest-savanna differentiation (**Fig. 2c**), with one exception. The common forest elephant haplotype *PLP-LOX12* (**Fig. 2c** and **Supplementary Figs. 1** and **2** online) occurred in two elephants (BE4059 and WA4013) from the Cameroon savanna. These two individuals were the only exceptions to complete separation between 655 and 114 *PLP* chromosome segments typed in savanna and forest populations, respectively. Excluding them, there would be one fixed nucleotide difference for *PLP* between forest and savanna haplotypes. Considering the three X-linked genes collectively, 1,762 of 1,764 (99.9%) savanna elephant chromosome segments had alleles typical of savanna elephants, whereas 293 of 293 (100%) chromosome segments from forest elephants in Dzanga Sangha, Lope and Odzala carried alleles typical of forest elephants (**Fig. 2** and **Supplementary Tables 1** and **2** online). These patterns confirm and extend evidence for species-level genetic separation between forest and savanna elephants[1–4].

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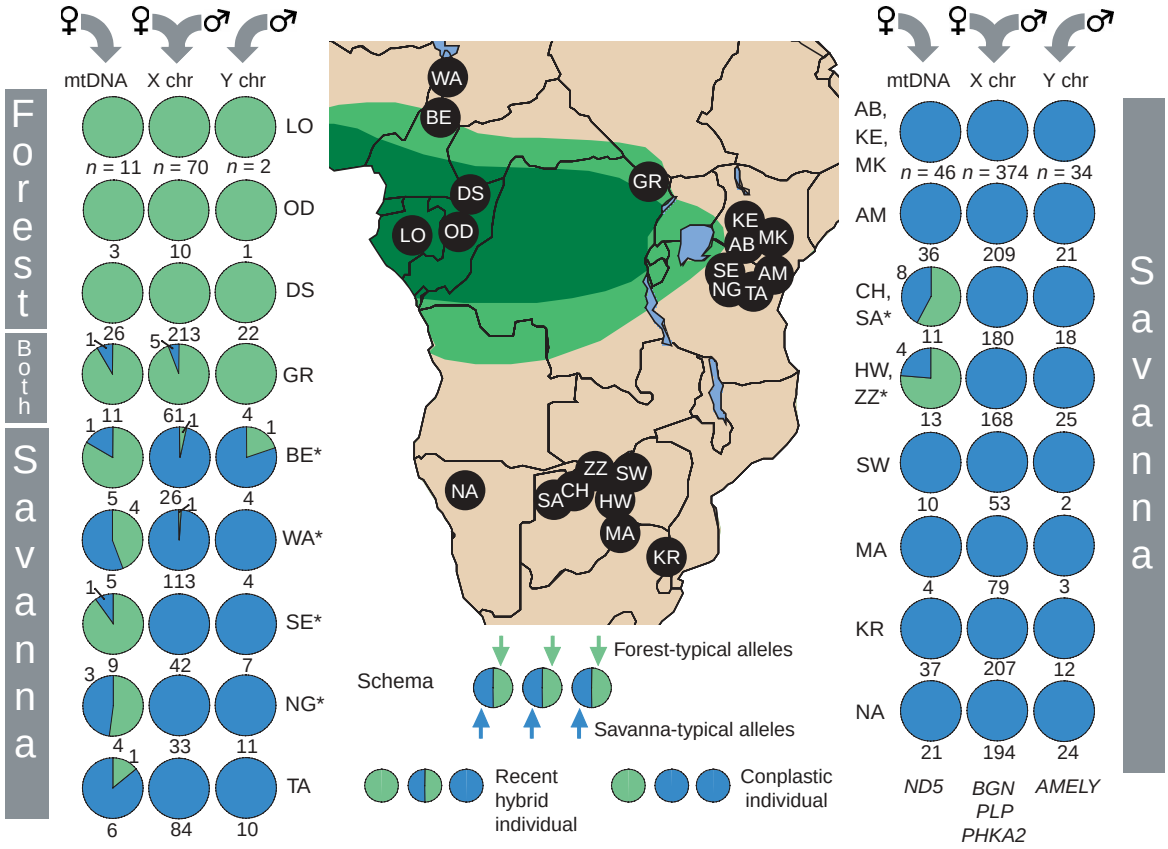

Figure 1 Map indicates locations of sampled elephant populations in Africa. Forest locations: DS, Dzanga Sangha; LO, Lope; OD, Odzala. Savanna locations: AB, Aberdares; AM, Amboseli; BE, Benoue; CH, Chobe; HW, Hwange; KE, central Kenya; KR, Kruger; MA, Mashatu; MK, Mount Kenya; NA, Namibia; NG, Ngorongoro; SA, Savuti; SE, Serengeti; SW, Sengwa; TA, Tarangire; WA, Waza; ZZ, Zambezi. The location Garamba (GR) is in the Guinea-Congolian/Sudanian transition zone of vegetation in Congo that includes a mixture of forest and secondary grasslands[14] suitable for both African elephant groups. Dark green represents tropical forests; light green is the forest-savanna transition zone of vegetation[14]. Pie charts indicate, by locale, the frequencies of species-typical genetic markers that are inherited maternally (left pie chart in each set of three), paternally (right pie chart) or biparentally (center pie chart). The maternally inherited marker is the mitochondrial *ND5* gene segment in **Figure 3b**; *ND5* clade I is indicated in blue, *ND5* clade II is in green. The paternally inherited marker is the Y-chromosome *AMELY* gene segment in **Figure 3a**: *AMELY* clade I is in blue, clade II is in green. The biparentally inherited nuclear markers combine the X-linked genes *BGN*, *PHKA2* and *PLP* shown in **Figure 2**, with savanna-typical haplotypes in blue and forest-typical haplotypes in green. Totals indicate the number of individuals (for mtDNA, Y chromosomes) or combined number of chromosome segments (biparentally inherited genes) examined. Cytonuclear dissociation was significant ($P < 0.05$) at locales labeled with an asterisk, where the proportion of forest-typical and savanna-typical alleles differed between mtDNA and biparentally inherited markers (**Supplementary Tables 1** and **3** online). Inset beneath the map are the expected gene pattern for a recent hybrid (left) and the conplastic pattern showing cytonuclear dissociation[17,22,23] (right).

To examine male lineages, we amplified a 1,551-bp intronic region of the Y-chromosome gene *AMELY*[13] in 205 males. We detected two distinctive Y-chromosome lineages for male elephants in Africa (**Fig. 3a**). Forest elephant males ($n = 25$) were all in *AMELY* clade II, whereas all but 1 of the 176 males in the savannas carried an *AMELY* clade I haplotype (**Fig. 3a**). The single sampled savanna male elephant to carry a Y chromosome typical of forest bulls was BE4059 (**Fig. 3a**), from Benoue in Cameroon, a savanna locale close to the forest-savanna habitat transition zone[14,15] (**Fig. 1**). He was also one of only two savanna individuals with a mixed forest elephant–savanna elephant nuclear genotype (**Fig. 2c**). Overall, the pattern and distribution of Y-chromosome haplotypes (**Fig. 3a**) resembled that of biparentally inherited nuclear genes in the distinctiveness of forest versus savanna populations[3,4].

To examine female lineages, we sequenced a 319-bp segment of the mitochondrial gene *ND5* in 281 African elephants (**Fig. 3b**). There was strong bootstrap support for two deeply divergent mtDNA lineages, clades I and II (**Fig. 3b**). All elephants in mtDNA clade I were savanna elephants. All forest elephants were in clade II (**Fig. 3b**), which also included savanna elephants from Cameroon, Tanzania, Botswana and Zimbabwe (**Fig. 3b**). Extreme cytonuclear dissociation (*i.e.*, lack of association between the presence of forest-typical mtDNA and the presence of forest-typical nuclear genes at a locale) was evident for these savanna elephant populations. In these populations, clade II mtDNA, typical of forest elephants, was carried by elephants with only savanna-elephant nuclear alleles.

Although twelve nucleotide sites were fixed between *ND5* clade I and clade II (**Supplementary Fig. 2** online), and 2.6 million years of evolution separate savanna from forest elephants[3,8], the forest-typical clade II mtDNA haplotypes found in savanna elephant populations were often identical in sequence to those of forest elephants (**Fig. 3b**). This suggests that a recent event (relative to the forest-savanna elephant split) is responsible for the discordant mtDNA phylogeographic pattern. The phylogeographic pattern and degree of cytonuclear discordance for the markers examined in African elephant populations are illustrated in **Figure 1**. Across Africa, biparentally

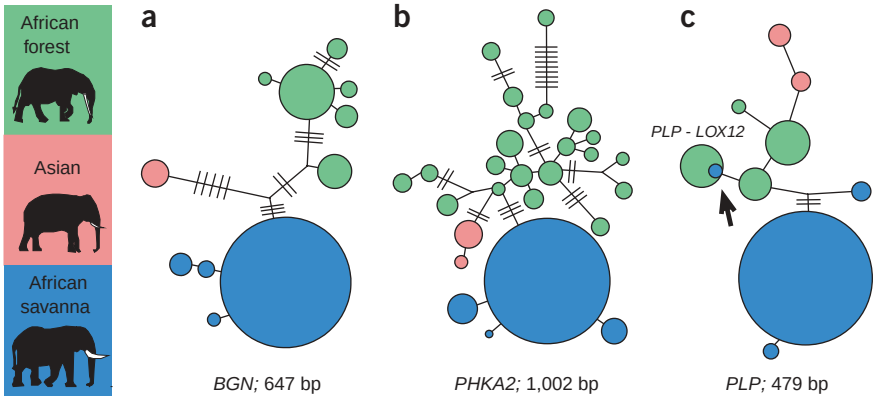

Figure 2 Haplotypes for three biparentally transmitted X-linked genes show almost complete differentiation among three elephant taxa. For each gene, haplotypes were assessed directly in males (single X chromosome sequenced); combinations of two haplotypes were then inferred for female sequences (two X chromosomes). Haplotypes found in Asian elephants are shown as red circles; blue circles represent African savanna elephant haplotypes; green circles are African forest elephant haplotypes. The size of the circles approximates the number of chromosomes with a particular haplotype. The almost complete lack of overlapping alleles between Asian, forest and savanna elephants is evident for each gene. (**a**) *BGN* haplotypes are completely distinct between forest and savanna populations ($n = 733$ African chromosomes with 10 unique haplotypes). (**b**) *PHKA2* proved to be the most diverse nuclear gene segment with 25 unique African haplotypes, yet the 596 African chromosomes examined were completely distinct between forest and savanna populations. (**c**) *PLP* haplotypes ($n = 794$ African chromosomes with 7 unique African haplotypes) were distinct between forest and savanna populations except for two individuals (BE4059, WA4013) in the savannas of Cameroon, each of which carried a *PLP-LOX12* haplotype (arrow) that was otherwise common in forest but absent among savanna elephants. Outside the mixed habitat zone of Garamba, these two individuals were the only elephants in which a mixed forest-savanna nuclear genotype was detected. Haplotypes are placed on unrooted maximum parsimony trees, separated by one step except where multiple steps are indicated by crossbars. Although multiple equally parsimonious trees were possible for the short and highly variable sequences, regardless of the gene tree topology the separation of haplotypes by taxa would remain evident. Gene trees with all haplotypes labeled, and corresponding sequence alignments, are presented in **Supplementary Figures 1** and **2** online.

transmitted genes show a pattern similar to that of the male-transmitted gene *AMELY*, whereas the phylogeographic pattern for mtDNA is quite disparate for the same populations and individuals (**Fig. 1**).

Forest-typical mtDNA (**Fig. 3b**) extends into savanna habitats distant from present-day forest habitats (**Fig. 1**), with a frequency as high as 90% among savanna elephants at some locales (*e.g.*, Serengeti; **Fig. 1**). The occasional dispersal of females from forest to savanna probably cannot completely account for the presence of forest-typical clade II mtDNA in such high proportions at great distances from the forest. Savanna elephant herds consist of nondispersing, closely related adult females (and young of both sexes) that share the same lineage of mtDNA[7,16]. Introgression of mtDNA from forest into savanna would imply progressive expansion of forest-derived female herds at the expense of savanna-derived female herds. It is questionable whether herds with forest elephant–derived mtDNA and savanna elephant–derived nuclear genes would enjoy a selective advantage allowing them to expand their range at the expense of savanna herds in which mitochondrial and nuclear genomes had coevolved for millions of years[17].

Forest and savanna elephants currently coexist in a relatively narrow habitat transition zone, largely converted to human use, that includes Garamba National Park[1,3,14,18] (**Fig. 1**). The location of this mixed vegetation zone has varied with climate change[14,19,20]. For part of the Holocene, forests extended beyond their current range both near Kilimanjaro and in Cameroon[19,20]. These extended forests probably harbored forest elephant herds, which may account for the current presence of forest-typical clade II mtDNA in these regions (**Figs. 1** and **3b**). In both Cameroon and Tanzania, the percentage of clade II mtDNA in savannas drops steeply with increasing distance from the current forest (*e.g.*, from Benoue to Waza, or from Serengeti to Ngorongoro to Tarangire; **Fig. 1**). The pattern in southern Africa is unusual: clade II mtDNA is found in areas of Botswana and Zimbabwe with low rainfall that are far removed from the tropical forest[14] (**Fig. 1**). But northern Botswana during the Pleistocene had one of the largest lakes in Africa, Lake Paleo-Makgadikgadi[21], indicative of wetter conditions[21] that may account for the presence of herds with forest-typical clade II mtDNA in the region. The late Pleistocene also saw the extinction of *Elephas* in Africa, which allowed range expansion for savanna *Loxodonta* populations[3,8], increasing opportunities for hybridization with forest *Loxodonta* populations.

But random hybridization between savanna and forest elephants cannot account for the dissimilar proportions of forest-typical and savanna-typical alleles among different genetic markers in the same populations and individuals. Hybrids would have a mix of forest and savanna alleles for nuclear genes; for example, first generation matings between forest and savanna elephants would produce offspring whose nuclear genotype is half forest (**Fig. 1**). Yet this is not the pattern that we observed; instead, forest nuclear alleles are completely absent in most savanna locales with high proportions of forest-typical clade II mtDNA (**Fig. 1**). For some savanna locales (**Fig. 1**), the proportion of forest alleles among the maternally inherited markers was significantly higher ($P < 0.05$, exact test) than the proportion of forest alleles for the biparentally inherited markers; at these savanna locales, the proportion of forest mtDNA alleles was also significantly higher ($P < 0.05$, exact test) than the proportion of forest alleles among the Y-chromosome sequences (**Supplementary Tables 1** and **3** online). This pattern would result after multiple generations of unidirectional hybridization of forest or hybrid females to savanna bulls[17,22,23]. Each backcross would dilute the proportion of forest nuclear alleles by half until the populations had overwhelmingly savanna-like nuclear genes but retained the maternally inherited forest-typical clade II mtDNA haplotype[17,22,23]. Ten generations of unidirectional hybridization and backcrossing would replace >99.9% of the forest nuclear genomic contribution[23] (**Supplementary Note** online).

In many cases of hybridization between distant taxa, the hybrids are inviable or sterile, with hybrids of the heterogametic sex (males, in the case of mammals) more frequently affected[24]. Hybrids may be subject to extrinsic effects, such as disruptions in adaptation to local environments, or intrinsic effects, such as developmental defects, sterility or physiologically reduced fertility[24]. Among elephants, sex differences in social and reproductive behavior suggest that an extrinsic mechanism[22,24] may contribute to the inferred failure of forest or hybrid males to successfully reproduce in savanna locales that

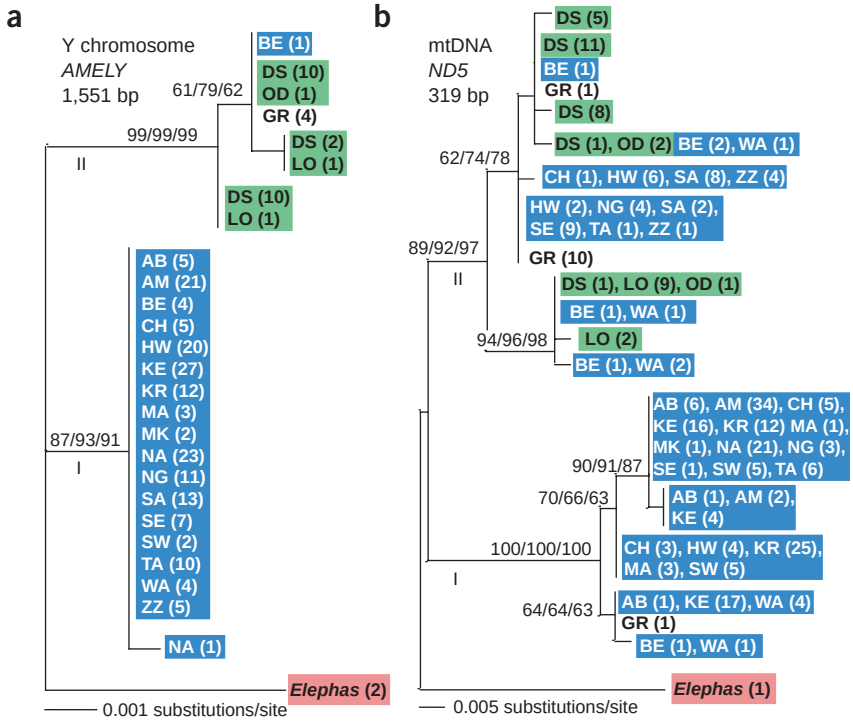

Figure 3 Phylogenetic relationships for Asian, African forest and African savanna elephant gene sequences inferred using maximum likelihood for (**a**) 1,551 bp of the paternally inherited Y-chromosome *AMELY* gene, including 205 male African elephants (–ln L = 2227.88057) and (**b**) 319 bp of the maternally inherited mitochondrial *ND5* gene, including 281 African elephants (–ln L = 658.63700). For both trees, the number of individuals with identical haplotypes is indicated in parentheses by locale (abbreviated as in **Fig. 1**), with Asian elephants shown in red, forest populations in green and savanna populations in blue. Individuals from Garamba, a mixed habitat zone[14], are shown without shading. Major clades (I and II) are labeled for each tree. Numbers at nodes indicate bootstrap support for (left to right) maximum likelihood, neighbor-joining and maximum parsimony methods, all of which produced trees with the same topology.

retain forest-typical clade II mtDNA. Female elephants are philopatric (nondispersing) and remain with their natal herd for life; males disperse at sexual maturity and mediate gene flow across herds[5,7,25]. Bulls periodically enter a condition of elevated testosterone called musth, in which they vigorously pursue opportunities to mate with estrous females and become aggressive towards competing males[5,7]. Older, larger male savanna elephants remain in musth for longer periods than younger, smaller males, and large males can suppress expression of musth in smaller males[5–7]. Because fully grown savanna bulls are almost twice as massive as forest bulls[1,2], and dominance and reproductive success are associated with larger male size[5–7], when forest and savanna elephant males come into contact, the larger savanna males probably out-compete forest males easily.

The occasional reproductive success of a forest or hybrid male is not precluded, although the different proportions of forest-typical alleles between mtDNA and nuclear markers suggest that larger savanna bulls out-reproduced smaller hybrid males for multiple generations, leading to replacement of the nuclear genome in herds that retained the ancestral maternal forest mtDNA. Although undetected forest nuclear alleles may have persisted, the complete lack of forest nuclear gene alleles among the 881 southern African and 742 eastern African savanna elephant X-chromosome segments examined is indicative of overwhelming dilution of any forest elephant contribution to herd nuclear genotypes. This conclusion receives support from previous studies that demonstrated genetic separation between forest and savanna elephants using autosomal markers[3,4]. Asymmetric hybridization seems to have produced a 'conplastic' cytonuclear pattern (**Fig. 1**) in many savanna elephants, in which the mitochondrial genome derives from a different lineage than the nuclear genes[17,22,23]. This pattern also implies that ongoing deforestation[14,15] may foster genetic replacement of forest elephants by opening their habitat to reproductive competition and aggressive hybridization by larger savanna males in those regions where both species persist.

The dearth of nuclear gene interchange between forest and savanna populations is indicative of a species-level distinction between the two groups[3,4]. In light of female philopatry and the matriarchal social structure of elephant herds, researchers have been generally cautious when applying mtDNA results to issues of elephant taxonomy[9,11,26]. Our findings suggest that caution was appropriate, because the history of female herd lineages exposed by mtDNA can be dissimilar to the nuclear DNA and morphometric patterns in the same individuals and populations[1,3,4]. This raises an important caveat to recent proposals for using mitochondrial sequences as a molecular 'bar code' for all living species[27,28] and as the basis for biodiversity assessment and taxon recognition. One report suggested that elephant systematics could have been resolved using this system[28], a conclusion that the present findings dispute. These results caution against minimizing the concept of species to a single mtDNA sequence.

METHODS

Data generation. Sample collection[29], laboratory techniques and analyses are described in detail in **Supplementary Methods** online. We obtained tissues in full compliance with specific Federal Fish and Wildlife Permits (endangered/threatened species and CITES Permits US 750138 and US 756611 to N.G.). We identified all genes by homology to GenBank entries. The three biparentally inherited genes that we sequenced (*BGN*, *PHKA2* and *PLP*) were X-linked but were not closely linked to each other in the genomes of eutherian taxa[13]. We selected X-linked genes as representative of biparentally inherited loci because the haplo-diploid inheritance of the X chromosome allows sequences to be readily haplotyped[3]; forest-savanna allelic separation has also been established for some autosomal loci[3,4]. Allele and haplotype totals listed exclude counts for Asian elephants (*Elephas maximus*) except where noted. A minority of *BGN* sequences were previously available[3]; all other sequences for *BGN* and other genes were newly determined.

Mitochondrial DNA. We sequenced the gene *ND5* instead of the mtDNA control region to avoid the greater potential for saturation of sites and the unreliable molecular clock in the control region. Indications that amplification of nuclear insertions of mtDNA had been avoided included unambiguous

fluorescent peaks and undisrupted open reading frames in sequences, supported by matching sequences produced for a subset of individuals from each clade that was amplified using multiple pairs of primers. The accuracy of the tree topology in **Figure 3b** was also established by 2.5 kb of mtDNA sequence for a representative subset of individuals (data not shown).

Garamba elephants. Garamba in Congo (Kinshasa) contains one of the few surviving elephant populations in intermediate habitat[15]. It is part of the Guinea-Congolian/Sudanian transition zone of vegetation that includes a mixture of forest and secondary grasslands[3,14]. Both savanna and forest elephants[1,18] as well as morphologically intermediate elephants[18] have been reported in Garamba. Genetic studies detected in our samples forest genotypes with some hybrids[3,4]. In this study, we compared elephant populations in savanna habitats to those in forest habitats; because Garamba includes both habitats, we did not include it in subtotals for forest or savanna, although we did include Garamba DNA segments when calculating overall totals for *Loxodonta*: 23 for *BGN*, 18 for *PHKA2*, 25 for *PLP*, 4 for *AMELY* and 12 for *ND5*. Three rare nuclear gene haplotypes detected only in Garamba are shown as forest haplotypes in **Figure 2**: each was a single nucleotide step from other haplotypes found in forest populations.

Divergence date estimates. The previous point estimate of divergence between forest and savanna elephants of 2.63 Mya was calibrated using a divergence date between *Elephas* and *Loxodonta* of 5 Mya[3,8]. Our estimate may be increased to 3.2 Mya to reflect a new calibration based on new *Loxodonta* fossils at least 6 million years old[30], depending on the phylogenetic position of *Elephas* relative to fossil *Loxodonta*.

GenBank accession numbers. AY823320-AY823387.

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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