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Genetic Evidence for Two Species of Elephant in Africa
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Elephants from the tropical forests of Africa are morphologically distinct from savannah or bush elephants. Dart-biopsy samples from 195 free-ranging African elephants in 21 populations were examined for DNA sequence variation in four nuclear genes (1732 base pairs). Phylogenetic distinctions between African forest elephant and savannah elephant populations corresponded to 58% of the difference in the same genes between elephant genera Loxodonta (African) and Elephas (Asian). High genetic distance, multiple genetically fixed nucleotide site differences, morphological and habitat distinctions, and extremely limited hybridization of gene flow between forest and savannah elephants support the recognition and conservation management of two African species: Loxodonta africana and Loxodonta cyclotis.

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Conservation strategies for African elephants have consistently been based on the consensus that all belong to the single species Loxodonta africana (1–3). Yet relative to African savannah elephants, the elephants in Africa’s tropical forests are smaller, with straighter and thinner tusks, rounded ears, and distinct skull morphology (2–11). Although forest elephants are sometimes assigned subspecific status and designated L. a. cyclotis, their degree of distinctiveness and of hybridization with savannah elephants has been controversial and often ignored (2–12). Recently, a comprehensive morphological comparison of metric skull measurement from 295 elephants (10, 11) and a provocative molecular report limited to a single individual (13) noted appreciable distinctions between forest and savannah specimens.

Here we report the patterns and extent of sequence divergence for 1732 nucleotides from four nuclear genes (14) among 195 African elephants collected across their range in Africa and from seven Asian elephants (Elephas maximus). African elephants were sampled, with biopsy darts (15, 16), throughout the continent, including individuals from 21 populations in 11 of 37 African elephant range nations (Fig. 1). Based on morphology (2–11) and habitat (17, 18), three populations were categorized as African forest elephants, whereas 15 populations in southern, eastern, and north-central Africa were categorized as savannah elephants (Fig. 1). DNA sequences from four nuclear genes, including short exon segments (used to establish homology to mammalian genes) and longer introns (which would evolve rapidly enough to be phylogenetically informative), were determined for all elephants (19). The genes include BGN [646 base pairs (bp)], CHRNA1 (655 bp),
GBA (100 bp), and VIM (331 bp), with sequence from all four genes obtained for 119 individuals. An alignment of variable sites and the composite genotypes are presented in supplemental information (20). Among 1732 bp, 73 sites were variable and 52 were phylogenetically informative. These nucleotide variants defined nine unique savannah genotypes among 58 individuals and 24 unique forest genotypes among 24 individuals. We observed nine genetically fixed nucleotide site differences between Asian and African elephants (GBN: 121, 155, 219, and 513 and CHRNA1: 011, 079, 274, 301, and 548) and one that approaches fixation (BGN 505). There were five fixed site differences between African savannah and forest elephants (GBN 304, 485, 508, 514, and 569) and two that were nearly fixed (CHRNA1 251 and GBA 20) (20).

Three methods of phylogenetic analysis (minimum evolution, maximum parsimony, and maximum likelihood) (21–23) revealed a concordant deep genetic division between the forest and savannah populations of African elephants (Fig. 2). The forest elephants of Dzanga-Sangha, Lope, and Odzala grouped together, separate from 15 savannah populations, which formed a distinct phylogenetic clade or lineage. An estimated 94% of the observed genetic variation (\( F_{ST} = 0.94, P < 10^{-5} \)) (24, 25) was due to differences between forest and savannah elephants and 6% to intragroup differences. Mantel tests (26) revealed only marginal association of genetic versus geographic distance (\( r = 0.19, P = 0.03 \)), and that association was attributed completely to forest versus savannah population differences (\( P > 0.05 \)) for forest or savannah populations tested separately.

Although forest and savannah elephants formed two genetically distinct groups, sequences from populations within the two categories could not be distinguished hierarchically. An estimated 94% of the population genetic structure is due to areas that are divergent (20) and the rest (6%) is due to intrapopulation differences (27).

The high level of genetic distinction is demonstrated by calculation of \( F_{ST} \) values among 24 populations (Fig. 2). The average number of within-group pairwise differences among 24 forest elephants was 1.74 as compared with a value of 0.06 among 58 savannah elephants (24, 25, 27). Each forest elephant had a unique composite genotype, whereas the 58 savannah elephants defined only nine distinct genotypes (20). Forest elephants displayed larger numbers of heterozygous nucleotide sites than did savannah elephants (an average of 3.54 heterozygous autosomal sites per individual in forest elephants versus 0.39 for savannah elephants) (20). These observations suggest a recent founder event in the history of the savannah metapopulation. A potential time venue for the bottleneck is indicated by fossil evidence, which suggests that the savannah elephant’s range greatly expanded at the end of the Pleistocene, after Elephas tene, the predominant African species, became extinct (3, 12).

The genetic and phylogenetic distinctiveness was evident without exception between 36 sampled forest elephants from three populations and 121 savannah elephants collected in 15 populations throughout sub-Saharan Africa. Each savannah population was genetically closer to every other savannah population than to any of the forest populations, even in cases where the forest population was geographically closer. Individuals from two “indeterminate” populations [Mount Kenya and Aberdare (Fig. 1)] contained exclusively savannah elephant genotypes (see Fig. 2, \( F_{ST} = 0.88, P < 10^{-5} \) in comparing both populations to three forest populations). Genotypes found in the third “indeterminate” population, Garamba, were diverse and predominantly nested within the forest elephant clade in the phylogenetic analyses. The forest populations (including Garamba) were genetically closer to each other than to any savannah populations, including several that were geographically close. A single exceptional Garamba individual, GR0021, contained five signature sequence sites that were diagnostic for savannah elephants (GBN 304T, 485T, 508G, 514G, and 569C), as well as a single site (GBA 79T) that was diagnostic for the forest elephants.

The high level of genetic distinction is demonstrated by calculation of \( F_{ST} \) values among savannah, forest, and Asian elephant populations as well as by the computation of genetic distances (average pairwise differences) among them (24, 25, 27). Highly significant differentiation is evident between savannah, forest, and Asian elephants (\( F_{ST} < 0.05 \)) but not between Garamba and forest elephants (\( P > 0.05 \)).
0.05). The genetic distance (average pairwise difference) between forest and savannah elephants is 9.0, which is 58% of the distance between Asian and African elephant genera (average = 15.5) (24, 25, 27). Tests for molecular evolutionary rate differentials did not reveal significant differences (P > 0.05) for the two African groups (24, 28, 29). Considering the estimation from fossil evidence for the divergence time between the two genera as 5 million years ago (12), the results suggest that forest and savannah elephants diverged approximately 2.63 (±0.94) million years ago (24, 27, 29), which is comparable to species-level distinction in other mammalian taxa, including elephants (12, 30, 31). This estimate should be considered as a maximum age, however, because the more recent genetic homogenization of the savannah elephants would inflate genetic distance as a consequence of a recent founder event.

Genetic distinctiveness between forest and savannah elephants is also apparent when individual gene variation is examined. For GBA, two variable sites in African elephants define three haplotypes ([C.C], [C.T], or [T.C] for nucleotide sites 20 and 79, respectively) that have large forest versus savannah frequency differences (Fig. 1, exact test P < 10^-5 for forest versus savannah). The predominant haplotype in savannah elephants is [T.C] (frequency = 0.96), whereas alternative [C.C] and [C.T] haplotypes comprise 100% of the forest elephants, suggesting that reproductive isolation exists between the two groups (Fig. 1). For VIM and CHRNA1, complete and exact haplotypes could not be determined for individuals heterozygous at two or more nucleotide sites, because gametic phase cannot be assessed (for example, for a two-locus genotype, does a double heterozygote G/C,T/A individual contain GT + CA or GA + CT haplotypes?). However, among forest and Garamba elephants, polymorphisms occurred at six nucleotide sites in VIM that were genetically monomorphic in savannah elephants (20). Similar differences in the occurrence of polymorphic nucleotide sites were apparent within CHRNA1: All sites that were variable among forest and Garamba elephants were fixed in savannah populations, whereas the two sites that were variable in savannah elephants were fixed in forest and Garamba elephants (20). Likewise, both CHRNA1 and VIM had an insertion/deletion variant limited to forest and Garamba elephants (20). The presence of these deletion variants in Dzanga-Sangha, Lope, and Garamba also is consistent with the recent occurrence of gene flow among these forest elephant populations across the Congolian forest.

The X linkage of BGN seen in other mammals (14) was affirmed in elephants by the presence of heterozygous nucleotide sites among females but not among the hemizygous males. Nineteen variable sites in BGN were used to identify 169 haplotypes from 55 males and 57 females. A minimum spanning phylogenetic network of the nine unique BGN haplotypes observed (Fig. 3) showed clear differentiation of a single distinct Asian haplotype (n = 13 chromosomes), two African savannah haplotypes (n = 53 chromosomes; including Aberdares and Mount Kenya), and six African forest haplotypes (n = 35 chromosomes; including one Garamba individual). For BGN, the number of nucleotide changes separating forest from savannah elephant haplotypes (six steps) was nearly as large as that separating either from the Asian elephant haplotype (seven steps). The BGN haplotypes present in the forest elephant populations were not found among savannah elephants, whereas haplotypes seen in the savannah elephants were not present in the forest populations (P < 10^-5, exact test of
the forest versus savannah haplotype frequencies) (24, 25, 32), suggesting a high degree of reproductive isolation between the forest and savannah populations. Taken together, the distinction affirmed by independent unlinked nuclear genes (Figs. 1 and 3) (20) offers strong support for the concept of appreciable genetic divergence between the African savannah and forest elephant populations.

There was no molecular genetic evidence of hybridization among 3 forest and 17 savannah elephant populations [defined a priori, plus Aberdares and Mount Kenya (Fig. 1)]. In Garamba, however, three individuals (GR0021, GR0035, and GR0037) showed genotypes with a combination of forest and savannah taxon-specific alleles, suggesting a history of limited hybridization in the ancestors of this population (20), as has been suggested by some (33), but not all (10, 11). GR0021 grouped with savannah elephants in the phylogenetic analysis, whereas animals GR0035 and GR0037 had largely forest genotypes (Fig. 2) except for the GB4 [T/C] haplotype, which is absent in forest elephants but predominant in savannah elephants (Fig. 1). The paucity of gene introgression between forest and savannah populations even near regions of potential physical contact [that is, in north-central Africa or near Garamba (Fig. 1)] suggests that hybridization in nature is rare and perhaps minimal by behavioral or physiological reinforcement. In this regard, no elephant from any population, including Garamba, displayed a predicted F2 hybrid genotype (that is, heterozygous at the genetically fixed sites between savannah and forest elephants), affirming the lack of gene flow or hybridization among the sampled elephants.

The molecular results of a pan-African phylogeographic elephant survey reported here offer support for the idea that a long period of adaptive evolution (estimated at 2.63 ± 0.94 million years) separated the savannah and forest elephant lineages. As such, the results strongly support recognition of species-level distinctions between African elephant taxa (5–11). Although reproductive isolation is the principal criterion for species recognition according to the Biological Species Concept (34), local hybridization or even the presence of a “hybrid zone,” as may have occurred in Garamba, would not preclude species recognition, because the genetic integrity of the parent species remains intact (34, 35). Hybrid zones that fail to spread or homogenize the genetic distinctiveness of contact species have been observed with scores of other species (35, 36). These considerations, along with the combined morphological, ecological, and molecular data, are cogent indicators that there should be species-level recognition for *Loxodonta africana* (Blumenbach 1797), the African savannah elephant (37) and *Loxodonta cyclotis* Matschie, 1900 (4), the African forest elephant. Given the rapid deple-

**Fig. 3.** Minimum spanning network depicting relationships among nine haplotypes observed for the X-linked *BGN* gene for Asian, African forest, and African savannah elephants. Hatch marks indicate the number of nucleotide differences separating each haplotype. Haplotypes were determined using 7 Asian (EMA), 74 African savannah (LAF) and 31 African forest elephants (LCY) for which the sex was known (a total of 55 males and 57 females). Haplotypes unique to each of the three taxa are identified by differences in shading; the number of chromosomes is indicated for each haplotype.

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19. DNA was extracted with a Qiaqan Qiamp kit. Previously described primer sequences (14) were used to amplify segments of four nuclear genes that in humans are on separate chromosomes: *BGN*, *CHRNA1*, GBA, and *VIM*. The following primers specific to the resulting elephant sequence were developed and used: CHRNA1-F2 (5’-CTCCTGCGGCTGAACTCCG-3’), CHRNA1-R3 (5’-CGCCTGGGAAAGAGGAGG-3’), VIM-F2 (5’-GGCTGCTCTCTGG-3’), and VIM-R2 (5’-TTGAAACCATTGTGAGAACTCCG-3’). Polymerase chain reaction using Taq Gold (Perkin-Elmer) consisted of a hot start at 95°C for 9.75 min, then 40 cycles of 15 s at 95°C and 30 s of annealing at 60°C (cycles 1 and 2), 58°C (cycles 3 through 8), 56°C (cycles 9 through 14), 54°C (cycles 15 through 20), or 52°C (cycles 21 through 40), and 60 s of extension at 72°C, with a final extension at 72°C for 5 min. Products were purified with Centricon concentrators (Amicon). ABI BigDye Terminator sequences were resolved on an ABI 3700 system. Homology of each elephant gene segment was established with the program NCBI BLAST 2.0 (39). All sequences for each gene segment were deposited in GenBank (accession numbers AF044919 through AF045493).

20. Supplemental Web material is available on Science Online at www.sciencemag.org/cgi/content/full/293/5534/1473/DC1. It is also available at the Laboratory of Genomic Diversity Web site at http://ldg.ncl.nih.gov.

21. DNA sequences were aligned with the software CLUSTALX (22) and visually inspected. Phylogenetic analyses of the concatenated data set (all four genes) were performed with heuristic searches (with random taxon addition and tree bisection-reconnection branch swapping) for maximum parsimony (MP), minimum evolution [neighbor joining (NJ)], and maximum likelihood (ML) methods implemented in PAUP*4.0b4 (23). MP analyses treated multistate characters as polymorphic and gaps as a fifth state. NJ analyses were performed with Kimura-2 parameter distances. ML analyses used empirical base fre-
In *Drosophila melanogaster*, the antennae, legs, genitalia, and analia make up a serially homologous set of ventral appendages that depend on different selector genes for their unique identities. The diversity among these structures implies that there is a common ground state that selector genes modify to generate these different appendage morphologies. Here we show that the ventral appendage that forms in the absence of selector gene activity is leglike but consists of only two segments along its proximo-distal axis: a proximal segment and a distal tarsus. These results raise the possibility that, during evolution, leglike appendages could have developed without selector gene activity.

Selector genes encode transcription factors that specify the identity of segments and appendages in insects and vertebrates (1, 2). The Hox genes are a subset of selector genes that are required for generating morphological differences along the antero-posterior axis of most animals. Studies in the fruit fly, *Drosophila melanogaster*, demonstrate that altering Hox function can cause one body part to be transformed into another. Perhaps in large part because they govern the development of entire body parts, changes in how Hox genes, and selector genes in general, were used during evolution have led to modifications in animal body plans throughout the animal kingdom (2, 3).

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**Fig. 1.** The ground state ventral appendage is a leglike appendage with two segments. (A) A wild-type (WT) T2 leg has five segments from proximal to distal: coxa (co), trocanter (tr), femur (fe), tibia (ti), and tarsus, which is subdivided into tarsal subsegments 1 to 5 (t1 to t5) and a distal claw (cl) (15). Five bristle types are indicated: bracted (green arrows), unbracted (red arrows), curved, spurs (sp), and apical (ap). The inset shows a closeup of the proximal femur where both bracted and unbracted bristles are present. The inset comes from a different wild-type leg. (B) A wild-type appendage consists of four segments, from proximal to distal: antennal segments 1 to 3 (a1 to a3) and arista (ar). (C) Antp

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