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## Genetic Evidence for Two Species of Elephant in Africa

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crease in the total continental flux of Sr or a change in its isotopic composition, or both. The contribution of G-B to the global cycle

$$\left(\frac{d\alpha_{\text{Sr-SW}}}{dt}\right) = \left(\frac{J_{\text{GB}}}{N_{\text{Sr}}}\right) \cdot (\alpha_{\text{Sr-GB}} - \alpha_{\text{Sr-SW}}) \quad (2)$$

is equal to  $0.82 \times 10^{-4} \text{ My}^{-1}$  for the low estimate of  $^{87}\text{Sr}/^{86}\text{Sr}$  and  $1.86 \times 10^{-4} \text{ My}^{-1}$  for the high estimate of  $^{87}\text{Sr}/^{86}\text{Sr}$  in Table 2. This rate of change is a factor of  $\sim 2.3$  to 5.3 higher than the observed average value of  $d\alpha_{\text{Sr-SW}}/dt \sim 0.35 \times 10^{-4} \text{ My}^{-1}$  for the past 40 My.

We also note that use of a  $^{87}\text{Sr}/^{86}\text{Sr}$  value of 0.711 for global river and continental flux creates an imbalance in the Sr cycle. To rectify this situation, we need to lower the continental flux isotopic composition to about 0.71049 [similar to the value proposed in (J)]. Also, the additional global continental Sr flux from groundwater would cause a rise in  $^{87}\text{Sr}/^{86}\text{Sr}$  of 0.0095 over 40 My if left unbalanced. This is higher by a factor of 7 than the observed rise over the past 40 My.

Thus, we conclude that the groundwater data have an enormous effect on the interpretation of the seawater Sr isotope balance. Although we do not claim that the new values presented in Table 2 should be considered as final, these data urge caution about overinterpreting Sr isotope data from a few local watersheds in this area. For example, trying to use the seawater Sr isotope curve to infer the detailed tectonic uplift history of the Himalayas as well as for estimating effects on global climate change still involves considerable uncertainty. Because of the highly variable nature of  $^{87}\text{Sr}/^{86}\text{Sr}$  in the G-B river system, reliable average values are difficult to estimate.

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21. Tritium-helium ages of 39 groundwater samples were determined and are being reported elsewhere [C. B. Dowling, R. J. Poreda, A. R. Basu, in preparation.] The  $^3\text{H}$ - $^3\text{He}$  isotopic ages of samples with higher contents of tritium (4 to 7 tritium units) are consistent with their origin during the nuclear test era. Tritiated water is found to a depth of 150 m in some areas, implying highly conductive sediments. The ratio of tritiogenic  $^3\text{He}^*$  to  $^3\text{H}$  gives a subsurface travel time for groundwater containing tritium. This velocity ranges from 3 m/year in the active flow system to  $<0.5$  m/year in the low-flow zone. Overall, the groundwater age data establish an average recharge rate of  $0.6 \pm 0.2$  m/year. In the highly conductive sediments of the Bengal Basin, the monsoon water is relatively quickly recharged and flushed through the sediments over wide areas of this flood plain. The horizontal hydraulic gradient was found to be very low at the sample collection sites (Fig. 1). The recharged water and the groundwater flowing beneath the river bottom ( $>30$  m) do not discharge to the G-B rivers and their tributaries; only the groundwater from the shallow part of the aquifer ( $<20$  m) will discharge to the G-B rivers and contribute to the base flow of the rivers. Thus, the groundwater flux to the oceans is estimated here on the basis of the average groundwater travel time below 30 m.
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25. We thank the Atomic Energy Center and the Water Development Board of Bangladesh, and the Central Groundwater Board, West Bengal, India for providing field assistance in water sample collection. The International Atomic Energy Agency (Vienna, Austria) sponsored this activity. This research was partially supported by NSF grants helpful. We also thank two anonymous reviewers for their helpful comments.

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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). Large 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*.

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 ap-

preciable 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),

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*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 (*BGN* 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 (*BGN* 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

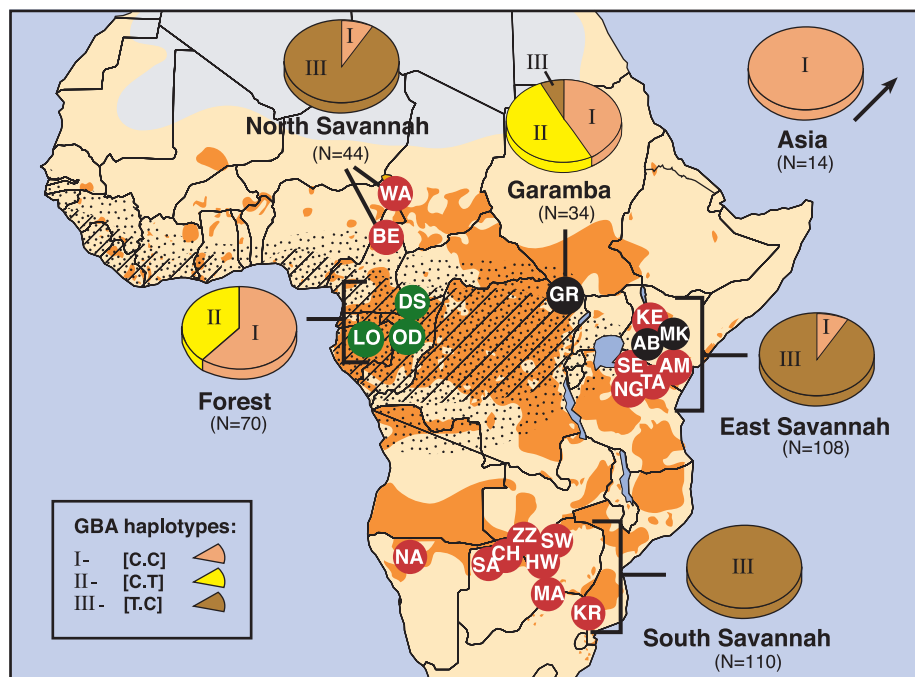
concordant deep genetic division between the forest and savannah populations of African elephants (Fig. 2). The forest elephants of Dzanga-Sangha, Lope, and Odzala grouped together, separate from 15 savannah populations, which formed a distinct phylogenetic clade or lineage. An estimated 94% of the observed 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 hierarchical analysis of molecular variance (AMOVA) (24,

25). For example, we could not genetically differentiate the forest elephants in Dzanga-Sangha from those of Lope ( $F_{ST}$   $P > 0.05$ ). Despite the extensive geographic distances separating them, the savannah populations in southern, eastern, and north-central Africa were genetically indistinguishable ( $F_{ST}$   $P > 0.05$ ). Forest elephants are genetically more diverse than savannah elephants (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 iolensis*, 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 Aberdares (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 (*BGN* 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 ( $F_{ST}$   $P < 10^{-5}$ ) but not between Garamba and forest elephants ( $P >$



**Fig. 1.** Locations of sampled African elephant populations. Circles indicate sampling locations and population abbreviations. Green circles are forest populations (the number of elephants sampled is given here in parentheses after the location): DS, Dzanga Sangha (17); LO, Lope (16); and OD, Odzala (3). Red circles are savannah populations: AM, Amboseli (6); BE, Benoue (8); CH, Chobe (5); HW, Hwange (5); KE, Central Kenya (9); KR, Kruger (10); MA, Mashatu (7); NA, Namibia (14); NG, Ngorongoro (10); SA, Savuti (6); SE, Serengeti (7); SW, Sengwa (6); TA, Tarangire (7); WA, Waza (14); and ZZ, Zambezi (7). Black circles are three populations that were not classified a priori in either category: AB, Aberdares (17); GR, Garamba (18); and MK, Mount Kenya (3). Garamba is located in the Guinea-Congolian/Sudanian transition zone of vegetation in Congo, which includes a mixture of forest and secondary grasslands (17) suitable for both African elephant groups. Savannah, forest, and morphologically intermediate elephants have been reported in Garamba (11, 33). The forests of Mount Kenya and Aberdares are currently isolated by surrounding bush (18), and both have elephants that more closely resemble the savannah morphological phenotype. However, these forests may have recently been contiguous with other forest habitat (17, 18) and retained relict forest elephants. Orange indicates current African elephant range (7); historic range is in bright yellow (10). The dotted pattern indicates the extent of tropical forest (hatched) and forest/savanna transitional vegetation zones (17). Pie charts indicate the combined population frequencies of *GBA* haplotypes: I, [C.C]; II, [C.T]; and III, [T.C] for nucleotide sites 20 and 79, respectively, in Asian, forest, Garamba, and three savannah regional populations.  $N$  = number of elephant chromosomes.

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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 ( $\pm 0.94$ ) 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 ( $n = 103$  chromosomes; including Aberdares and Mount Kenya), and six African forest haplotypes ( $n = 53$  chromosomes; including one Garamba in-

dividual). 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

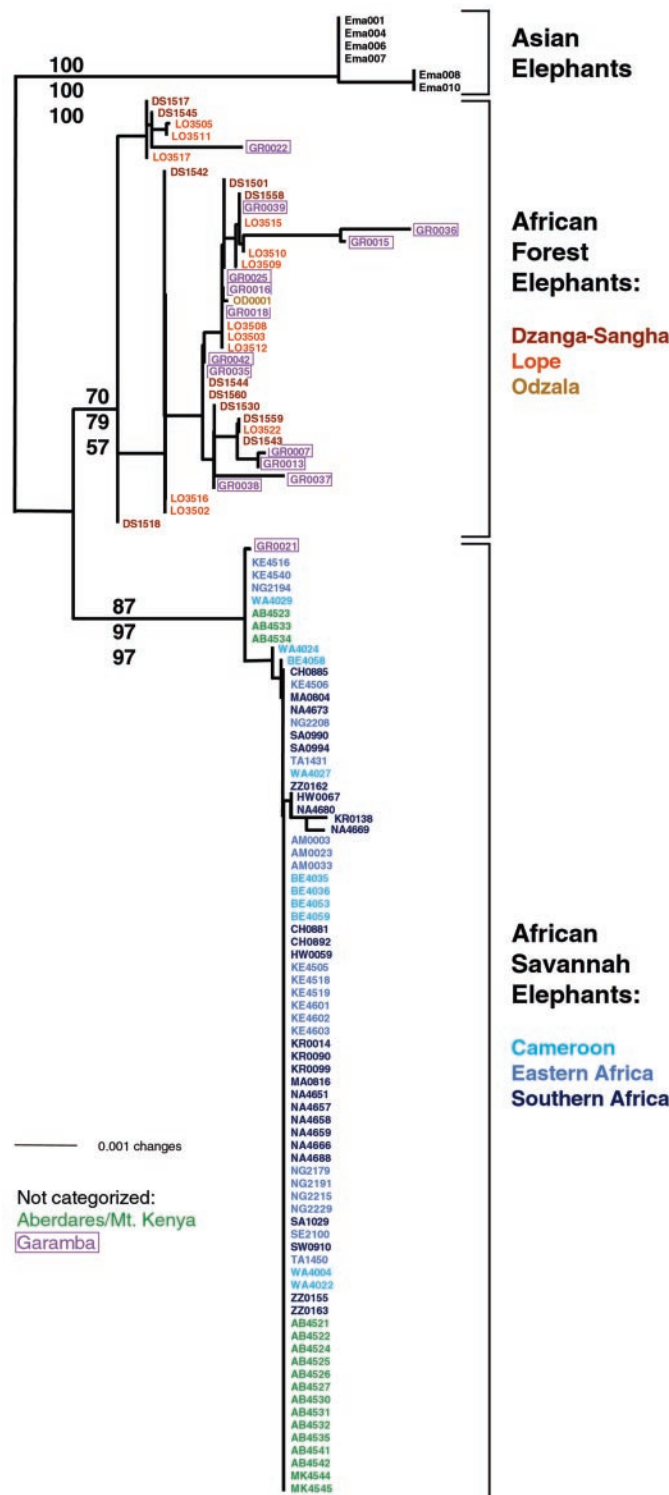


Fig. 2. Phylogenetic relationships for Asian, African forest, and African savannah elephants inferred from combined analyses (21–23) of 1732 bp (*BGN*, *CHRNA1*, *GBA*, and *VIM*); the two-letter codes for African elephant populations are given in Fig. 1. Asian elephant individuals are coded “Ema.” The minimum evolution (NJ) tree is shown. Concordant trees were obtained by MP (tree length was 248 steps; CI = 0.927, RI = 0.934) and ML ( $-\ln L. = 2774.53539$ ) analyses, which produced the same topology in defining the three groups. Bootstrap resampling support (100 iterations) is listed on branches for NJ (top), MP (middle), and ML (bottom) analyses for nodes supported by all three methods.

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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 *GBA* [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 minimized by behavioral or physiological reinforcement. In this regard, no

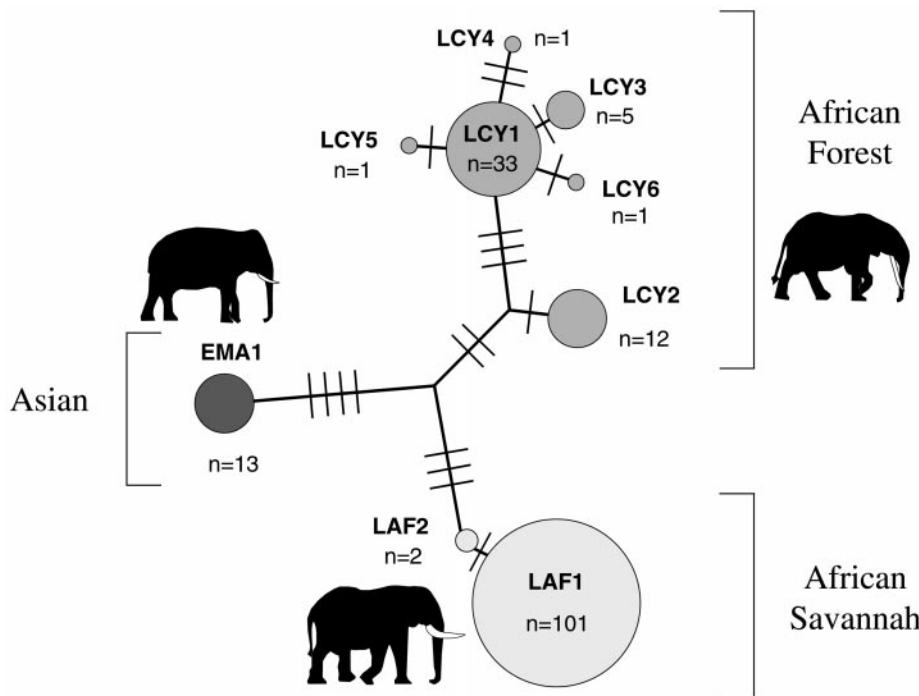
elephant from any population, including Garamba, displayed a predicted  $F_1$  hybrid genotype (that is, heterozygous at the genetically fixed sites between savannah and forest elephants), affirming the lack of gene flow or hybridization among the sampled elephants.

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  $2.63 \pm 0.94$  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-

tion of both forest and savannah elephant numbers in the past century and the ongoing destruction of their habitats, the conservation implications of recognition and species-level management of these distinct taxa are considerable (1, 10, 38).

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19. DNA was extracted with a Qiagen Qiaamp 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'-GCTCTGGG CTGGAAATCC-3'), *CHRNA1-R3* (5'-CGCCTGGGAAAGAAGAGG-3'), *VIM-F2* (5'-CGCA TCTGGAGTCCCTGG-3'), and *VIM-R2* (5'-TTGAACCCAATTGTGCAGGAA-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 377 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 AY044919 through AY045493).
20. Supplemental Web material is available on Science Online at [www.sciencemag.org/cgi/content/full/293/5534/1473/DC1](http://www.sciencemag.org/cgi/content/full/293/5534/1473/DC1). It is also available at the Laboratory of Genomic Diversity Web site at <http://lged.nci.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-



**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.

# The Ground State of the Ventral Appendage in *Drosophila*

Fernando Casares\* and Richard S. Mann†

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.

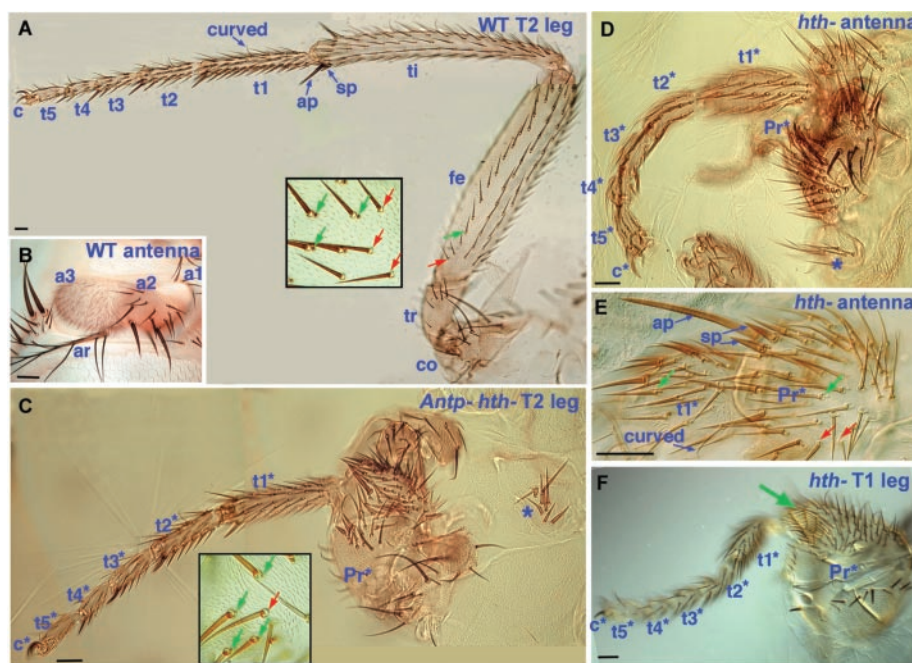
Selector genes encode transcription factors that specify the identity of segments and appendages in insects and vertebrates (1, 2). The Hox genes are a subset of selector genes that are required for generating morphological differences along the antero-posterior axis of most animals. Studies in the fruit 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).

quencies and estimated values for the shape parameter for among-site rate variation ( $\alpha = 0.119$ ) and the transition/transversion ratio (1.89). Bootstrap resampling support was based on 100 iterations.

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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), trochanter (tr), femur (fe), tibia (ti), and tarsus, which is subdivided into tarsal subsegments 1 to 5 (t1 to t5) and a distal claw (c) (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 antenna consists of four segments, from proximal to distal: antennal segments 1 to 3 (a1 to a3) and arista (ar). (C) *Antp<sup>-</sup> hth<sup>-</sup>* T2 leg. Most of this appendage is mutant ( $y^-$ ). The recovered tarsal segments (t1\* to t5\*) and single proximal segment (Pr\*) are indicated. The inset shows a region of a similar appendage with bracted and unbracted bristles. The asterisk [also in (D)] indicates a proximal plate with unbracted bristles that is typically associated with the ground state. (D) An *hth<sup>-</sup>* antenna results in an indistinguishable appendage morphology as seen in (C). Most of this appendage is mutant ( $y^-$ ). (E) A high-magnification view of part of the t1\* and Pr\* segments of an *hth<sup>-</sup> y<sup>-</sup>* antenna. The same bristle types are observed in *Antp<sup>-</sup> hth<sup>-</sup>* T2 legs. (F) An *hth<sup>-</sup>* T1 leg with proximal fusions. Transverse row bristles (arrow), which are indicative of a first leg identity, are observed.