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The evolution and phylogeography of the African elephant inferred from mitochondrial DNA sequence and nuclear microsatellite markers

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Recent genetic results support the recognition of two African elephant species: *Loxodonta africana*, the savannah elephant, and *Loxodonta cyclotis*, the forest elephant. The study, however, did not include the populations of West Africa, where the taxonomic affinities of elephants have been much debated. We examined mitochondrial cytochrome *b* control region sequences and four microsatellite loci to investigate the genetic differences between the forest and savannah elephants of West and Central Africa. We then combined our data with published control region sequences from across Africa to examine patterns at the continental level. Our analysis reveals several deeply divergent lineages that do not correspond with the currently recognized taxonomy: (i) the forest elephants of Central Africa; (ii) the forest and savannah elephants of West Africa; and (iii) the savannah elephants of eastern, southern and Central Africa. We propose that the complex phylogeographic patterns we detect in African elephants result from repeated continental-scale climatic changes over their five-to-six million year evolutionary history. Until there is consensus on the taxonomy, we suggest that the genetic and ecological distinctness of these lineages should be an important factor in conservation management planning.

Keywords: forest elephant; savannah elephant; mammal evolution; molecular scatology; speciation

1. INTRODUCTION

Populations of the African elephant (*Loxodonta africana*) once ranged from the Mediterranean to the farthest southern regions of the continent (Barnes *et al.* 1999). In 1979, the total population was estimated at 1.3 million (Spinage 1994), but poaching for ivory and competition with humans for habitat have reduced this number to between 400 000 and 500 000 (Barnes *et al.* 1999). Today, populations are only found south of the Sahara in fragments of their former habitat.

The oldest *Loxodonta* fossils were found in Uganda and dated at 5.5–6.0 Myr old (Tassy 1995). According to Maglio (1973), the earliest species was *L. adaurora*, which originated in East Africa and radiated throughout the sub-Saharan regions. During the early Pleistocene, a larger and more specialized congener, *L. atlantica*, arose and expanded into northern and southern Africa. The first fossils attributed to *L. africana* may indicate descent from *L. adaurora*, although transitional forms are unknown (Maglio 1973; for alternative hypotheses see Beden (1983) and Kalb *et al.* (1996)). The decline and disappearance of *L. adaurora* and *L. atlantica* coincided with the appearance in Africa of *Elephas recki* and *E. iolensis*, relatives of the Asian elephant. With the disappearance of *Elephas* from Africa in the late Pleistocene, *L. africana* became the only continent-wide elephant species.

The taxonomic designations of the forms of *L. africana* have been much debated (Maglio 1973; Spinage 1994; Grubb *et al.* 2000). During the colonial era, it was fashionable for sport hunters to have species named after them when they had financed collecting expeditions for museums; at least 18 subspecific designations resulted from this practice (Spinage 1994). Ansell (1971) suggested that the genus could be divided into a forest elephant group with two subspecies: (i) the living forest elephant *L. a. cyclotis*; and (ii) the extinct north African elephant *L. a. pharaohensis*; and a savannah elephant group with four subspecies: (i) the southern African bush elephant *L. a. africana*; (ii) the elephant of Tanzania, Kenya, southwestern Somalia and Uganda *L. a. knockenhaueri*; (iii) the 'pointed ear' bush elephant of the northeastern Sudanese region *L. a. oxyotis*; and (iv) a form found formerly in northern Somalia and currently in western Ethiopia, *L. a. orleansi*. Until recently, these two groups have generally been recognized as subspecies: the African savannah elephant *L. a. africana*, and the forest elephant *L. a. cyclotis*, found in the forests of western and Central Africa. As compared with the savannah form, forest elephants are significantly smaller, have longer, thinner and straighter tusks, smaller and more rounded ears, a flatter forehead region (Martin 1991) and a larger number of toenail-like structures (Sikes 1971).

Recent morphological (Grubb *et al.* 2000) and genetic studies have indicated that the forest elephant represents a different gene pool from the savannah elephant, supporting proposals that the two forms may actually be different species. Barriel *et al.* (1999) analysed cytochrome *b* sequence from extant and extinct members of the Elephantidae, including one forest elephant from Sierra Leone. The West African forest elephant was found to be highly divergent from African savannah elephants, occupying the basal position in the clade. Roca *et al.* (2001) surveyed four nuclear introns (1732 bp) and found

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multiple fixed genetic differences between forest elephants and savannah elephants. The genetic distance they estimated between the two forms, calibrated with the estimated 5 Myr divergence between African and Asian elephants, suggests that African forest and savannah elephants diverged *ca*. 2.63 Myr ago.

However, genetic divergence between forest and savannah elephants is only one part of the story of the evolution of *L. africana*. First, the taxonomic affinity of West African elephants was not examined by Roca *et al.* (2001). Elephants from this region were considered 'indeterminate' by Frade (1955), while Groves (2000) suggested that the same forms of forest and savannah elephants found elsewhere on the continent coexist and occasionally interbreed in this region. Second, in eastern and southern African savannah populations, highly divergent mtDNA haplotypes have been found to coexist (Georgiadis *et al.* 1994; Tiedemann *et al.* 1998; Nyakaana & Arctander 1999), while other haplotypes are shared by very distant populations. One explanation for this might be that there has been secondary contact between groups of elephants that diverged in allopatry. Georgiadis *et al.* (1994) rejected this explanation on the basis that there is no evidence of geographical barriers that could isolate populations for a sufficient time to produce such marked divergence. Instead, they attributed their findings to large, long-term effective population sizes and a recent range expansion. Studies of other species with similar distributions, however, have uncovered patterns of divergence between regions of Africa that indicate significant differentiation in the absence of physical barriers, possibly as the result of climatic changes and associated shifts in vegetation (Arctander *et al*. 1999; Cobb *et al*. 2000; Van Hooft *et al.* 2000; Flagstad *et al.* 2001).

Continent-wide studies of African elephants have not been carried out, in part because of the difficulties involved in collecting tissue samples from populations of elephants in the inaccessible rainforests. Recent advances in molecular genetic techniques have made it possible to use DNA extracted from faeces to study these populations. Here, we combine the results of our in-depth study of the phylogeography of western and Central African forest and savannah elephant populations with mtDNA sequences from eastern and southern African savannah elephant populations to infer the patterns and processes of the evolution of today's African elephant.

2. MATERIAL AND METHODS

(a) *Field samples and DNA extraction*

Dung samples were collected at ten locations (figure 1, sites 1–10). We collected between 23 and 50 samples at each location (table 1*a*), spreading our efforts across each area to avoid sampling the same herds repeatedly. Faeces were collected (*ca*. 20 g) from each dung pile, boiled in the collection tubes to avoid transporting pathogens and preserved using a buffer of 20% DMSO saturated with NaCl (Amos *et al.* 1992), 100 mM of Tris (pH 7.5) and 0.25 M EDTA.

Amplification products for the mtDNA fragment and microsatellite loci were provided by the Addo Elephant Project for six individuals from Addo Elephant Park, South Africa. We also obtained aliquots of DNA from the Frozen Zoo collection of the Zoological Society of San Diego for three Kenyan elephants and five Asian elephants formerly, or currently, in North American zoos.

DNA extraction procedures were performed using a modified version of the protocol and reagents of Boom *et al.* (1990). Extractions and amplifications were performed in separate UV-sterilized enclosures used only for low copy-number samples. We centrifuged 1.5 ml of the preserved dung for 15 min and discarded the supernatant. After adding lysis buffer L6 to produce a volume of 1.5 ml, the sample was vortexed and incubated overnight at 60 °C. We centrifuged to pellet the debris, transferred 750 µl of the supernatant into a new tube containing 250 µl of fresh L6 buffer and 50 µl of silica suspension, and incubated for 1 h with shaking. After centrifuging for 3 min, we discarded the supernatant, washed the silica twice with 1 ml of wash buffer and once with 1 ml of 70% ethanol. The pellet was dried and the DNA was eluted twice with 100 µl of purified water. Each group of extractions was accompanied by control extraction blanks.

(b) *Mitochondrial DNA*

(i) *Amplification and sequencing*

A 593 bp fragment of mtDNA, including the 3′ end of the cytochome *b* gene, the transfer RNAs for threonine and proline and 358 bp of the control region, was amplified using primers MDL3 and MDL5 (Fernando *et al.* 2000). Amplifications were performed in a 25 µl volume containing 2.0 µl of DNA extract, 1.5 µl of reaction buffer (Promega, Madison, WI, USA), 0.4 µm of forward primer, 0.4 µm of reverse primer, 0.5 mm of dNTP mix and 0.5 units of *Taq* DNA polymerase (Promega, Madison, WI, USA). The profile consisted of a single denaturation step at 94 °C for 3 min, followed by 40 cycles of 94 °C denaturation for 1 min, 1 min of primer annealing at 60 °C and 1.5 min of primer extension at 72 °C. Control extraction blanks were included in the first set of amplifications, and all reactions included controls to which no DNA was added.

Due to the degradation of DNA, the entire 593 bp region could not be amplified in some samples. For these, we amplified the region using two overlapping fragments. The primers AFDL1 (5′TTACACCATTATCGGCCAAATAG-3′) and AFDL2 (5′-TGACACATTGATTAAACAGTACTTGC-3′, annealing temperature 55 °C for this primer pair) amplified a 400 bp region from the 3′ end of the cytochrome *b* gene through the 5′ end of the control region, and AFDL3 (5′-CTTCTT AAACTATTCCCTGCAAGC-3′) and AFDL4 (5′GTTGA TGGTTTCTCGGAGGTAG-3′, annealing temperature 58 °C for this primer pair) amplified a 377 bp fragment including the 3′ end of tRNA proline and the 5′ end of the mitochondrial control region. Amplification products were purified using the QiaQuick PCR purification kit (Qiagen, Valencia, CA, USA) and automated sequencing of both strands was performed by the Molecular Pathology Shared Resource, UCSD Cancer Center.

When preliminary analysis of the data revealed highly divergent haplotypes within West African populations, we were concerned that we might be detecting nuclear integrations of mitochondrial sequences (Greenwood & Pääbo 1999). To test for this, we selected two individuals of each haplotype, cloned fresh PCR products into a sequencing vector (Topo TA Cloning Kit for Sequencing, Version C, Invitrogen, Carlsbad, CA, USA) and sequenced 10 clones for each individual. We predicted that if a nuclear integration of this mitochondrial DNA fragment (Lopez *et al.* 1994) was present, we would find two or more distinct sequences per individual (Greenwood & Pääbo 1999).

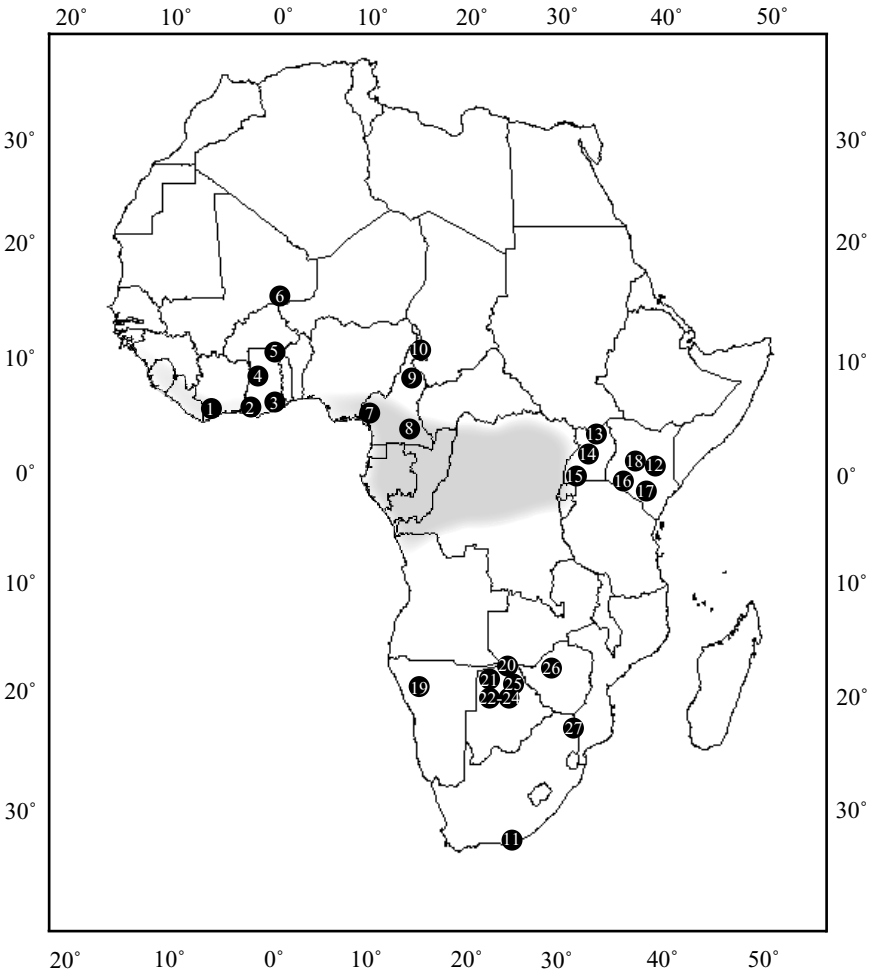

Figure 1. The sources of samples and sequences used in this study. The present-day forest zone is indicated by shading. (1) Taï National Park, Côte d'Ivoire; (2) Bia National Park, Ghana; (3) Kakum National Park, Ghana; (4) Mole National Park, Ghana; (5) Red Volta Valley, Ghana; (6) Gourma Region, Mali; (7) Banyang Mbo Forest Sanctuary, Cameroon; (8) Dja Forest Reserve, Cameroon; (9) Benoue National Park, Cameroon; (10) Waza National Park, Cameroon; (11) Addo Elephant Park, South Africa; (12) North American zoo elephants; (13) Kidepo Valley National Park, Uganda; (14) Murchison Falls National Park, Uganda; (15) Queen Elizabeth National Park, Uganda; (16) Masai Mara National Reserve, Kenya; (17) Amboseli National Park, Kenya; (18) Samburu National Reserve, Kenya; (19) Khorixis, Namibia; (20) West Caprivi, Namibia; (21) Chobe National Park, Botswana; (22) Kwando, Botswana; (23) Ngwasha, Botswana; (24) Nunga Valley, Botswana; (25) Sibuyu Forest Reserve, Botswana; (26) northern Zimbabwe; (27) Kruger National Park, South Africa.

However, for each individual we only detected the original sequence.

(ii) *Data analysis*

Sequences were aligned using CLUSTAL W Multiple Sequence Alignment Program v. 1.7 (Thompson *et al.* 1994). The average number of pairwise differences (π) within and between the sampled populations and the nucleotide diversity were estimated in ARLEQUIN 2.000 (Schneider *et al.* 2000).

To determine which method of phylogenetic inference was appropriate for this dataset, we used a hierarchical likelihood-ratio test in MODELTEST 3.06 (Posada & Crandall 1998). This program compares tree scores for 64 nested inference models using the Akaike Information Criterion (AIC, Akaike 1974), as well as generating an estimate of proportion of invariable sites (I) and the gamma distribution shape parameter. Phylogenetic analyses were performed in PAUP* 4.0b10Altivec (Swofford 1998), using the neighbour-joining (model HKY (Hasegawa *et al.* 1985), as suggested by MODELTEST), maximum-likelihood and parsimony criteria with the observed transition : transversion ratio. The phylogenetic signal was assessed by evaluating the skew of the tree-length distribution (g_1) of 10 000 random trees in PAUP* (Hillis & Huelsenbeck 1992). Support for the resolved clades was assessed with 1000 bootstrap replicates.

(c) *Microsatellite DNA*

(i) *Amplification and scoring of alleles*

Using four microsatellite loci that could be amplified in each of our sampled populations, we genotyped ten individuals (defined as a sample with a unique four-locus genotype) per population. The loci selected were *LA5*, *LA6* (Eggert *et al.* 2000), *Laf MS01* and *Laf MS02* (Nyakaana & Arctander 1998). Amplifications were carried out in two steps. The first reaction was conducted in a 5 µl volume containing 1.5 µl of DNA extract, 0.5 µl of reaction buffer (Promega, Madison, WI, USA), 0.4 µm of forward primer, 0.4 µm of reverse primer, 0.5 mm of dNTP mix and 0.5 units of *Taq* DNA polymerase (Promega, Madison, WI, USA). The profile consisted of a denaturation step at 94 °C for 3 min, followed by 40 cycles of 94 °C denatur-

Table 1. Samples included in this study.
(FR, forest reserve; NP, national park.)

site		habitat type	no. of samples	haplotypes	sites with which haplotypes are shared
(*a*) Samples sequenced for the 593 bp fragment of mtDNA. Unique haplotypes for this fragment are italicized. GenBank accession numbers: AF27638–27683					
1	Tai NP	forest	30	*Tai6, Tai17, Tai19, Tai29*	none
2	Bia NP	forest	37	*Bia3, Bia48, Bia64, Bia69*	none
3	Kakum NP	forest	50	*K68, K102, K381*	none
4	Mole NP	savannah	50	Mole3, Mole9, Mole13, *Mole33*	Gourma region, Red Volta Valley
5	Red Volta Valley	savannah	36	RVV15, *RVV22*	Mole NP
6	Gourma region	savannah/desert	23	Mali2, Mali7, Mali14, *Mali28*	Mole NP, Benoue NP, Waza NP
7	Banyang Mbo FS	forest	36	*Bmbo1, Bmbo6, Bmbo16, Bmbo37, Bmbo43*	none
8	Dja FR	forest	36	*Dja34, Dja36, Dja39*	none
9	Benoue NP	savannah	37	*B1*, B7, B8, B36	Waza NP, Gourma region
10	Waza NP	savannah	50	Waza10, Waza15, Waza27	Benoue NP, Gourma region
11	Addo Elephant Park	savannah	6	*Addo1, Addo5*	none
12	N. American zoos	savannah	3	*Af8, Af9, Af10*	none
(*b*) Mitochondrial control region haplotypes (388 bp from GenBank representing eastern and southern African populations) added to the above to form the combined dataset					
13	Kidepo Valley NP			*KV1, KV2, KV7, KV8, KV17, KV28*	none
14	Murchison Falls NP			*MF1, MF5*	none
15	Queen Elizabeth NP			QE1, QE4, *QE13*	N. American zoos, Addo Elephant Park
16	Masai Mara NP			*MM4, MM19, MM20*	none
17	Amboseli NP			*AM1, AM2, AM10, AM12*	none
18	Samburu NR			*SA8*	none
19	Khorixis			*KH2*	none
20	W. Caprivi			*WC2, WC4, WC6, WC13*	none
21	Chobe NP			*Bot2, Bot4, Bot6*	none
22	Kwando			*Bot 9*	none
23	Ngwasha			*Bot15*	none
24	Nunga Valley			*Bot 16*	none
25	Sibuyu FR			*Bot 21*	none
26	Northern Zimbabwe			*Zbe1, Zbe2, Zbe3, Zbe4 Zbe5, Zbe6,*	none
27	Kruger NP			*KG1, KG2*	none

ation for 1 min, 1 min of primer annealing at 1 °C below the optimal annealing temperature and 1.5 min of primer extension at 72 °C. Immediately following the first reaction, samples were reamplified by adding 5 µl of a labelled PCR mix containing the reagents listed above with half of the forward primer labelled with 32P-γdATP. The profile was the same as above except that the annealing temperature was 1 °C above the optimal temperature for the locus. Control extraction blanks were included in the first set of amplifications for each locus and all reactions included controls to which no DNA was added.

Alleles were separated in a 6% polyacrylamide gel, visualized by autoradiography and scored by comparison with an M13 length standard and two control samples of savannah elephants. For each locus, samples that were scored as heterozygotes were confirmed in a second reaction, and samples that were scored as homozygotes were confirmed in at least two additional reactions. If there was any indication in any of the three results for putative homozygotes that there might be a second allele, we followed the multiple tubes approach of Taberlet *et al.* (1996) and analysed an additional four positive amplifications before scoring the genotype.

(ii) *Microsatellite data analysis*

We tested for departures from Hardy–Weinburg equilibrium (HWE) using a modified version of the Markov chain method of Guo & Thompson (1992), as implemented in GENEPOP v. 3.2a (Raymond & Rousset 1995). Testing for genotypic disequilibrium between each pair of loci in each population was done using a Fisher exact test in GENEPOP, and a global test was performed for each pair of loci across populations. All results were evaluated for significance after application of the sequential Bonferroni correction (Rice 1989).

It has been shown that distance measures that use the product of the allele frequencies shared between populations reconstruct the phylogeny of closely related organisms better than methods that use the Stepwise Mutation Model (Goldstein & Pollock 1997). Thus, relationships between populations for microsatellite data were inferred using the computer program POPULATIONS 1.2.24 (Langella 2000) to generate the shared allele distances (D_{AS}; Jin & Chakraborty 1993), the Cavalli-Sforza chord distances (D_C; Cavalli-Sforza & Edwards 1967), and the support for the resolved clades using 100 bootstrap replicates. The distance information was imported into TREEVIEW (Page 1996).

(d) *Additional mitochondrial DNA sequences*

To investigate the genetic relationships between populations at the continental level, we combined our sequences with mtDNA control region sequences from GenBank. These sequences (table 1*b*; figure 1), which were generated by Silvester Nyakaana, represent populations from eastern and southern Africa and include those he used in his study of Ugandan populations (Nyakaana & Arctander 1999). The combined dataset includes 388 bp (30 bp of tRNA proline and 358 bp of control region) and was aligned and analysed separately, using the methods shown above for mtDNA. In addition to bootstrap analysis, support for the resolved clades was assessed by examining the Bayesian posterior probabilities using MRBAYES (Huelsenbeck & Ronquist 2001), using the criteria recommended for non-coding sequences.

3. RESULTS

(a) *Field samples*

(i) *Sequence analysis*

Sequencing revealed 73 variable sites with 68 transitions (Ts) (including two insertions/deletions at these sites) and five transversions (Tv) (see electronic Appendix A available on The Royal Society's Publications Web site). Of these, 16 were in the cytochrome *b* gene (13 Ts, 3 Tv), five transitions were found in the tRNA threonine sequence, four transitions were found in the tRNA proline sequence and the remaining 48 variable sites (46 Ts including one insertion/deletion, two Tv) were in the control region. Forty-one haplotypes were detected, 28 of which were found in only one location, while 13 were shared between various savannah locations. There were no significant differences in haplotype number between forest and savannah locations ($\bar{X}_{savannah} = 3.3 \pm 0.8$, $\bar{X}_{forest} = 3.6 \pm 0.9$, Mann–Whitney $U = 14.5$, $p = 0.61$).

Between African and Asian elephants, HKY (Hasegawa *et al.* 1985) divergence corrected for within-species differences was 6.22 bp in 101 bp of cytochrome *b* sequence. Assuming that the genera diverged 5 Myr ago, as indicated by fossil evidence, the corrected rate of divergence at cytochrome *b* is 1.2% per million years. This value is low in comparison with some other mammals, but is in agreement with Garcia-Rodriguez *et al.* (1998) who estimated 2% Myr^{-1} for the dugong, and Fleischer *et al.* (2001) who estimated cytochrome *b* divergence at 1.3% Myr^{-1} for Asian elephants. The mean pairwise difference between African elephant haplotypes was 15.6 ± 7.1 bp, and the total nucleotide diversity was 0.026.

(ii) *Phylogenetic analysis of mitochondrial DNA*

An analysis of 10 000 randomly generated trees indicated a significant phylogenetic signal in the data ($g_1 = -0.669$, $p < 0.01$). Figure 2 depicts the relationships between the field samples and the out-group. Groupings resolved using the neighbour-joining, maximum-parsimony and maximum-likelihood criteria were in complete agreement. There are two highly divergent clades of West African elephants, each of which receives relatively strong support in the bootstrap analysis. Within each clade there are haplotypes from both forest and savannah habitats. Also resolved are two highly divergent clades of savannah elephants, one whose range includes East and Central Africa and another with a West, Central and South African distribution. Bootstrap support for each of these clades is 100%. The fifth clade, with weak bootstrap support, includes all forest elephant haplotypes from Central Africa, as well as a single haplotype (Benoue 7/Waza 15) shared between two Central African savannah sites. The lack of bootstrap support for the deeper branches of the tree indicates that the early branching patterns are unclear and suggests that some groups may have diverged almost simultaneously.

(iii) *Analysis of microsatellite markers*

Allele frequencies at all loci are shown in table 2. Genotypic disequilibrium was not detected between loci in any population. Significant departures from HWE were detected at locus *LA6* in the Waza NP population and at locus *Laf MS01* in Tai NP. Since both of these popu-

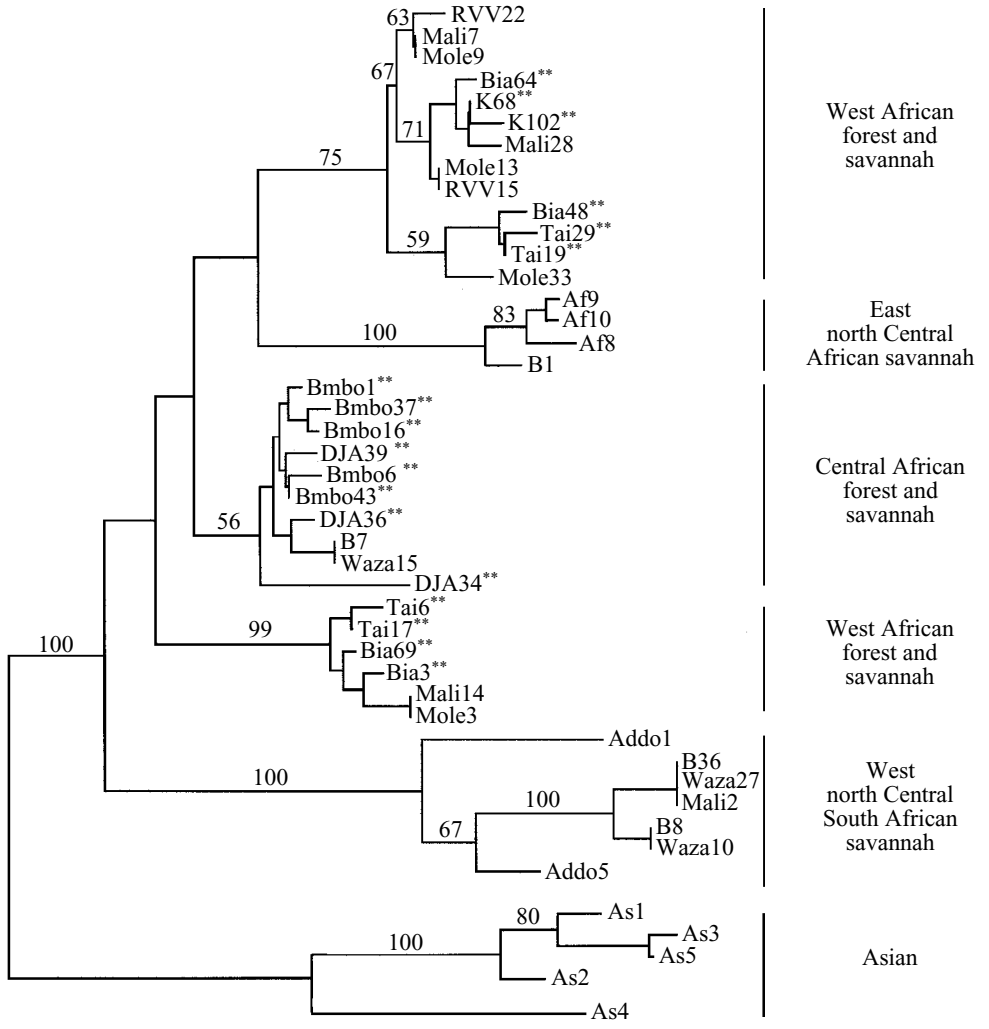

Figure 2. Analyses of field samples from sites 1–12; haplotypes from forest populations are indicated by two asterisks. Neighbour-joining tree of 593 bp of mtDNA sequence (101 bp cyt *b*, tRNA Thr, tRNA Pro, 358 bp control region) with bootstrap support shown above the branches.

lations contained individuals with highly divergent mitochondrial haplotypes, we reanalysed the data after breaking down the populations by mitochondrial haplotype. This analysis detected no significant departures from HWE. While it appears that forest populations may have higher allelic diversity than savannah populations, the difference is not statistically significant ($\bar{X}_{\text{savannah}} = 13.0 \pm 4.9$, $\bar{X}_{\text{forest}} = 18.4 \pm 4.2$, Mann–Whitney $U = 20.5$, $0.20 < p < 0.10$, test conducted without including North American zoos (NA zoo) and Addo which had unequal sample sizes).

Figure 3*a*,*b* shows the results of the phylogenetic analysis of the four microsatellite markers at the population level. Since allele sizes may not be expected to be conserved over the 5 Myr divergence time between African and Asian elephants, trees are unrooted. The branching patterns reveal two groups of savannah elephants, one with a north Central, southern and western African distribution (Addo National Park (NP), Benoue NP, Mali, Waza NP) and one with an East and West African distribution (NA zoo, Mole NP, Red Volta Valley (RVV)). There are also two groupings of forest elephants, one that includes Central African populations (Banyang Mbo Forest Sanctuary, Dja Forest Reserve) and one that includes west African populations (Bia NP, Tai NP). The forest elephants of Kakum NP do not clearly group with either of these in the D_{AS} analysis, and group with the Central African forest elephants in the D_{C} analysis.

(b) *Control region analysis on the combined dataset*

In the combined control region dataset, there were 65 variable sites, with 60 transitions, three transversions and two insertions/deletions. Between African and Asian elephants the HKY distance, corrected for within-species differences, was 13.98 in 388 bp. Calibrating this with the estimated 5 Myr split between the two genera, the rate of divergence at the control region is 2.8% Myr^{-1}. This value is higher than that of Fernando *et al.* (2000) who estimated control region divergence at 1% Myr^{-1} for Asian elephants. Ten substitutions (eight Ts, two Tv) differed only between African and Asian elephants. There were 73 haplotypes in the 27 locations, 53 of which were unique. Eight were shared between locations of savannah elephants, and two were also found in forest elephant populations in West Africa. The mean pairwise difference

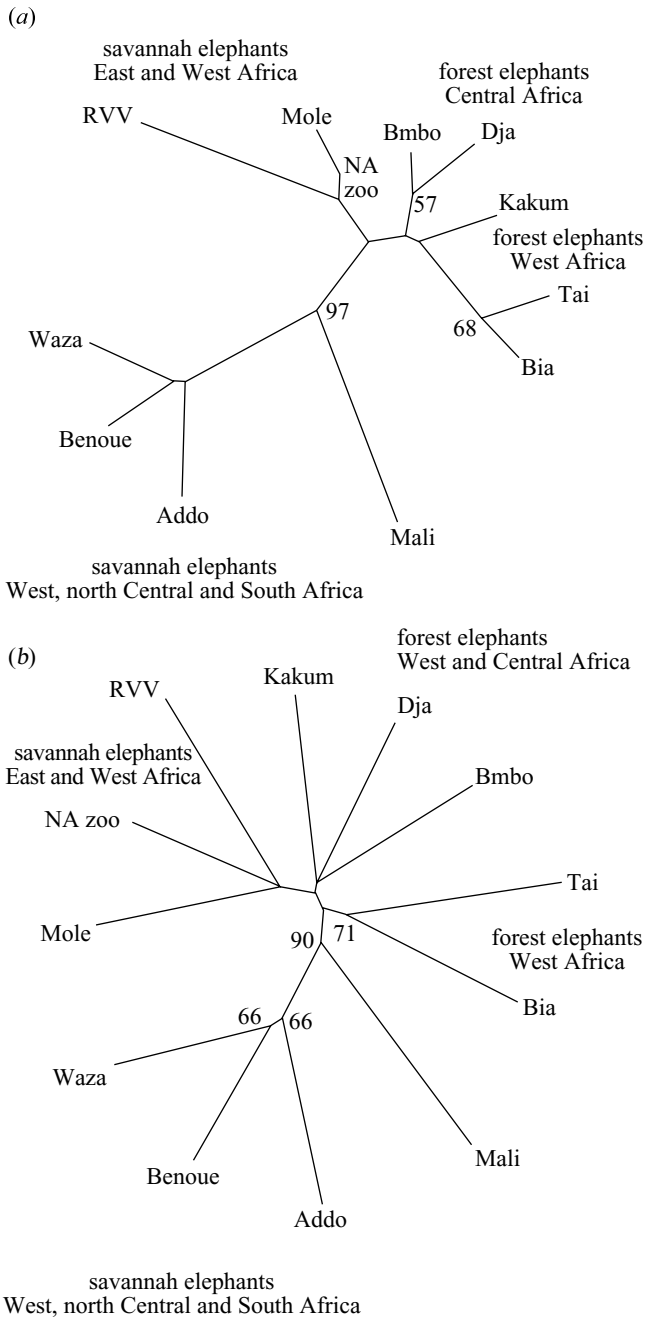

Figure 3. Analysis of four microsatellite loci for field samples from sites 1–12. (*a*) Unrooted neighbour-joining tree of the shared allele distances, D_{AS}, with bootstrap support shown at the nodes. (*b*) Unrooted neighbour-joining tree of the chord distances, D_C, with bootstrap support shown at the nodes. NA, North American; RVV, Red Volta Valley.

between haplotypes was 13.1 ± 5.9 bp, and the total nucleotide diversity was 0.034.

Evaluation of 10 000 random trees indicated significant phylogenetic signal in this dataset ($g_1 = -0.239$, $p < 0.01$). ModelTest 3.06 suggested that the appropriate model for analysing the data was HKY (Hasegawa *et al.* 1985) including invariant sites ($I = 0.6906$) and Γ ($g = 0.7025$). In the phylogenetic analysis, the out-group is split, presumably representing the two deeply diverged clades of Asian elephants reported by Fernando *et al.* (2000) and Fleischer *et al.* (2001). The monophyly of *L. africana* was strongly supported (figure 4), and groupings resolved using the neighbour-joining, maximum-parsimony (135 steps, CI = 0.4963) and maximum-likelihood (−LnL = 1271.839 65) analyses were in complete agreement. As in the previous analysis, two clades (1 and 3) comprise savannah haplotypes that are deeply diverged but have a broad geographical overlap. Clade 2 includes a mixture of West African haplotypes with no partitioning between forest and savannah habitats, and clade 5 contains all other West African haplotypes except Mali2. Clade 4 is made up of Central African forest elephants and, as in the analysis of the field samples, includes a single haplotype shared between two north central savannah sites (Benoue 7 and Waza 15). This haplotype was found in populations in northern Cameroon, where elephants with intermediate phenotypes have been reported (M. Tchamba, personal communication). Although its inclusion in a clade of Central African forest elephants might be interpreted as evidence of present-day hybridization between the two forms, it could also be explained by the isolation of forest elephants at the periphery of their range during historical forest retractions.

In the minimum spanning network (figure 5), sequences of Asian elephants are most similar to the forest elephants of central Africa. From the Central African forest, elephants appear to have moved into West Africa once, and into the savannahs of southern and eastern Africa twice. The number of fixed differences between the haplotypes in the Central African forest and those in West Africa (clade 5) is five, which is equal to the number of fixed differences between the Central African forest elephants and the savannah elephants of clade 1, supporting the notion that these groups may have diverged almost simultaneously. From the savannah elephants of clade 1, a second colonization of West Africa is seen. Each of the savannah groupings shows a star-like pattern, indicating possible radiation of populations from these regions.

4. DISCUSSION

(a) *Phylogeographic patterns*

Unlike Roca *et al.* (2001), who detected multi-locus nuclear DNA differences between African forest and savannah elephants, we do not find reciprocal monophyly between these two groups at mtDNA, and observe evidence of deeply diverged lineages that do not correspond with either two species or subspecies. Our analysis of four nuclear microsatellites reveals two groups of forest elephants, one in West Africa and one in Central Africa. Although the affinity of the easternmost West African population (Kakum NP) differs between the two analysis methods, this population clearly contains microsatellite genotypes more similar to forest than to savannah populations. Analysis of the microsatellite loci also indicates two genetically distinct groups of savannah elephants with little or no geographical structuring.

This pattern of deep divergence between broadly sympatric clades corresponds with the phylogeographic category II of Avise (2000); when coupled with fixed differences between nuclear loci (Roca *et al.* 2001), it is rare. It is usually the more highly variable mtDNA that detects deep divergences and reciprocal monophyly, while the more slowly evolving nuclear loci do not. Most explanations for such a pattern invoke selection on some of the genetic markers (Avise 1994), a factor that is unlikely to

Table 2. Allele frequencies at microsatellite loci for field samples.
(Ten individuals per population were scored, except in North American zoos (NA zoo, $n = 3$) and Addo Elephant Park ($n = 6$).)

locus/allele	Bmbo	Dja	Tai	Bia	Kakum	Mole	RVV	Mali	Benoue	Waza	NA zoo	Addo
LA5												
187	—	—	—	0.10	—	—	—	—	—	0.09	—	—
191	—	—	—	—	—	—	—	1.00	—	0.46	—	—
193	—	—	—	—	—	—	—	—	—	0.05	—	—
195	0.15	—	—	—	—	—	—	—	—	—	—	—
197	—	—	0.40	0.30	—	—	—	—	—	—	—	—
199	—	—	0.60	—	—	—	—	—	—	—	—	—
203	0.25	—	—	—	—	—	—	—	—	—	—	—
215	—	—	—	—	—	—	—	—	—	—	0.17	—
217	—	0.05	—	—	—	—	—	—	—	0.05	0.33	—
219	0.30	—	—	—	0.60	—	0.50	—	0.65	0.23	—	1.00
221	0.10	0.17	—	0.60	0.40	0.50	0.40	—	0.15	0.05	0.33	—
223	0.20	0.67	—	—	—	0.17	0.10	—	—	—	0.17	—
225	—	0.11	—	—	—	—	—	—	—	0.05	—	—
227	—	—	—	—	—	0.33	—	—	0.20	—	—	—
229	—	—	—	—	—	—	—	—	—	0.05	—	—
LA6	—	—	—	—	—	—	—	—	—	—	—	—
156	—	0.25	0.10	0.28	—	0.20	—	—	—	0.18	—	—
158	0.30	0.15	0.05	0.06	0.15	0.65	0.80	—	—	0.09	0.83	—
160	0.20	0.05	—	0.17	—	—	—	—	—	—	—	—
162	—	—	0.05	—	—	0.15	0.20	—	—	—	—	—
164	—	—	—	—	—	—	—	—	0.50	0.46	0.17	1.00
166	—	0.30	—	—	—	—	—	—	—	—	—	—
168	—	—	—	—	—	—	—	—	—	—	—	—
170	0.25	—	—	—	—	—	—	—	—	—	—	—
172	—	0.05	—	—	0.15	—	—	1.00	0.50	0.27	—	—
178	—	—	—	0.11	—	—	—	—	—	—	—	—
192	0.25	0.15	0.80	0.39	0.70	—	—	—	—	—	—	—
LafMS01	—	—	—	—	—	—	—	—	—	—	—	—
172	—	—	—	—	—	—	—	—	—	—	—	0.25
178	—	—	0.05	—	—	—	—	—	—	—	—	—
180	0.05	—	—	—	—	—	—	—	—	—	—	—
182	—	—	0.10	—	—	—	—	—	0.50	0.96	—	0.67
184	0.10	0.10	0.05	0.28	—	—	0.50	0.20	—	—	—	—
186	0.80	0.90	0.40	0.22	0.75	0.50	0.10	0.60	—	—	0.83	—
188	0.05	—	0.35	0.28	—	0.20	—	0.20	—	—	—	—
190	—	—	—	0.06	—	0.30	0.40	—	—	—	0.17	—
192	—	—	—	0.17	0.25	—	—	—	—	—	—	—
194	—	—	—	—	—	—	—	—	—	0.05	—	0.08
196	—	—	0.05	—	—	—	—	—	0.10	—	—	—
198	—	—	—	—	—	—	—	—	0.40	—	—	—
LafMS02	—	—	—	—	—	—	—	—	—	—	—	—
130	0.05	0.05	0.07	—	—	—	—	—	—	—	—	—
132	—	—	—	0.14	—	—	—	—	—	—	—	—
134	—	0.05	0.29	—	—	—	—	—	—	—	—	—
136	0.05	0.05	—	—	—	—	—	—	—	—	0.17	—
138	—	0.05	—	0.07	0.05	—	0.50	—	—	—	—	—
140	0.20	0.30	0.29	—	0.50	0.25	—	—	0.10	—	0.50	0.25
142	—	0.15	—	—	0.35	—	—	—	0.05	0.05	—	—
144	—	—	0.07	0.07	—	—	0.50	—	—	—	—	0.25
146	0.20	—	—	—	—	0.10	—	—	0.10	0.18	—	—
148	0.15	—	—	0.07	—	0.25	—	—	—	0.05	—	0.33
150	0.05	0.10	0.07	0.29	—	0.15	—	0.70	0.70	0.50	—	0.17
152	0.15	0.20	—	0.14	0.10	—	—	—	0.05	0.18	0.17	—
154	—	—	0.14	—	—	0.10	—	—	—	—	0.17	—
156	—	0.05	0.07	0.21	—	—	—	—	—	—	—	—
158	0.15	—	—	—	—	—	—	—	—	—	—	—
160	—	—	—	—	—	0.15	—	—	—	—	—	—
162	—	—	—	—	—	—	—	0.30	—	0.05	—	—

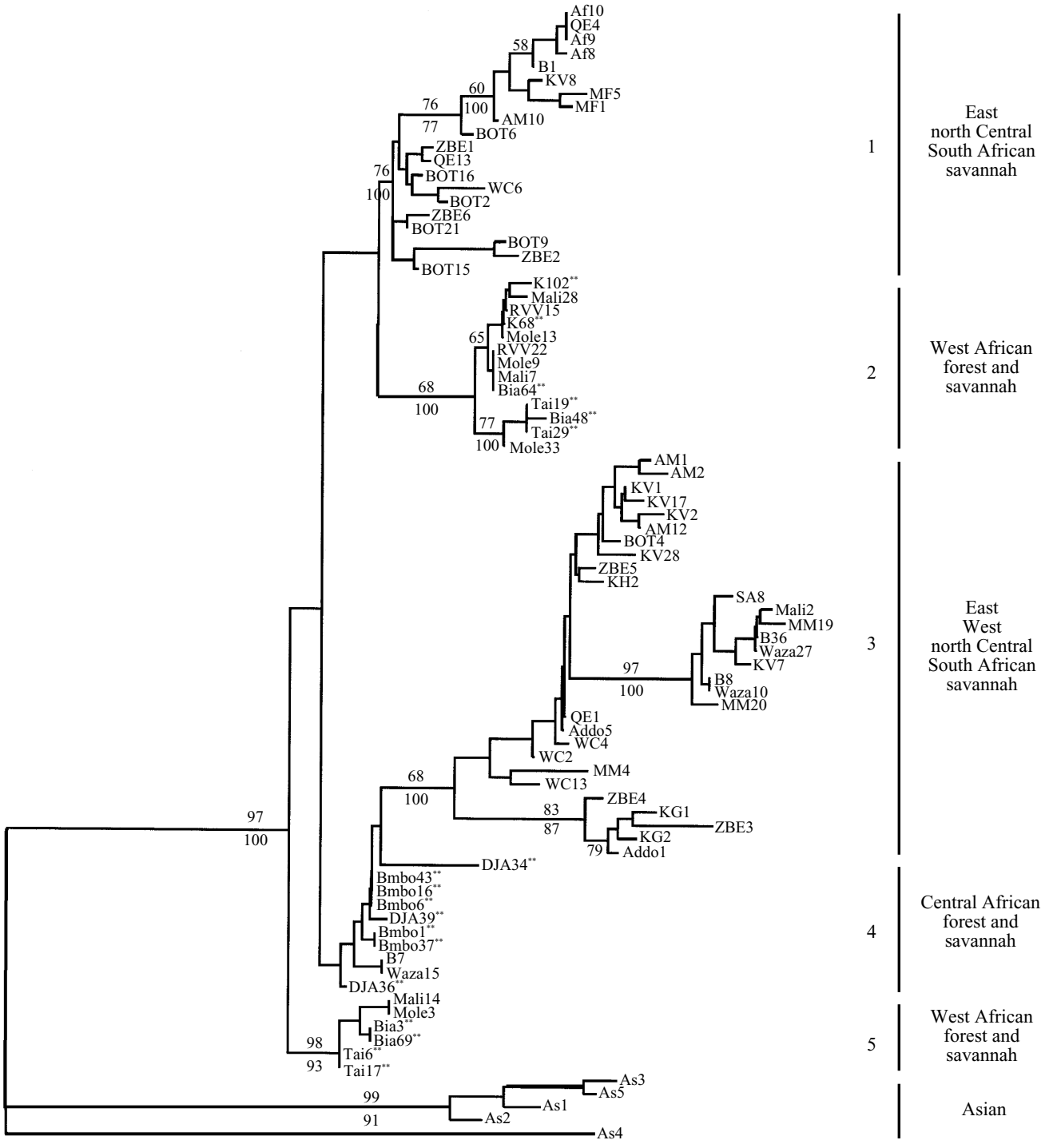

Figure 4. Analysis of mtDNA control region sequences for the combined dataset, haplotypes from forest populations are indicated by two asterisks. Neighbour-joining tree with bootstrap support shown above and Bayesian posterior probabilities shown below the nodes.

apply to the mitochondrial control region, the nuclear microsatellites or nuclear introns used by Roca *et al.* (2001).

(b) *Patterns in other African taxa*

Other African mammals have been found to have highly divergent mtDNA haplotypes within the same population. Gagneux *et al.* (1999) found highly divergent mtDNA haplotypes in populations of the western chimpanzee (*Pan verus*), the bonobo (*P. paniscus*) and the western lowland gorilla (*Gorilla g. gorilla*). In kob (*Kobus kob*), Birungi & Arctander (2000) found two reciprocally monophyletic groups of mtDNA haplotypes that differ by 9.8% within East African populations. West African kob were nested within one of the clades. In East African black-backed jackals, Wayne *et al.* (1990) found haplotypes that differed by 8.0% in the same population.

In taxa where continental-level patterns can be surveyed, results have indicated that geographical distance may not predict which populations are more closely related. Cobb *et al.* (2000) found lower F_{ST}-values for eight microsatellite loci between *Drosophila teissieri* popu-

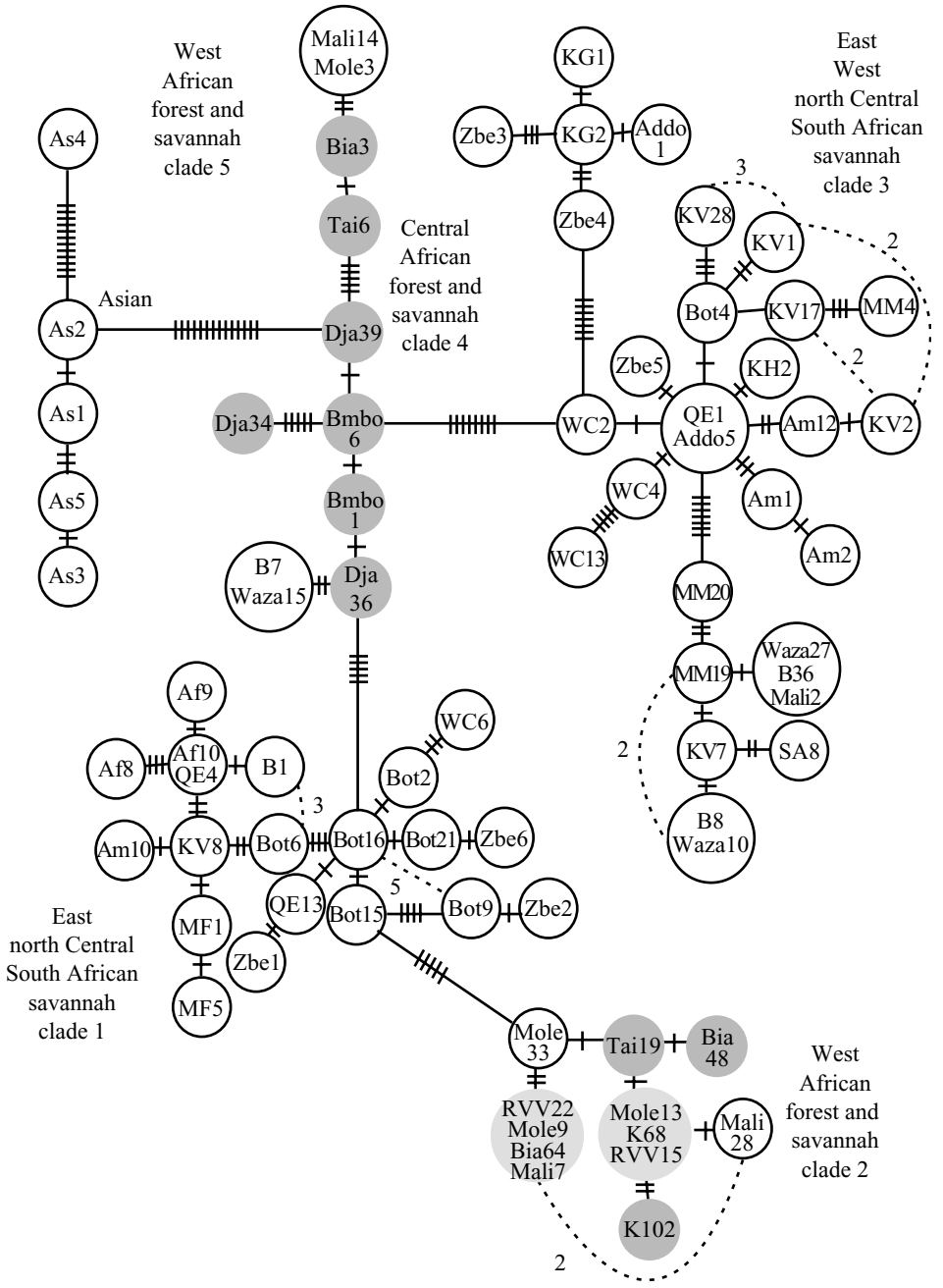

Figure 5. Minimum spanning network for control region sequences with the number of differences between taxa shown. Alternate links are indicated with dashed lines and numerals for the number of differences. Haplotypes from forest populations are shown in dark grey and those found in both forest and savannah populations are in light grey.

lations in East and West Africa than between East and Central Africa. Differentiation at microsatellite loci between eastern and southern African populations of African buffalo (*Syncerus caffer*) was found to be much lower than differentiation between eastern and Central African populations, supporting the subspecific designation of the central African animals. In hartebeest (*Alcelaphus buselaphus*; Arctander *et al.* (1999)), West and Central African populations group together in a single clade, while eastern and southern populations contain haplotypes from two reciprocally monophyletic clades. Girman *et al.* (1993) found that eastern and southern populations of African wild dogs were reciprocally monophyletic at mtDNA, but differed by only 1% between regions. Comparing morphology, they found that Central African wild dogs were either intermediate to those in eastern and southern Africa in principal components analysis (where body size was on the first axis) or fell completely outside of a group of eastern and southern animals that showed overlap in a canonical discriminatory analysis of cranial and dental characters. These studies support the regional pattern of refugia suggested by Arctander *et al.* (1999) for bovids, in which animals survived climatic fluctuations in separate areas of eastern, western and southern Africa.

Our results agree with earlier reports of highly divergent mitochondrial haplotypes within populations of eastern and southern African savannah elephants (Georgiadis *et al.* 1994; Nyakaana & Arctander 1999). Although this could have arisen through high gene flow among popu-

lations with large, long-term effective population sizes, as suggested by Georgiadis *et al.* (1994), it could also result from the admixture of lineages that evolved in allopatry. Our finding of deeply divergent haplotypes in both the forest and savannah elephant populations of West Africa, where large, long-term effective population sizes are less likely to have existed, has led us to consider allopatry in conjunction with episodes of climate change as a third factor in understanding the mtDNA and microsatellite patterns.

(c) *Pliocene and Pleistocene climate in the African tropics*

Deep-sea sedimentary sequences indicate that between 5 and 3.5 Myr ago, equatorial Africa was warmer and more humid than today (Morley 2000). Wet tropical climates and vegetation similar to modern rainforest was found as far as 10° N in Cameroon and 12–14° N in West Africa (Maley 1996), in areas that are now dry savannah. Habitat in northeastern Africa was a mosaic of savannah and forest, while in the south, palaeontological evidence indicates a shrubland–grassland–forest mosaic (Partridge *et al.* 1995).

Between 3.5 and 3.4 Myr ago, conditions began to change (Morley 2000). Deep-sea cores extracted off West Africa contain evidence of a marked increase in windborne dust, indicating that the formation of the Sahara desert had begun (Maley 1980). The rainforest began to contract as climate patterns similar to those of today became established (Maley 1996). Pollen data from the Niger delta show that savannahs existed north of the forest area around the Gulf of Guinea. These observations, combined with evidence of increased savannah vegetation in eastern Africa (Cerling 1992), indicate the extension of savannahs and open environments in tropical Africa and a contraction of the humid forests.

Deep-sea cores taken from both western and eastern Africa reveal a major shift of climatic variability *ca*. 2.8 Myr ago (deMenocal & Bloemendal 1995). For the first time, high-latitude ice sheets became large enough to affect the sub-tropical African climate (deMenocal & Rind 1993), resulting in episodes of coolness and dryness associated with high-latitude hypothermal phases. The major cooling event that began 2.8 Myr ago was has been implicated as an important factor in the turnover of bovid lineages (Vrba 1995), small mammals (Wesselman 1995) and possibly hominids (Kimbel 1995).

(d) *Origin and diversification of the African elephant*

Our results indicate that the earliest members of *L. africana* may have inhabited the forests of Central Africa. This may explain the paucity of fossil evidence of this species until the Pleistocene, as remains are poorly preserved in this habitat (Hare 1980). The warmer and wetter conditions prior to 3.5 Myr ago would have provided a suitable habitat for early populations to colonize areas of forest and forest–savannah mosaic across the continent south of 10° N, limited primarily by competition with *Elephas* species and with *L. adaurora*. In the northern part of the range, elephants inhabited forests, while populations in the southern part of the range expanded into the shrubland–grassland–forest mosaic.

As conditions began to change, the habitat in the southern part of the range became progressively cooler, dryer and more open. Elephants that adapted to these changes may have become the first savannah-dwelling members of *L. africana*. Isolation was reinforced by the cooling event 2.8 Myr ago, resulting in two groups of elephants: clades 3 and 4 in figure 4. Sequence divergence between these two clades averages 3.7%; at 1.2% Myr^{-1}, their coalescence time is 3.1 Myr ago. After the cooling event at 2.8 Myr, repeated episodes of cooling and drying followed by warmer and more humid conditions caused the forests to contract and expand, disrupting the geographical ranges of numerous species. During at least one of these periods, elephants from the Central African forests may have colonized the shrubland–grassland–forest mosaic south and east of the forest zone. Populations that moved to the east colonized areas of the former habitat of *Elephas* and *L. adaurora*, which disappeared from Africa by the mid-Pleistocene. As they expanded to the south, populations would have admixed with the earlier radiation of savannah elephants, and both lineages expanded into the areas of southeastern and eastern Africa.

The minimum spanning network indicates that the most recent events may have involved expansion of populations across areas north of the Central African forest zone and into West Africa. Haplotypes that are most similar to the first savannah radiation are present in the Central African savannahs, while the most common ones in West Africa are more similar to savannah elephants from the second radiation. This suggests that these events happened at different times, that one or the other lineage has been lost through lineage sorting, or that in the northern regions, the two radiations of savannah elephants did not recognize each other as conspecifics, while in the south and east they admixed freely. After the second invasion of West Africa, the elephants in that region became isolated from populations elsewhere on the continent and have since diverged in allopatry. Clades 1 and 2 each differ from clade 4 by an average of 2.8%, making their estimated time of divergence 2.3–2.4 Myr ago.

(e) *Taxonomy of the African elephant*

Our phylogenetic analysis of *L. africana* reveals several deeply diverged lineages that do not correspond well with current taxonomy. The patterns detected using both mtDNA and four microsatellite loci are more complex and defy attempts to divide the species into reciprocally monophyletic groups. Nevertheless, three broad groups may be recognized for their genetic, geographical and ecological differences.

The first of these is made up of the forest elephants of Central Africa. The group that includes these haplotypes is at the base of the African elephant radiation. The presence of haplotypes closely related to these animals in Central African savannah populations is evidence that there has been a limited amount of dispersal out of these forests since *ca*. 2.3 Myr ago. However, there is no evidence of immigration into the Central African forests, and there is no question that these elephants are ecologically and morphologically very different from elephants in the nearby savannahs.

The second group includes the forest and savannah elephants of West Africa. Genetically, these regional

populations do not divide into separate forest and savannah forms, but retain ancestral Central African forest elephant haplotypes together with more derived Central African savannah elephant haplotypes (as evidenced by the placement of Mali2). Our analysis indicates that these West African populations are now genetically distinct from other forest and savannah elephants and have been on a different evolutionary trajectory for more than 2 Myr. As human conversion of habitat now precludes significant gene flow into these populations, they should be considered a separate taxon.

The third genetically recognizable group includes the savannah elephants of Central, eastern and southern Africa. These populations share the mitochondrial haplotypes of two genetically distinct radiations of ancestral forest elephants. This conclusion is supported by our limited survey of nuclear DNA microsatellites as well as by Roca *et al.* (2001), who found little or no genetic differentiation between savannah populations in four nuclear introns. It will be most interesting to establish whether the two haplotype groups mate with each other at random in admixed populations. Nyakaana & Arctander (1999) detected individuals from clades 1 and 3 in Uganda and found only weak subdivision between two of their three population samples in microsatellites, suggesting present-day introgression of the mtDNA lineages. Unless these genetic clusters can be firmly established, based on both mtDNA haplotypes and nuclear markers, these widespread savannah elephants should also be recognized as a genetically and ecologically distinct taxon.

5. CONCLUSIONS

Our finding of three recognizable taxa of African elephants is based on insufficient genetic data to warrant formal taxonomic revision of the species at this time. Further studies are needed to better understand the effects of matrilineal structure and climate change on the evolutionary history of the African elephant. The West African populations must be examined at nuclear loci to provide comparable estimates of genetic differences between forest and savannah elephants at the continental level. If the level of genetic differentiation between the three taxa identified here is confirmed to reflect several million years of divergence, it will be appropriate to treat them as species in recognition of their long independent evolutionary trajectories.

Populations in the Central African forests and in West Africa are generally smaller and more isolated than those in savannahs. They are also severely threatened by competition for habitat with humans and by poaching for meat and ivory. By recognizing the genetic and ecological distinctness of these populations, we highlight the importance of their separate conservation management (Woodruff 2001).

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