

A case study of apparent conflict between molecular phylogenies: the interrelationships of African elephants

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Accepted 31 August 2004

Abstract

Recent molecular phylogenies of the African elephants suggest that there is an evolutionary structure within *Loxodonta africana*. Some nuclear results (Roca et al., 2001) support the separation of the forest African elephant subspecies *L. a. cyclotis* as a species distinct from the savannah elephant *L. a. africana*, on the basis of the recognition of both forming highly divergent (reciprocally monophyletic) clades. Conversely, a mitochondrial survey (Eggert et al., 2002), while admitting a geographic partitioning of the genetic structure within African elephants, suggests retaining the status quo. They recognize three diagnosable entities (western, central and south-eastern Africa) with non-overlapping ranges within *L. africana sensu lato*. In order to address these conflicting views (historical fragmentation and speciation or isolation by distance, respectively), we have sequenced two datasets of 1961 bp (for 50 elephants) and about 3700 bp, respectively (for 20 elephants) of the mitochondrial DNA for both forms of elephants (*cyclotis* and *africana*). They span the cytochrome *b* gene, the control region and several RNAs. When compared with former mtDNA data, they provide the most comprehensive view of the African elephant phylogeny (78 mtDNA haplotypes, of which 44 are new) and provide the first insight into populations from the Democratic Republic of Congo. The genetic diversity of mtDNA was appraised and the stability of alternative phylogenetic trees was investigated. Our results are inconsistent with both those prior studies. They revealed two highly divergent molecular clades referred to as F and S, that do not conform to the morphological delineations of *cyclotis* and *africana*. A non-negligible proportion of specimens of *L. a. africana* display haplotypes prevailing in forest elephant populations (clade F). The geographic distribution of clades and areas of their co-occurrence support the hypothesis of incomplete isolation between forest and savannah African elephant populations, followed by recurrent interbreeding between the two forms. We state that the conclusions of prior studies resulted from insufficient character and/or geographic sampling. We conclude that there is no satisfying argument which can recognize two or more species of African elephants. We briefly comment on the meaning of such an attitude in a conservation viewpoint.

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Despite its status as the largest extant land animal, little is known about the genetic diversity and structure of the African elephant *Loxodonta africana* (Blumenbach 1797). Georgiadis et al. (1994) provide an overview of the genetic diversity in south-eastern populations of savannah elephants through RFLP and microsatellite analyses. Local diversity studies have also endeavoured to evaluate the long-term effects of poaching and other

anthropogenic disturbances (Nyakaana and Arctander, 1999; Whitehouse and Harley, 2001). The debate over the systematics of African elephants started when Barriel et al. (1999) produced the first mitochondrial cytochrome *b* sequence of a forest elephant (from Sierra-Leone) that suggested a deep division between this specimen and other African elephants. Pending a broader sampling of the populations of both forest and savannah forms, they assumed that all their African specimens belonged to a single species: *Loxodonta africana*. Since then, *Loxodonta a. cyclotis* (Matschie 1900) was elevated to species status by Roca et al. (2001)

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and Comstock et al. (2002) through the analyses of genetic variation for six nuclear introns and 16 microsatellites, respectively. This old view was formerly resurrected by morphology—i.e., cranial measurements (Groves et al., 1993; Grubb et al., 2000).

This conclusion seemed to have put an end to the debate of the last century over the systematic status of forest African elephants. Indeed, when discovered by Matschie in 1900, they were described as either a potential species, or a regional race of Cameroon (Matschie, 1900). Matschie advocated the usefulness of hydrographical basins in order to subdivide African elephants into distinct units. He thus contributed to the profusion of new taxa to be defined by the turn of the 20th century, so that the taxonomy of the African elephant quickly became extravagant, the most meagre morphological evidence being used to acknowledge a new form (Lydekker, 1907). Up to 22 forms of *Loxodonta* were described that were finally assigned either to the savannah or the forest elephant—see Laursen and Bekoff (1978) for a review. Morphologists have addressed this question for decades according to their personal taxonomic perspectives. Some have considered that, although displaying a smaller size, smaller round ears—responsible for their designation as “*cyclotis*”—more toenail structures on both feet, thin down-pointing tusks and a flatter back and forehead, forest elephants belong to the same species—i.e., *Loxodonta africana*—as savannah elephants with whom they assumed were reproductively compatible (Backhaus, 1958; Carroll, 1988; Cousins, 1996). Many cases of intermediate morphology have supported this view, which had become prevalent (Laursen and Bekoff, 1978). Conversely, the “splitter” attitude led other authors to put forest elephants apart on the basis of the same anatomical distinctiveness (Frade, 1931; Frade, 1933; Allen, 1936; Petter, 1958). More doubtful morphological characters—extent of hair-covering, color of the skin, carriage of head—have been put forward to support this division.

Surprisingly, as the view of two species is meeting a larger audience, Eggert et al. (2002) have revived the question by publishing a mitochondrial phylogeny of the African elephants where they are split into three clusters inconsistent with the current taxonomy (Fig. 1): west forest and savannah elephants first, central forest elephants second, and central, eastern and southern savannah elephants third. Though casting doubt on the recognition of two species of elephants, they remain indecisive on systematic grounds while focusing on conservation management planning. They notably found a geographic partitioning of molecular diversity within the African elephant at the continental level that is consistent with the view of a single species displaying isolation between western, central and other populations due to climatic changes during the Pleistocene period. However these results are at odds with those of Roca

et al. (2001) who maintained that the genetic partitioning within the African elephants was due to the persistence of two reproductively distinct units, seen as forest elephants on the one hand and savannah elephants on the other (Fig. 1). Such a discrepancy in the genetic structuring of molecular markers is unexpected, even though the geographic samplings are not similar in both cases. Moreover, nuclear markers display a stronger structure than do the mitochondrial sequences when the opposite is expected: the elephants are known to have a strong social structure governed by a matrilineal organization of herds. Juvenile males are excluded from the herds when they reach teen age, and live solitary (Moss, 2000; Nyakaana et al., 2001). Migratory patterns are male-biased because the herds show a philopatric behavior (Nyakaana and Arctander, 1999). These features lead us to expect a low rate of intra-population genetic variation and a strong level of inter-population differentiation, particularly for mtDNA.

However we suspect that the contradiction between the two types of molecular markers is possibly an artefact due to insufficient molecular sampling by previous researchers (Nyakaana and Arctander, 1999; Eggert et al., 2002; Nyakaana et al., 2002). To shed more light on this issue we sequenced longer fragments of the mitochondrial genome spanning all published sequences (Fig. 2): one protein coding gene (cytochrome *b*), one ribosomal gene (12S rRNA), three transfer RNA genes (tRNA-Thr, tRNA-Pro, and tRNA-Phe), and the mitochondrial control region were sequenced. We analyzed two arrays: 1961 bp for 48 African elephants and ~3700 bp for 18 African elephants, referred to here as short and long fragment, respectively (Fig. 2B).

We also assumed that a prior knowledge of the sample might have been insufficient in the former analyses, leading to unsupported conclusions: both Roca et al. (2001) and Eggert et al. (2002) considered that the taxonomy of the elephants could be assessed through their geographic origin. However, it is now obvious that the two forms, if named “forest” and “savannah”, are not strictly allopatric within Africa, their ranges overlapping in many areas (Laws et al., 1975; Barnes, 1987; Barnes et al., 1995), and their frontier is difficult to establish in western Africa (Groves, 2000). To overcome this problem, we examined the morphotype of the specimens that were sequenced in order to establish whether they could be clearly assigned to the forest or savannah forms. Of 48 African specimens, 18 were morphologically identified as forest elephant whereas 28 others were of the savannah form. However, two specimens could not be definitely diagnosed due to their intermediate morphology.

The populations of forest African elephants have been reduced in size and their habitat has been much fragmented since they were studied by Matschie (Douglas-Hamilton, 1987). The current distribution of

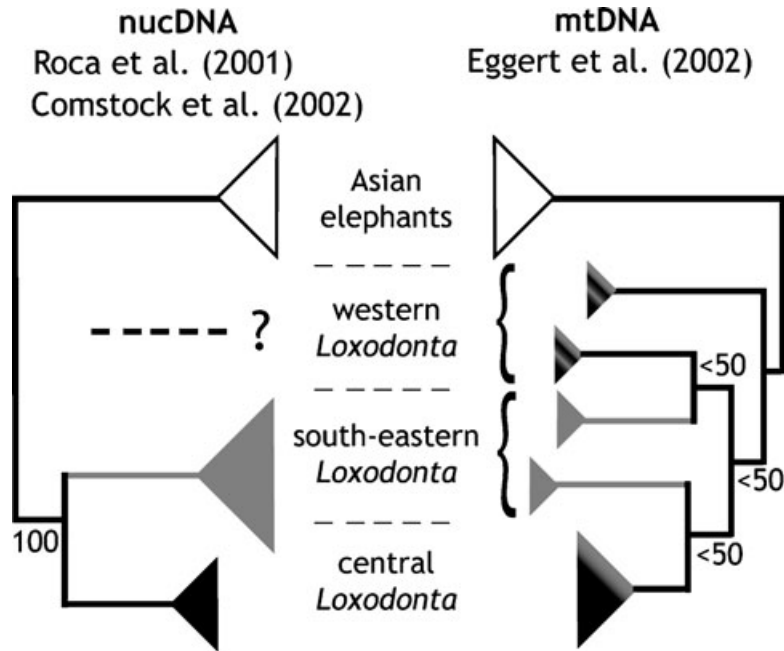


Fig. 1. Synoptic display of main contradictions between the phylogenetic results of Roca et al. (2001) and Comstock et al. (2002) for nuclear DNA, and of Eggert et al. (2002) for mitochondrial DNA. *Loxodonta africana africana* is displayed in gray, *Loxodonta africana cyclotis* in black.

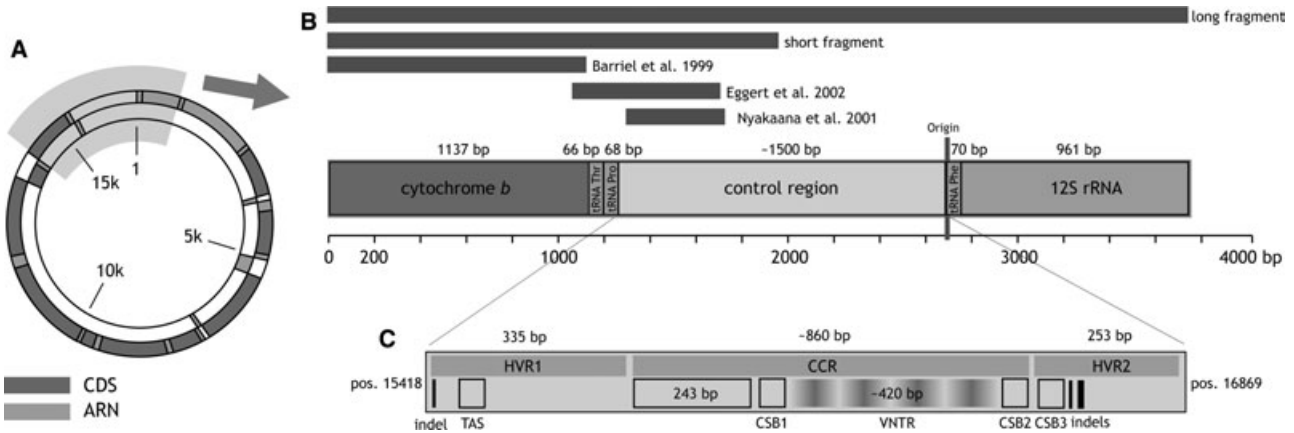


Fig. 2. Molecular scope. (A) Portion of mtDNA chromosome sequenced. (B) Display of the two arrays analyzed (short and long fragments) according to formerly published datasets and to mitochondrial markers. (C) Close-up to structure of the mitochondrial control region; conserved regions are boxed.

forest elephants encompasses all the equatorial forest-covered land of Africa, but their densities vary extensively throughout this range. Indeed, the last IUCN census (Barnes et al., 1999) recorded no remnant population in Sierra Leone, the most western part of the elephant range, noting that all western populations were at critically low levels, while more than 20 000 individuals are thought to inhabit the Congo basin. However, these latter populations suffered during the recent decades of the endemic warfare across these areas and drastically lack a protected range (Höft, 2000). Until today, no molecular data have been produced from this

very central part of the range of the forest African elephant which potentially harbors one-fifth of their total population. We address here the mitochondrial affinities of these populations and emphasize their relevance.

Materials and methods

Taxonomic sample

Two different types of samples were collected for our molecular analyses (Table 1). Hair and blood samples

Table 1
Specimens included in the phylogenetic analyses

Specimen	taxon label	Provider	Geographic	Sample type	Short*	Long*	Reference
Origin							
Laa SouthAfrica1	<i>Loxodonta africana africana</i>	Basel Zoo (Switzerland)	South Africa	Hair		AF132528	Modified from (Debruyne et al., 2003b)
Laa SouthAfrica2	<i>L. a. africana</i>	Thoiry Zoo (France)	South Africa, Natal Parc	Hair		AF132529	Modified from (Debruyne et al., 2003b)
Laa SouthAfrica3	<i>L. a. africana</i>	Basel Zoo (Switzerland)	South Africa	Hair	AY741320		This study
Laa Namibia1	<i>L. a. africana</i>	Thoiry Zoo (France)	Namibia	Hair	AY741325		This study
Laa Namibia2	<i>L. a. africana</i>	Annéville Zoo (France)	Namibia	Hair	AY741326		This study
Laa Uganda1	<i>L. a. africana</i>	Basel Zoo (Switzerland)	Uganda	Hair	AY741323		This study
Laa Uganda2	<i>L. a. africana</i>	Berlin Tiergarten (Germany)	Uganda	Hair	AY741077		This study
Laa Uganda3	<i>L. a. africana</i>	Annéville Zoo (France)	Uganda	Hair	AY741324		This study
Laa Zimbabwe1	<i>L. a. africana</i>	Colchester Zoo (England)	Zimbabwe	Hair	AY741321		This study
Laa Zimbabwe2	<i>L. a. africana</i>	Colchester Zoo (England)	Zimbabwe	Hair	AY741329		This study
Laa Zimbabwe3	<i>L. a. africana</i>	Berlin Tiergarten (Germany)	Zimbabwe	Hair			This study
Laa Zimbabwe4	<i>L. a. africana</i>	Circus Krone (Germany)	Zimbabwe	Hair	AY741069		This study
Laa Zimbabwe5	<i>L. a. africana</i>	Circus Krone (Germany)	Zimbabwe	Hair	AY742799		This study
Laa Zimbabwe6	<i>L. a. africana</i>	Circus Krone (Germany)	Zimbabwe	Hair	AY741071		This study
Laa Zimbabwe7	<i>L. a. africana</i>	Berlin Tiergarten (Germany)	Zimbabwe	Hair	AY741067		This study
Laa Zimbabwe8	<i>L. a. africana</i>	Berlin Tiergarten (Germany)	Zimbabwe	Hair	AY741075		This study
Laa Zimbabwe9	<i>L. a. africana</i>	Berlin Tiergarten (Germany)	Zimbabwe	Hair	AY741068		This study
Laa Zimbabwe10	<i>L. a. africana</i>	Berlin Tiergarten (Germany)	Zimbabwe	Hair	AY742800		This study
Laa Tanzania1	<i>L. a. africana</i>	Annéville Zoo (France)	Tanzania	Hair	AY741070		This study
Laa Tanzania2	<i>L. a. africana</i>	Annéville Zoo (France)	Tanzania	Hair			This study
Laa Zambia1	<i>L. a. africana</i>	Colchester Zoo (England)	Zambia	Hair	AY741076		This study
Laa Mozambique1	<i>L. a. africana</i>	Nürnberg Zoo (Germany)	Mozambique	Hair			This study
Laa IvoryCoast1	<i>L. a. africana</i>	No. 1961–129, MNHN (France)	Ivory Coast	Cranial bone	AY741327		This study
Laa Angola1	<i>L. a. africana</i>	No. 22268, MRAC (Belgium)	Angola, Luiza (DRC frontier)	Cranial bone	AY741072		This study
Laa Sudan1	<i>L. a. africana</i>	No. A2253, MNHN (France)	Sudan (ex-southern Egypt)	Cranial bone	AY741073		This study
Laa Botswana1	<i>L. a. africana</i>	Dresden Zoo (Germany)	Botswana	Hair	AY741074		This study
Laa Chad1	<i>L. a. africana</i>	MNHN (France)	Chad, Zakouma Reserve	Vertebral disc	AY741080		This study
Laa DR11	<i>L. a. africana</i>	No. 25421, MRAC (Belgium)	Southern DRC, Kamemba	Bone	AY741078		This study
Lac SierraLeone1	<i>L. africana cyclotis</i>	Vincennes Zoo (France), MNHN	Sierra-Leone	Hair		AF132530	Modified from (Debruyne et al., 2003b)
Lac Liberia1	<i>L. a. cyclotis</i>	No. 36288, MRAC (Belgium)	Liberia	Bone	AY741079		This study
Lac Gabon1	<i>L. a. cyclotis</i>	Lope Reserve (Gabon)	Gabon, Lope Reserve	Muscle		AY359278	Modified from (Debruyne et al., 2003b)
Lac Gabon2	<i>L. africana pumilio</i>	No. 1950–728, MNHN (France)	Gabon, coast near Aloombé	Cranial bone	AY359265		(Debruyne et al., 2003b)
Lac Congo1	<i>L. a. pumilio</i>	No. 1956–192, MNHN (France)	Congo Brazzaville	Cranial bone	AY359266		(Debruyne et al., 2003b)
Lac Congo2	<i>L. a. cyclotis</i>	No. 1906–450, MNHN (France)	Congo Brazzaville	Cranial bone	AY359268		(Debruyne et al., 2003b)
Lac Cameroon1	<i>L. a. pumilio</i>	No. 1956–194, MNHN (France)	Cameroon, Yambong	Cranial bone	AY359267		(Debruyne et al., 2003b)
Lac Cameroon2	<i>L. a. cyclotis</i>	No. 1928–122, MNHN (France)	Cameroon	Cranial bone	AY359269		(Debruyne et al., 2003b)
Lac CAR1	<i>L. a. cyclotis</i>	Fribourg Museum (Switzerland)	Southern CAR, Ubangi river	Hyoid bone	AY359272		(Debruyne et al., 2003b)
Lac DR1	<i>L. africana fransseni</i>	No. 3396, MRAC (Belgium)	Western DRC, Mai-Ndombe Lake	Tooth	AY359277		((Debruyne et al., 2003b))

Lac DRC2	<i>L. a. cyclotis</i>	No. 8188, MRAC (Belgium)	Eastern DRC, Panga Na Bodio	Cranial bone	AY359270	(Debruyne et al., 2003b)
Lac DRC3	<i>L. a. cyclotis</i>	No. 8203, MRAC (Belgium)	Northern DRC, Uele n'Dungu	Cranial bone	AY359271	(Debruyne et al., 2003b)
Lac DRC4	<i>L. a. pumilio</i>	No. 7692, MRAC (Belgium)	Southern DRC, Moma	Cranial bone	AY359275	(Debruyne et al., 2003b)
Lac DRC5	<i>L. a. pumilio</i>	No. 9524, MRAC (Belgium)	Southern DRC, Moma	Cranial bone	AY359276	(Debruyne et al., 2003b)
Lac DRC6	<i>L. a. pumilio</i>	No. 20330, MRAC (Belgium)	Northern DRC, Bosobolo	Cranial bone	AY359273	(Debruyne et al., 2003b)
Lac DRC7	<i>L. a. pumilio</i>	No. 20331, MRAC (Belgium)	Northern DRC, Bosobolo	Cranial bone	AF517566	(Debruyne et al., 2003b)
Lac DRC8	<i>L. a. pumilio</i>	No. 20332, MRAC (Belgium)	Northern DRC, Bosobolo	Cranial bone	AY359274	(Debruyne et al., 2003b)
Lac DRC9	<i>L. a. pumilio</i>	No. 20333, MRAC (Belgium)	Northern DRC, Bosobolo	Cranial bone	AY359279	Modified from (Debruyne et al., 2003b)
Lax DRC13	<i>L. africana</i>	No. 37441, MRAC (Belgium)	Eastern DRC, Kanyatsi	Bone	AY741081	This study
Lax DRC17	<i>L. africana</i>	No. 35790, MRAC (Belgium)	Southern DRC, Katanga	Bone	AY742802	This study
Emi India1	<i>Elephas maximus indicus</i>	Vincennes Zoo (France)	India	Blood	AF132520	Modified from (Debruyne et al., 2003b)
Emi Burmal	<i>E. m. indicus</i>	La Palmyre Zoo (France)	Burma	Hair	AF132521	Modified from (Debruyne et al., 2003b)

*Accession number (GenBank) of each sequence is given in the relevant "Short" or "Long" fragment column.

(taken in EDTA) were collected from 26 wild-born animals from European zoological gardens. Tissues from a poached animal in Lope Reserve (Gabon) were added to our sample. In complement, 23 samples of bone or tooth were derived from 22 specimens from Museum collections and one from a carcass found in the field in Zakouma Reserve (Chad). These samples from central and western Africa were used to overcome the difficulty of sampling free-ranging elephants in remote and/or depleted populations. All specimens were individually identified as belonging to *africana* (savannah form) or *cyclotis* (forest form) through morphology, pedigree and museum records. *Loxodonta pumilio* and *Loxodonta fransseni*, the surrogate dwarf forms of forest elephants, were formerly synonymized with *cyclotis* by Debruyne et al. (2003b). For living elephants this characterization was made using classical features: height, form of the ears and the head, shape and set of the tusks, and curvature of the spine. This precise acknowledgment of the "morphological type" is seen as a decisive preliminary step to further addressing molecular affinities: geographic origin sole may lead us to overlook the extent of hybridization in regions where different forms co-occur. Actually 18 specimens were determined as *cyclotis* (Lac in figures and tables) and 28 as *africana* (Laa). The last two were considered to be of intermediate morphotype (Lax), as they display mixed morphological characters. Twenty out of 37 nations forming the current range of African elephants were thus examined. Two Asian elephants were added as outgroup taxa and represent the two main clades formerly acknowledged within *Elephas maximus* Linné 1758 by Fernando et al. (2000) and Fleischer et al. (2001).

Molecular scope

In this paper, we focus exclusively on mitochondrial DNA (mtDNA). Its high level of variability suggested that it might reveal more topological resolution than nuclear markers. Indeed, in their study of 1732 bp of intronic sequences, Roca et al. (2001) obtained 73 polymorphic sites (4% of total), most of which affected the divergence between Africa and Asia. Debruyne et al. (2003b) stated that a level of polymorphic sites as high as 10% (two-thirds of which were African elephants alone) exists within the cytochrome *b* gene and control region. We assume that, even if built on 195 specimens, the nuclear affinities are underestimated relative to the mitochondrial ones. Furthermore, mtDNA is a haploid, maternally inherited, non-recombining molecule, which is of the greatest interest in getting rid of tokogenetic effects in infra-specific analyses for such a matriarcal taxon (Birky, 1989; Avise, 1994).

Along the mitochondrial chromosome, we selected a ~3700 bp fragment spanning the cytochrome *b* gene

(*cyt. b*), control region (CR), 12S rRNA gene, and three tRNAs (Fig. 2B). These contiguous markers allowed comparisons with published datasets (Barriel et al., 1999; Nyakaana and Arctander, 1999; Eggert et al., 2002). Because of the difficulty of amplifying the 3' extremity of the control region (Hauf et al., 1999), the second array (long fragment) was only sequenced for 18 African elephants.

MtDNA extraction, PCR reactions and sequencing protocols

Hair, blood and muscle samples were treated with a standard CTAB extraction (Winnpeninckx et al., 1993). Bone samples were submitted to a modified phenol/chloroform protocol (Hassanin et al., 1998). Some of the bone fragments have been preserved for more than a century, and their maximum length of PCR product was often within 300 bp. Therefore, a set of 28 elephantid specific primers (see the Appendix) were designed from the entire mitochondrial sequence of *Loxodonta africana* (Hauf et al., 1999) with the OLIGO program (version 4.0; Rychlik and Rhoads, 1989). PCR reactions were as previously published (Debruyne et al., 2003b) with profiles as follows: 3 min at 94 °C of initial denaturation, 10 cycles of 94 °C denaturing for 40 s, annealing temperature (50 up to 55 °C according to the couple of primers) for 80 s, and 72 °C extension for 40 s. The annealing temperature was further raised 5 up to 10 °C and the annealing time reduced to 40 s for another 25 cycles, with the other parameters as before. A step of 2 min at 72 °C ended the thermal cycling program. Purified products (MinElute columns; Qiagen) were sequenced on a CEQ2000 automated sequencer (Beckman).

Sequence and VNTR alignments

All sequences, read on both strands, were authenticated with the BLASTN program (Altschul et al., 1997) in order to rule out any sequencing of Numt (Greenwood and Pääbo, 1999). They were entered into a database using the MUST package (version 2000; Philippe, 1993). The sequences were aligned manually following two guidelines: keeping the number of gaps minimal and maximizing transitions relative to transversions. Only three indels were found in the control region and unambiguously aligned within conserved blocks. Two single-nucleotide gaps were found to distinguish some of the savannah elephants from the remainder, whereas a five-nucleotide indel near the 3' end of the control region was diagnostic for Asian/African dichotomy (Fig. 2C). Gaps were treated as a fifth character state.

Variable Number Tandem Repeats (VNTR) of the central conserved region (CCR; Fig. 2C) were excluded

from the matrix as they vary greatly in the structure of the motifs and in the number of repeats, which prevents them from being used in a parsimony analysis for two main reasons: (i) Homology hypotheses might be assessed between identical motifs nested in super-motifs, themselves nested in phrases, but the contiguous repetition of motifs precludes the recognition of (somehow) orthologous units. (ii) The turning of any motif into another and especially the gain and loss of motifs cannot be coded without heavy *a priori* about the model of evolution of these sequences (see Results). For these reasons, VNTR were only mapped *a posteriori* on the long dataset phylogenetic tree (Grandcolas et al., 2001).

Phylogenetic analyses

Phylogenetic analyses were performed using PAUP* (version 4.0b10; Swofford, 1998). The options of heuristic search were as follows: step-wise addition, 100 random sequence additions, TBR branch-swapping on best trees only. The robustness of the topologies was explored through Bremer Scores (BS; Bremer, 1988) and bootstrap replications (1000 replicates in parsimony). Partitioned Bremer Support (PBS; Baker and DeSalle, 1997) was investigated with TREEROT (version 2.0; Sorenson, 1999).

In order to make direct comparisons with the ML analyses of Eggert et al. (2002), the selection of an evolutionary model for maximum likelihood analysis was completed with MODELTEST (version 3.06; Posada and Crandall, 1998) using the default options: Jukes and Cantor distance (Jukes and Cantor, 1969) for a neighbor joining starting tree, best-fitting model chosen with Akaike Information Criterion (AIC; Akaike, 1974). Maximum likelihood analyses were performed using the same heuristic options as parsimony (but with 100 bootstrap replicates).

Results

Analysis of the short fragment dataset (1961 bp; 50 terminals)

The short sequences dataset revealed 212 variable sites defining 50 unique haplotypes, which is far in excess of that found in nucDNA. The level of variability is not constant along the whole sequence assayed, and Table 2 summarizes these features. The *cyt. b* gene displays a level of variability which is as great as the hypervariable regions of the CR, with mutational events distributed mainly within third codon positions: 109 polymorphic rather than 25 in first codon position and only 13 in the second position. The parsimony analysis yields 229 433 parsimonious trees of 338 steps. The overall homoplasy is low: $CI_{\text{uninf}} = 0.59$ (excluding

Table 2
Variability, tree length, and homoplasy content for each marker analysed

Sequence	Marker	Length	Variable	Ti	Tv	Informative	CTL _C	CTL _S	CI _{uninf}	RI
50 sequences	Cytochrome <i>b</i>	1137	147 (13%)	144	6	118 (10%)	205.1	204	0.69	0.96
	tRNA Thr, Pro	134	7 (5%)	7	0	6 (4%)	8.7	8	0.86	0.98
	HVR1 (CR)	333	48 (14%)	49	3	39 (12%)	108.2	98	0.46	0.85
	CCR (CR)*	357	10 (3%)	8	3	6 (2%)	16.0	12	0.86	0.98
	Total	1961	212 (11%)	207	13	169 (9%)	338	322	0.59	0.93
20 sequences	HVR2 (CR)	348	30 (9%)	20	11	19 (5%)	40	39	0.70	0.86
	tRNA Phe	70	2 (3%)	1	1	2 (3%)	2	2	1.00	1.00
	rRNA 12S	961	37 (4%)	32	8	28 (3%)	41	41	0.97	0.98
	Total	1379	69 (5%)	53	21	49 (4%)	83	82	0.84	0.95

Column titles correspond to: the number of sequences involved (Seq.), the marker and its length, number of variable sites, minimum numbers of transitions (Ti) and transversions (Tv), number of informative sites, consensus tree length in combined analysis (CTL_C) or separate analysis (CTL_S), and homoplasy indices for separate analyses: CI excluding uninformative sites and RI. *VNTR were not considered.

uninformative sites), RI = 0.93. The strict consensus, presented on Fig. 3 with supports, is only partially unresolved in crown.

The model selected with MODELTEST was HKY + I+G with the following parameters: Ti/Tv = 24.8399, base frequencies are A = 0.3011, C = 0.2703, G = 0.1347, and T = 0.2939, proportion of invariable sites I = 0.7669, and shape parameter of gamma law α = 0.752. The most likely tree under this model—from now on referred to as the “ML tree”—is in general agreement with the parsimony consensus. Discrepancies are: (i) minor differences in terminal nodes which are not discussed, and (ii) making the clade (Lac Liberia1 (Lac Icoast1, Lac Sleone1)) a supported sister group (bootstrap = 80) of (Laa Chad1 (Lac Congo1 (Lac Gabon1, Lac Gabon2))).

The most striking feature relies on the deep divergence of two clades, whose reciprocal monophyly is indisputable. The most divergent group (clade S; Fig. 3) displays a BS of 23 and comprises 23 sequences exclusively of the *africana* form. The second group (clade F; BS = 14) comprises all the sequences of the *cyclotis* form, but also the haplotypes of the two intermediate specimens as well as five savannah sequences. Relative to this tree, it is obvious that the prevalent taxonomic units—*africana* and *cyclotis* forms, whatever their category—are non-monophyletic.

Within clade F, the sister group relationship between two clades ((B) and (E); Table 3) is unclear: retrieved in ML analysis, it is found as an unsupported alternate to the grouping of clades (E) and (R) in some parsimonious trees. (B) is never found as the sister-group of (R). The division of clade F into three main clades (nodes (B) (E), and (R); Table 3) is robust and each of them is also highly structured. Conversely, most of the collapsed nodes belong to the clade S: except the first dichotomy between two sets of sequences—one of southern and the other of ubiquitous individuals—it exhibits a poor internal structuring due to the lack of sequence divergence (soft polytomy) rather than homoplasy (hard polytomy).

The three kinds of markers employed, namely: the cyt. *b* coding gene, tRNAs and CR, provide differential support for the nodes of the consensus tree as exemplified by PBS scores (Table 3 and Fig. 3). Deep nodes are mainly supported by the cyt. *b*, especially the dichotomy between F and S clades. The few informative tRNA characters (Table 2) do not provide much support to any node ($-1 < \text{PBS} < 1$). The control region rather exhibits singular supports in crown (e.g., nodes (E) or (M), Table 3). However, it contradicts several supported nodes, with a striking lack of support for deep nodes within clade F: nodes (P) (Q) and (R) in Table 3. It appears that HVR1 contains the most homoplastic data involved in our analysis: their indices are the lowest observed in the separate analyses, and in the combined analysis, they account for more than 10 supernumerary steps of the consensus length (CTL_C-CTL_S, Table 2). However the conflict in phylogenetic signal between CR and other markers is always moderate (lowest PBS = -1.3, Table 3).

Analysis of the long fragment dataset (3340 bp; 20 terminals)

The second dataset investigated is 3340 bp long, VNTR motifs apart. It yields two parsimonious trees of 344 steps (CI_{uninf} = 0.75; RI = 0.92) which differ for only one node: Laa Zimbabwe2 is the sister-group of either (Laa Zimbabwe4, Lax DRC17), or the ((Laa Zimbabwe4, Lax DRC17), (Laa Zambia1, Lac DRC9)) cluster. The former grouping (displayed in Fig. 4) is in total agreement with the analyses of the short fragment and identical to the ML tree for the long dataset—TrN+I+G model (Tamura and Nei, 1993). Both trees retrieve clades S and F, whose support is comparable or greater (BS = 37 and 13, respectively). The substructure of these clades is broadly the same, considering the extent of sequences examined. A grouping of Lac Gabon1 with (Laa Icoast1, Lac Sleone1) is depicted, as suspected with the short fragment.

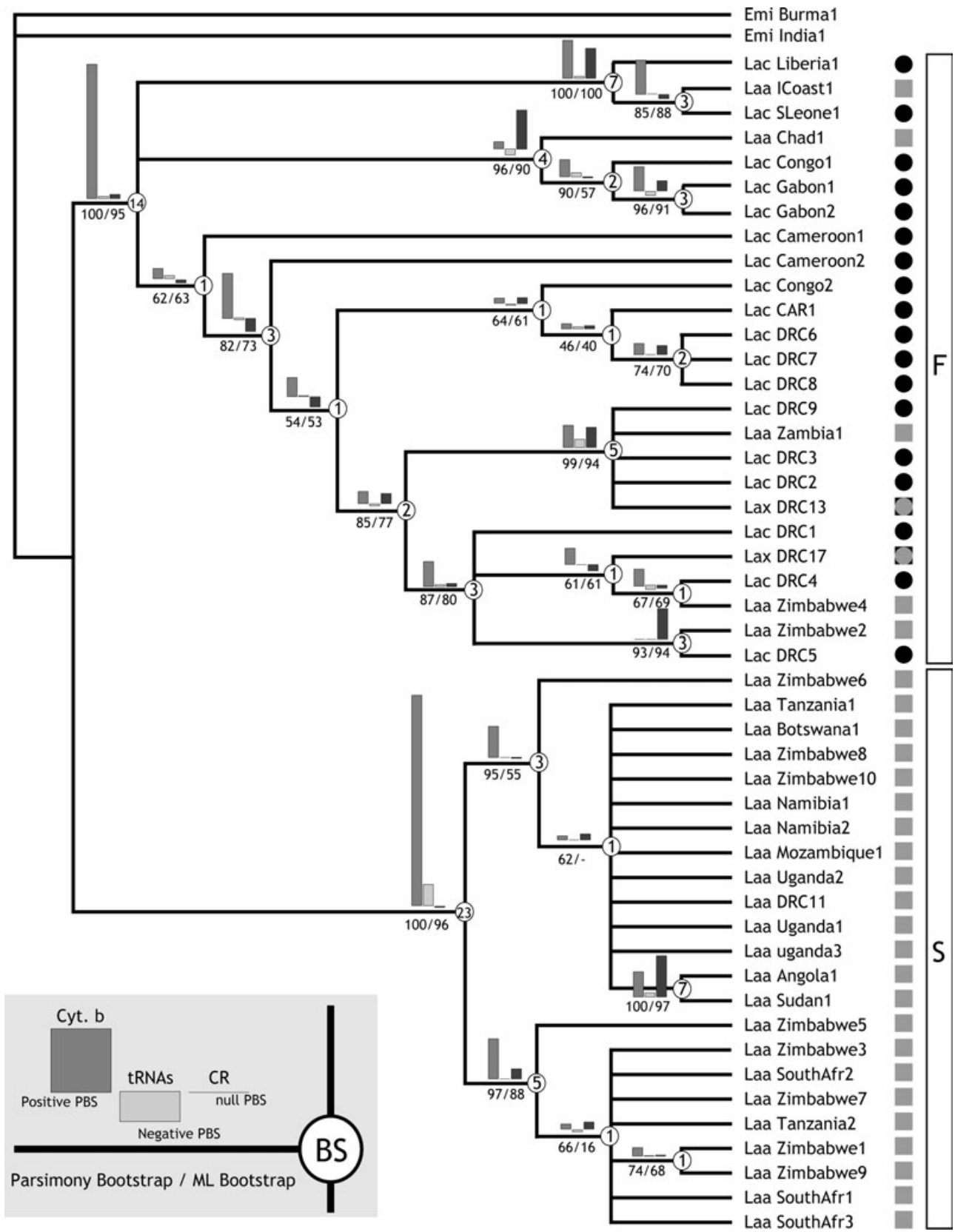


Fig. 3. Strict consensus, for the short fragment analysis, of 229 433 parsimonious trees of 338 steps for 50 sequences (see Table 2 for indices). Taxon labels are described in Table 1. Black dots are for forest elephants and gray squares are for savannah elephants; gray dots on a black background are for intermediates. Bremer support (BS) is indicated at the node next to the associated PBS along three partitions: Cyt. *b* gene, tRNA Thr Pro together (tRNAs), and 5' part of the CR. Bootstrap proportions are indicated below branches.

Mapping of tandem repeats

The VNTR motifs of the CR have been coded on their own, consistent with the following procedure: the four alternative di-nucleotides were replaced by a single numerical motif (AC = 1, AT = 2, GC = 3 and GT = 4) so that forming a six-nucleotide super-motif consisted of a combination of three numbers put into square brackets (Fig. 5). Only a few super-motifs explain the pattern observed, with a high number of repetitions for [113] and [214]. These super-motifs cluster locally in complex phrases (coded as κ , λ , μ , and other Greek characters in Fig. 5), of which the number of repetitions is also distinctive.

Every nested category of motifs is subject to its own variation. Any alignment that respects the optimality

criterion of parsimony for motifs most may not provide a parsimonious explanation of the evolution for the super-motifs or phrases. Pending an objective coding of these features, we chose not to discuss this point here: the alignment displayed on Fig. 5 was constructed to minimize the number of transformations for phrases rather than other nested categories. This arbitrary choice of the level where parsimony should be applied does not allow any inference on the homology of motifs nor super-motifs, especially where contiguous repeats are observed (Fig. 5). Along with Grandcolas et al. (2001) who state that these types of characters might be secondarily mapped on a parsimonious tree constructed from an independent data matrix, we provide a preliminary test for the homology of the phrases (Fig. 4).

Four main groups can be acknowledged within African sequences (Fig. 5): the first one comprises all sequences from clade S, and the three others divide clade F into a western group (Lac Gabon1, Laa Icoast1, Lac Sleone1) and two groups relating to central Africa: (Laa Zambia1, Lac DRC9), and (Laa Zimbabwe2, Laa Zimbabwe4, Lax DRC17). The distribution of the changes is in good agreement with the parsimonious tree (Fig. 4): The shifts in phrase patterns are mostly

Table 3
Partitioned Bremer Support for short fragment analysis

	Node	BS	PBS cyt. <i>b</i>	PBS tRNAs	PBS CR
Clade F	(A) = (Laa Icoast1, Lac Sleone1)	3	+3.4	–	–0.4
	(B) = (Lac Liberia1 (A))	7	+3.8	+0.2	+3.0
	(C) = (Lac Gabon1, Lac Gabon2)	3	+2.4	–0.4	+1.0
	(D) = (Lac Congo1 (C))	2	+1.7	+0.4	–0.1
	(E) = (Laa Chad1 (D))	4	0.7	–0.6	+3.9
	(F) = ((B) (E) (R))	14	+13.4	+0.2	+0.4
	(G) = (Lac DRC6, Lac DRC7, Lac DRC8)	2	+1.1	–	+0.9
	(H) = (Lac CAR1 (G))	1	+0.5	+0.2	+0.3
	(I) = (Lac Congo2 (H))	1	+0.5	–0.1	+0.6
	(J) = (Laa Zambia1, Lac DRC9, Lac DRC2, Lax DRC13, Lac DRC3)	5	+2.2	+0.8	+2.0
	(K) = (Lac DRC4, Laa Zimbabwe4)	1	+1.7	–0.4	–0.3
	(L) = (Lax DRC17 (K))	1	+1.6	–	–0.6
	(M) = (Lac DRC5, Laa Zimbabwe2)	3	–	–	+3.0
	(N) = (Lac DRC1 (L) (M))	3	+2.5	+0.2	+0.3
	(O) = ((J) (N))	2	+1.2	–0.2	+1.0
	(P) = ((I) (O))	1	+1.9	+0.1	–1.0
	(Q) = (Lac Cameroon2 (P))	3	+4.5	–0.2	–1.3
	(R) = (Lac Cameroon1 (Q))	1	+1.0	+0.3	–0.3
	Clade S	(S) = ((V) (Y))	23	+21.0	+2.1
(T) = (Laa Angola1, Laa Sudan1)		7	+2.5	+0.4	+4.1
(U) = (Laa Tanzania1, Laa Botswana1, Laa Zimbabwe8, Laa Zimbabwe10, Laa Namibia1, Laa Namibia2, Laa Mozambique1, Laa Uganda2, Laa DRC11, Laa Uganda1, Laa Uganda3 (T))		1	+0.4	–	+0.6
(V) = (Laa Zimbabwe6 (W))		3	+3.1	–	–0.1
(W) = (Laa Zimbabwe1, Laa Zimbabwe9)		1	+0.8	+0.1	+0.1
(X) = (Laa Zimbabwe3, Laa SouthAfr2, Laa Zimbabwe7, Laa Tanzania2, Laa SouthAfr1, Laa SouthAfr3 (W))		1	+0.5	–0.2	+0.7
(Y) = (Laa Zimbabwe5 (X))		5	+4.0	–	+1.0

Negative PBS are in bold. The numerical values are reported in Fig. 3 using vertical bars.

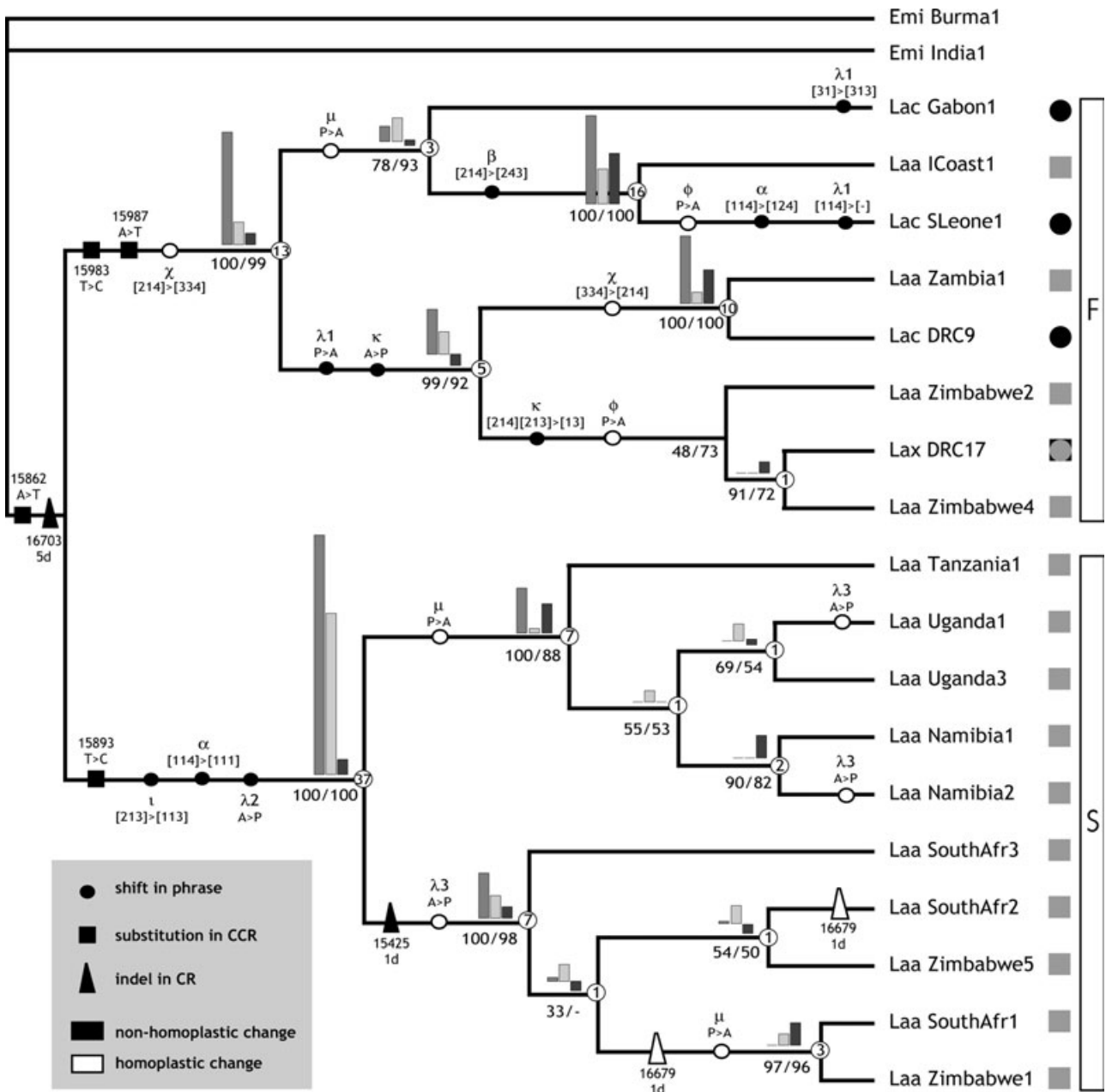


Fig. 4. One of the two parsimonious trees (344 steps, $CI_{\text{uninf}} = 0.75$, $RI = 0.92$) for the long fragment analysis (20 sequences; 3340 bp). Indication of support follows the same rule as in Fig. 3. One parsimonious optimization of the transformations of VNTR phrases is reported on branches. Gaps in the control region and fixed substitutions of the central conserved domains are also mapped. Homoplastic changes are displayed as white symbols.

optimized as non-homoplastic, supporting the main dichotomies formerly put forward.

Test of published data (592 bp; 91 terminals)

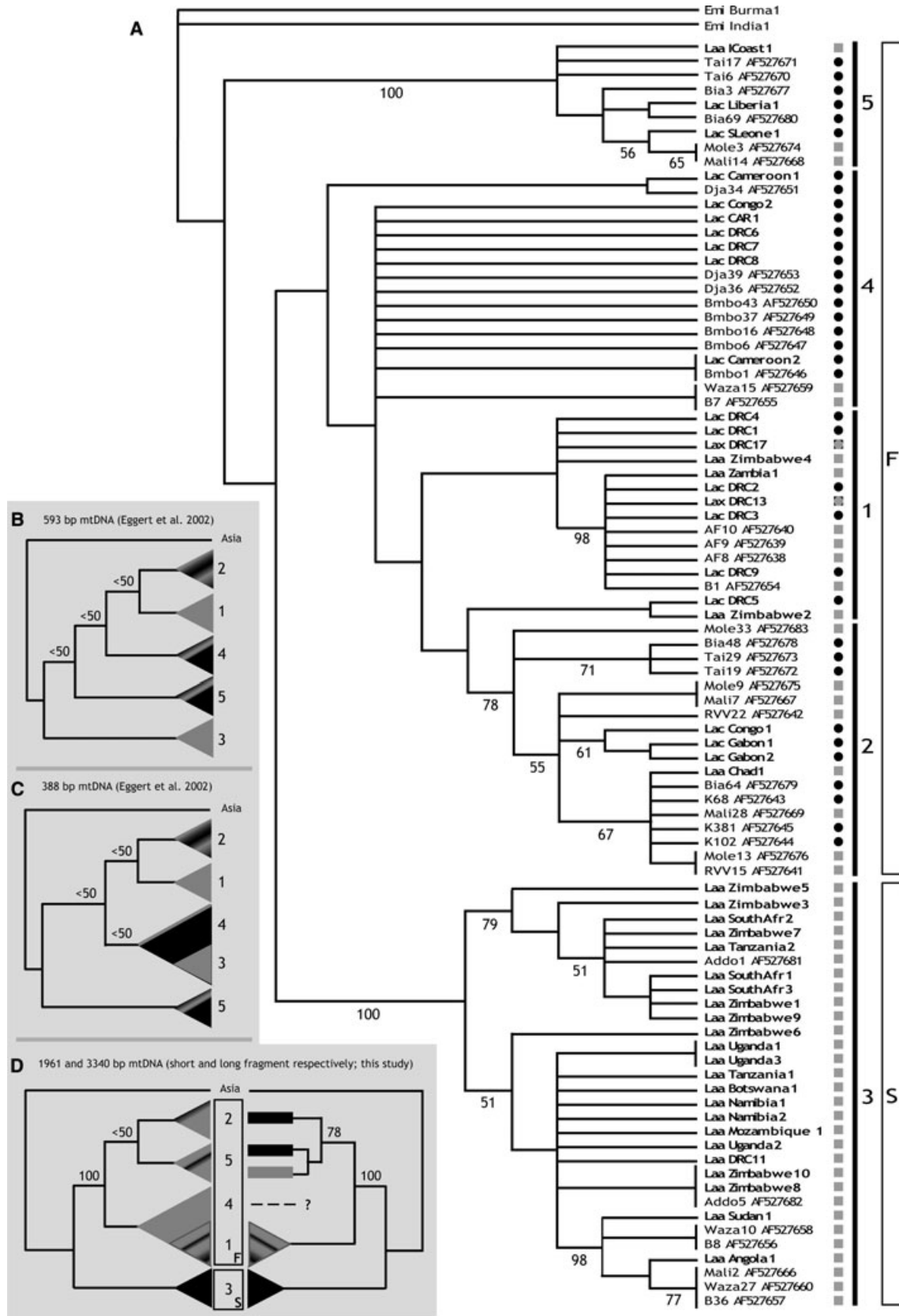
To test for the distribution of new mitochondrial haplotypes with the published data (Eggert et al., 2002; Fig. 2), we performed an analysis of a 592 bp fragment (82 variable and 64 informative sites): 91 sequences yielded 80 different haplotypes of which 9 are present

more than once. Figure 6A displays the strict consensus of 54 385 parsimonious trees of 163 steps ($CI_{\text{uninf}} = 0.48$; $RI = 0.93$) which is poorly resolved (56 nodes collapsed). A few nodes are present along with the partition observed by Eggert et al. into five main haplogroups—numbered 1–5, according to their nomenclature.

Two out of 5 haplogroups are found to be paraphyletic in Fig. 6A. (i) Lac DRC5 and Laa Zimbabwe2 from haplogroup 1 rather cluster with group 2 here. The

	α	β	γ	δ	ϵ	ϕ	λ_1	λ_2	λ_3	η	ι	ρ
Laos SouthA03	[111]	[214]	[214]	[213]	[113]	[114]	31 [113] [114]	31 [113] [114]	31 [113] [114]	[214] [213] [113] [213]	x2	[213] [213]
Laos Zimbabwe5	[111]	[214]	[214]	[213]	[113]	[114]	31 [113] [114]	31 [113] [114]	31 [113] [114]	[214] [213] [113] [213]	x2	[213] [213]
Laos SouthA02	[111]	[214]	[214]	[213]	[113]	[114]	31 [113] [114]	31 [113] [114]	31 [113] [114]	[214] [213] [113] [213]	x2	[213] [213]
Laos SouthA01	[111]	[214]	[214]	[213]	[113]	[114]	31 [113] [114]	31 [113] [114]	31 [113] [114]	[214] [213] [113] [213]	x2	[213] [213]
Laos Zimbabwe1	[111]	[214]	[214]	[213]	[113]	[114]	31 [113] [114]	31 [113] [114]	31 [113] [114]	[214] [213] [113] [213]	x2	[213] [213]
Laos Uganda3	[111]	[214]	[214]	[213]	[113]	[114]	31 [113] [114]	31 [113] [114]	31 [113] [114]	[214] [213] [113] [213]	x2	[213] [213]
Laos Nambias2	[111]	[214]	[214]	[213]	[113]	[114]	31 [113] [114]	31 [113] [114]	31 [113] [114]	[214] [213] [113] [213]	x2	[213] [213]
Laos Nambias1	[111]	[214]	[214]	[213]	[113]	[114]	31 [113] [114]	31 [113] [114]	31 [113] [114]	[214] [213] [113] [213]	x2	[213] [213]
Laos Uganda1	[111]	[214]	[214]	[213]	[113]	[114]	31 [113] [114]	31 [113] [114]	31 [113] [114]	[214] [213] [113] [213]	x2	[213] [213]
Laos Tanzania1	[111]	[214]	[214]	[213]	[113]	[114]	31 [113] [114]	31 [113] [114]	31 [113] [114]	[214] [213] [113] [213]	x2	[213] [213]
Laos Zambia1	[114]	[214]	[214]	[213]	[113]	[114]	31 [113] [114]	31 [113] [114]	31 [113] [114]	[214] [213] [113] [213]	x2	[213] [213]
Lac DRC9	[114]	[214]	[214]	[213]	[113]	[114]	31 [113] [114]	31 [113] [114]	31 [113] [114]	[214] [213] [113] [213]	x2	[213] [213]
Laos Zimbabwe2	[114]	[214]	[334]	[213]	[113]	[114]	31 [113] [114]	31 [113] [114]	31 [113] [114]	[214] [213] [113] [213]	x2	[213] [213]
Laos Zimbabwe4	[114]	[214]	[334]	[213]	[113]	[114]	31 [113] [114]	31 [113] [114]	31 [113] [114]	[214] [213] [113] [213]	x2	[213] [213]
Lac DRC17	[114]	[214]	[334]	[213]	[113]	[114]	31 [113] [114]	31 [113] [114]	31 [113] [114]	[214] [213] [113] [213]	x2	[213] [213]
Lac Gabon1	[114]	[214]	[334]	[213]	[113]	[114]	31 [113] [114]	31 [113] [114]	31 [113] [114]	[214] [213] [113] [213]	x2	[213] [213]
Laos Icoast1	[114]	[243]	[334]	[213]	11	[113]	31 [113] [114]	31 [113] [114]	31 [113] [114]	[214] [213] [113] [213]	x2	[213] [213]
Lac Sleone1	[124]	[243]	[334]	[213]	11	[113]	31 [113] [114]	31 [113] [114]	31 [113] [114]	[214] [213] [113] [213]	x2	[213] [213]
Emi Indai1	[114]	[214]	224	[213]	1	[113]	31 [113] [114]	31 [113] [114]	31 [113] [114]	[214] [213] [113] [213]	x2	[213] [213]
Emi Burma1	[114]	[214]	224	[213]	1	[113]	31 [113] [114]	31 [113] [114]	31 [113] [114]	[214] [213] [113] [213]	x2	[213] [213]
HAI for L. a.	[114]	[214]	[274]	[213]	[113]	[114]	31 [113] [114]	31 [113] [114]	31 [113] [114]	[214] [213] [113] [213]	x2	[213] [213]

Fig. 5. One possible alignment of VNTR motifs that maximizes the number of hypotheses of homology between sequences. “HAI” refers to the construction of a sequence for an Hypothetical Ancestral Haplotype of the African elephants, used to derive the apomorphic traits of Fig. 4. Phrases motifs are framed. The homology between the three λ motifs (1, 2, 3) among sequences is uncertain, as well as the homology of repeated hexa-nucleotides along the fragment: the most simple hypothesis to explain the data was set for minimizing independent changes.



composition of haplogroup 1 differs from that found by Eggert et al. (Fig. 6): it consists of sequences of both forms of elephants and their morphological intermediates (Lax) from a broad area of central to austral Africa. These haplotypes are intermingled without any systematic consistency, but a geographic clustering rather arises. (ii) Two sequences of elephants from Cameroon of the haplogroup 4 emerge earlier in the tree than the remainder. This paraphyletic assemblage of most plesiomorphic sequences, is composed of only two savannah representatives and 15 forest elephants from a narrow area ranging from western Cameroon up to northern Democratic Republic of Congo.

Haplogroup 3 fits the delineations of our clade S, comprising only sequences of ubiquitous savannah elephants. Each of the haplogroups 1, 2, 4 and 5 is composed of sequences from both forms of elephants. Although nested within our clade F in our previous analyses (Fig. 6D), these four haplogroups are set apart for this fragment, because group 5 is the sister group to all other African elephants, making the “clade F” paraphyletic (Fig. 6A) as in Eggert et al.’s findings.

However, there is little agreement in the present nesting of these groups with previous results of Eggert et al. (2002) on the same fragment (593 bp; Fig. 6B) or for a narrower array (388 bp; Fig. 6C): only haplogroups 1 and 2 cluster together in every case, contrasting with our analyses of short and long fragments (Fig. 6D), where haplogroups 2 and 5 of western specimens from both forms of elephants are sister groups.

Inner structure of the five haplogroups (388 bp; 132 terminals)

When the most comprehensive sample of sequences from African elephants is used (Fig. 7A), it provides a good estimate of the inner diversity and distribution of every of the five proposed haplogroups, but their interrelationships become fully unresolved (data not shown). Table 4 shows that clade S (= haplogroup 3) remains formed by savannah haplotypes alone, in spite of the enlargement of the sampling. However, this haplogroup only embodies a short majority of savannah elephant sequences (57%), the remainder lying in clade F. The statement that more than one half of the sequences from clade F belong to savannah elephants (and intermediates), and that 1/7 of its haplotypes are shared by both

savannah and forest elephants violates the assumption that they might belong to exclusive taxonomic units (Table 4).

The geographic distribution of the haplogroups conveys that: (i) within clade F, the distribution of haplogroups 5, 2, 4 and 1 is clinal from west to east and south, spanning largely beyond the current forest habitat range (Fig. 7B). (ii) The savannah populations show a mosaic structure: they all display S haplotypes mixed with F haplotypes originating from their closest forest elephant neighbors.

Discussion

*Phylogenetic relationships between *L. a. africana* and *L. a. cyclotis**

Our results provide an original viewpoint of the divergence pattern within African elephants. As Roca et al. (2001) showed on nuclear grounds, we uncover a deep division between two divergent clades, namely clade S and clade F (Fig. 3). This division is deemed very reliable, considering the low level of homoplasy in the dataset and the robustness of these two nodes in bootstrap and Bremer support (Figs 3 and 4). However, our phylogeny does not support the reciprocal monophyly of the *cyclotis* and *africana* forms. Despite the clade S only being represented by the savannah form, the F clade is more intriguing: it consists of an assemblage of all sequences of the *cyclotis* form intermingled with five savannah elephants and two intermediates. In point of fact, the comparison of the prevalent taxonomy, assessed through morphology with mitochondrial results, only shows that a subset of *L. a. africana* is monophyletic (i.e., haplogroup 3), but that *L. a. africana* as a whole, as well as *L. a. cyclotis*, affects a polyphyletic arrangement.

This pattern could be regarded as evidence for very rare hybridization events between the two forms. Nevertheless, other published sequences (Eggert et al., 2002; Nyakaana et al., 2002) contribute to confirm and even reinforce the heterogeneity of clade F; when a more comprehensive dataset is examined, from one-third (Fig. 6) up to one-half (Table 4) of clade F is constituted by *africana* haplotypes which are intermingled with *cyclotis* haplotypes without any systematic consistency, but rather with a high geographic consistency. This therefore depicts a protracted gene flow between the two forms wherever their ranges intersect.

The combination of the data has its drawbacks: the more sequences are taken into account, the less common information we have at our disposal—from 50 terminals with 1961 bp up to 132 terminals with only 388 bp—so that the structuring of these clades becomes somehow unresolved. Thus, the phylogenetic affinities between the main nested groups within *L. africana* (namely, haplo-

Fig. 6. (A) Strict consensus of 54 385 trees (163 steps; $Ci_{unif} = 0.48$; $RI = 0.93$) for 91 sequences (80 different haplotypes) of 592 bp. Sequences by Eggert et al. (2002) are indicated with their respective accession numbers. New haplotypes are in bold type. (B and C) Relationships of the five main clades in previous analyses. Bootstrap values are indicated at the main nodes. (D) Synoptic display of our former results on other fragment lengths examined.

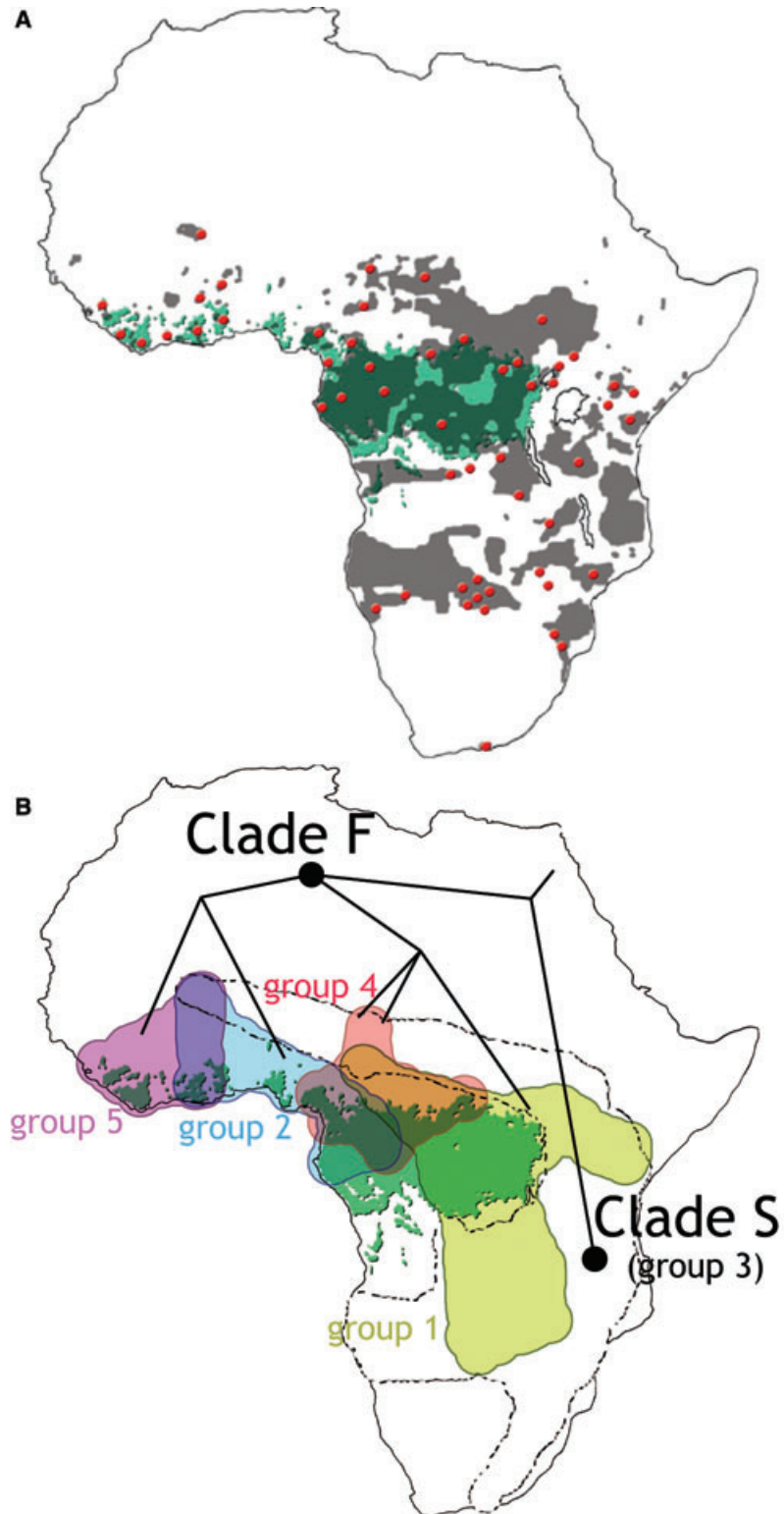


Fig. 7. African distribution of elephants, with display of equatorial rainforest in green. (A) Extant range of *Loxodonta* (after Barnes et al., 1999), with each red dot representing a sampling area of any haplotypes examined. (B) Distribution of clade S (black outline) and the four haplogroups (colored) forming clade F.

Table 4
Haplotype diversity of the total sample of published and new mitochondrial sequences sharing 388 bp (mainly HVR1) of our short fragment

Clade	Haplogroup	N haplotypes	Total (<i>n</i> = 132)	Laa (<i>n</i> = 93)	Lac (<i>n</i> = 37)	Lax (<i>n</i> = 2)
F	5	05 [0]	07% (09)	03% (03)	16% (06)	–
	4	10 [0]	13% (17)	02% (02)	41% (15)	–
	2	12 [2]	17% (22)	13% (12)	27% (10)	–
	1	21 [5]	23% (31)	25% (23)	16% (06)	100% (2)
	(1, 2, 4, 5)	48 [7]	60% (79)	43% (40)	100% (37)	100% (2)
S	3	33 [0]	40% (53)	57% (53)	–	–

In column “N haplotypes”, the total number of haplotypes per haplogroup is reported, with the number of haplotypes shared by both Laa and Lac in square brackets. In next columns, the absolute number of sequences (*n*) in every category is indicated in parentheses.

groups 1–5 and clades F and S) are explored with our short and long datasets (Figs 3 and 4), whereas the broader geographic samples are used to assess the extent and distribution of mitochondrial variability (Fig. 6 and Table 4).

Differences between mtDNA surveys

The basal dichotomy between clades S and F disagrees with the former mitochondrial phylogeography of Eggert et al. (2002) which rather recognized a division between five “clades” (haplogroups 1–5 here) having experienced a radiation. Nevertheless, we demonstrate that a differential character sampling is mainly responsible for this difference. The partitioned Bremer analysis shows that: (i) the monophyly of clade F is almost exclusively supported by the cyt. *b*, and (ii) the first dichotomies within clade F are even contradicted by the CR (Figs 3 and 4). Eggert et al.’s dataset was composed, to a large extent, of control region marker and tRNAs (83% of their total sequence of 593 bp). When the same array is examined for our sample, the monophyly of clade F is not retrieved (Fig. 6A). However, this monophyly is strongly supported by the total data (BS = 13), and it comes along with the distribution of VNTR of the control region. The lack of support for clade F based on the analyses of small fragments is therefore seen as a spurious result due to the relative inconsistency of the control region and the limited molecular sampling. Likewise, the explosive diversification of *L. africana* promoted by Eggert et al. (2002), based on the lack of resolution between haplogroups 1–5, is fairly contradicted by the strong structuring found within clade F in our analyses.

Another point of disagreement relates to the composition of group 1 within clade F, which was shown by Eggert et al. (2002) to comprise only savannah elephants, while we determined that, like the other groups nested in clade F, both forest and savannah elephants—and intermediates—are represented (Fig. 6). The sampling of Congo Basin (DRC) populations demonstrates that this “savannah clade” within clade

F was artificial due to an imperfect sampling of the forest range of elephants.

Criticism over methodologies at the phylogeographic level

Eggert et al. (2002), who studied 593 and 388 bp of mtDNA, advocated for pluralism in their phylogenetic approach as they performed parsimony, neighbor-joining (NJ) and maximum-likelihood (ML) analyses (the last two accordingly to MODELTEST). When comparing the results of all methods, the authors only stated that the “groupings resolved using the neighbor-joining, maximum-parsimony and maximum-likelihood criteria were in complete agreement” (Eggert et al., 2002, p. 1997). However, they stated that the inability of the parsimony method to group all specimens in a strict hierarchical dichotomous tree (as exemplified in our consensus Fig. 6), was due to the relative inefficiency of this method relative to NJ or ML. As a consequence, only fully resolved NJ trees were presented in their paper, the low support of internal nodes (bootstrap all below 50%) being recognized as evidence for a radiating event at the root of the *Loxodonta* tree. They validated their approach by examining the skewness of distribution of their trees. Yet, a simple shift in their taxonomic (45–84 sequences) and character sampling (593–388 bp) yielded a thoroughly different topology (Fig. 6B, C), which is inconsistent with a “significant phylogenetic signal” (Eggert et al., 2002 p. 1997).

This attitude is fairly common in phylogeographic studies where the level of divergence of taxa examined is rather low. How may we explain Eggert et al.’s results? The global estimate of the skewness in the distribution of topologies may not reflect that the phylogenetic structure of the data is very high. In the present case, this result is due in some great extent to the phylogenetic structure between the ingroup and the outgroup on the one hand, and between the sequences of clade F or clade S on the other. When these latter clades are examined separately for the “skewness” of the distribution of random trees, the result is far less conclusive, exemplifying that many arrangements are parsimonious

or likely within these clades (data not shown). This result demonstrates the limited use of the skewness evaluation in determining the phylogenetic structure of data matrices, which can be altered by the presence of divergent sequences. The skewness analysis does not locate the phylogenetic structure it may detect in the trees that are reconstructed, but it is likely that most of this overall structure is caused by the comparisons with outgroup terminals and/or only a few supported nodes among the ingroup.

Our parsimony trees show a very limited amount of overall homoplasy in the data, as well for the short (RI = 0.93) as for the long fragment assayed (RI = 0.92). Our ML analyses have also reveal that, with underlying evolutionary models thought to best explain the data distribution in our matrices, the results we obtain are in strong agreement with parsimony results. Under such conditions, nodes that are retrieved in ML analyses but are absent from parsimony consensus are prone to be artefacts due to the lack of a phylogenetic signal in terminal and short internal branches. As a consequence, we assume that the parsimony analysis provides the minimal, but valid, phylogenetic information for this dataset. The subsequent dichotomies displayed in the NJ and ML trees of Eggert et al. (Fig. 6B, C) are simply regarded as irrelevant.

Non-representativeness of former nuclear results

Although recovering a deep division between two clades, our results are not in agreement with the nuclear results of Comstock et al. (2002) and Roca et al. (2001). We observed “leaky” mtDNA boundaries while they intended to demonstrate a clear-cut division between the *cyclotis* and *africana* forms. Their nuclear pattern did not warrant the high level of introgression of F haplotypes within savannah elephants that makes these forms polyphyletic in our analyses. This incompatibility is mainly caused by a differential sampling of the diversity of African elephants. By focusing on a limited extent of the actual range of African elephants, Roca et al. (2001) are likely to have overlooked the molecular structure of the forest and savannah subspecies:

(i) Most of their sampling locations dealt with populations which in our study showed either exclusively S haplotypes (southern Africa) or exclusively F haplotypes (Gabon/Cameroon/Congo). However several studies have questioned that only “forest” elephants might be found in the latter area (Blancou, 1951; Fay and Agnagna, 1991; Barnes et al., 1995), so that Roca et al. (2001) may have identified savannah elephants (or intergrades) as *cyclotis*, as they displayed the same genotypes as the local “true” forest elephants. Such a procedure could only result in the recognition of two highly divergent units in a phylogenetic framework,

although this was based on the geographic structuring of the molecular diversity rather than on the taxonomic differentiation of African elephants.

(ii) Only one sampling location, the Garamba National Park at the north-eastern border of DRC, is seen as evidence of interbreeding between *cyclotis* and *africana*, based on their nuclear data (Roca et al., 2001). However, here again they overlooked the actual mixing of both forms, because they considered this population as uniform and belonging to *cyclotis*, as these specimens cluster within their “forest” clade. However, the identification of the morphotype for Garamba elephants is still highly controversial (Backhaus, 1958; Hillman Smith, 1990). In conjunction with our statement that haplogroup 1 (clade F) predominates in this area (Fig. 3), even within savannah elephants (Nyakaana and Arcander, 1999), it leads to a grouping of Garamba and western forest elephants, with the exclusion of other savannah populations as portrayed in Roca et al.’s tree (see Fig. 2 in Roca et al., 2001). This procedure thus prevented the uncovering of intergradation in the areas where it was most likely to occur.

(iii) The lack of any representatives from the western populations of Africa or the Congo basin is also a major limitation of this nuclear study, as far as it mostly sampled populations for which a dichotomous pattern: *cyclotis* versus *africana*, is awaited, even for mtDNA, due to their geographic location. When examining a more comprehensive sample of savannah populations, Nyakaana et al. (2002) showed that the mtDNA structuring at the continental scale was twice as great as that observed for microsatellite loci, which contradicts the clear-cut pattern of nucDNA obtained by Roca et al. (2001) and Comstock et al. (2002).

For these reasons, we consider that all their results might have been misinterpreted or circular at the very least, their only identification of the *cyclotis* and *africana* morphotypes being performed consistently with the molecular partitioning into two clades (a posteriori), rather than with any independent source of data (a priori).

How many species of elephants in Africa?

Our results contradict somewhat the hypothesis of two species that was advocated by Roca et al. (2001) on the basis of the reciprocal monophyly of both forms and their high level of genetic divergence.

We have shown that the monophyly of the two taxa *cyclotis* and *africana* was not retrieved with mitochondrial data (Figs 3 and 6). The polyphyly of the forest and savannah haplotypes is actually best explained by recurrent hybridization wherever both forms coexist. This interbreeding has had a major impact on the reciprocal integrity of extant forest and savannah elephants. In all western Africa, most of the identified

africana elephants display F haplotypes, even in very remote subdesertic populations, as in Mali (Fig. 6). Likewise, in eastern (Kenya) and southern Africa (Botswana, Zimbabwe), F haplotypes are at least as frequent as S haplotypes among savannah elephants. This confirms the pattern depicted by the data of Georgiadis et al. (1994), which conveyed the division of these populations into two sympatric divergent groups. Thus, the hybrid zone between *africana* and *cyclotis* is not fairly “narrow”. This interbreeding process among the forest and savannah forms rejects their specific status based on the so-called “biological species concept” (Mayr, 1942), even when relaxed to account for hybridization (Strickberger, 1996, pp. 228–232, 557–560). Likewise, these taxa do not conform to “evolutionary” (Wiley and Mayden, 2000) concepts, nor to a “cohesive species concept” (Templeton, 1989): when a topological consensus of morphological and mitochondrial data is drawn, the tree is fully unresolved, indicating that no repeated pattern of variation can be found within African elephants which behave as a single unit (though not panmictic) rather than two valid species.

From a phylogenetic viewpoint, the conclusion is also obvious. The polyphyletic relationships between *africana* and *cyclotis* (Figs 3 and 4) conveys that no competing “phylogenetic species concept” is suitable for these taxa: they form neither monophyletic species (Mishler and Theriot, 2000) nor diagnosable species (Cracraft, 1983; Wheeler and Platnick, 2000), insofar as the morphological and molecular patterns are totally incongruent and do not provide any consistent picture of what might differentiate forest and savannah elephants.

Conceptual and operational appeal are separated in species definitions. We agree with de Queiroz when he stated that “Although real differences underlie alternative species definitions, there is really less disagreement about species concepts than the existence of so many definitions seems to suggest” (de Queiroz, 1998, p. 57). Taxonomy provides rules for determining what taxon might be recognized as a valid species, but the actual application of its rules is left to its practitioners. If all species concepts are not theoretically and operationally exchangeable, all rely on a set of successive criteria that might be fulfilled by a taxon or not (De Queiroz, 1998). The relevance of the specific identification of a taxon may thus be questioned when different criteria promote different taxonomic schemes. Here we rather demonstrate that, in the light of different criteria based on pattern (phylogenetic and evolutionary definitions of species) or not (biological definition of species) we are bound to recognize the same taxonomic units within African elephants that are a single species with two parapatric subspecies that do interbreed wherever their ranges intersect.

We may be looking at speciation processing within African elephants, but acknowledging *L. a. cyclotis* and

L. a. africana as valid species seems premature, as they do not conform to any species concept.

The illusion of divergence for taxonomic purposes

One of Roca et al.’s (2001) recurrent arguments for elevating *cyclotis* and *africana* at the species level is that they form two entities (therein acknowledged as clades) separated by 58% of the molecular divergence between *Loxodonta africana* and *Elephas maximus*, the Asian elephant. This argument even has priority over pattern, in their view. Nevertheless, if one states as Wu does, that “speciation is the stage where the populations will not lose their divergence upon contact and, furthermore, will be able to continue to diverge” (Wu, 2001, p. 854), one must acknowledge that the phylogenetic pattern should always prevail, in contrast to what is described in the different phenetic species concepts: the magnitude of DNA sequences variation conveying no information to attest to the completion of speciation.

Witnessing that two clades (here S and F) display a very high level of molecular divergence is useless unless these clades are included along with other sources of phylogenetic characters as morphology. In the opposite case, it becomes tricky to establish what pattern should prevail between the morphology and the molecules—and among them, such-and-such marker (Baum and Shaw, 1995). If one attempts to conflate all available sources of information on the taxonomy of African elephants, the only defensible attitude assumes that Africa harbors a single species of elephant.

The usefulness of divergence for conservation purposes

Conservation and taxonomic arguments often conflate when dealing with elephants’ relationships, which is deemed a dead end in systematics. Taxonomy provides guidelines for conservation surveys, but it is not itself aiming at defining the units to be protected. Whether it is non-significant for species recognition, the extent of sequence divergence below the species level is indeed useful for conservation purposes. It allows us to determine the relevant Evolutionary Significant Units (ESUs; Ryder, 1986) by comparing the level of molecular singularity of a group (should it be a clade or not being a matter of discussion; see Fraser and Bernatchez, 2001) with its level of endemism and immediate probability of extinction.

Here we propose that the four haplogroups nested within clade F should be considered as four ESUs. Indeed, we have shown that their genetic diversity is unevenly distributed within the two clades F and S (Figs 3 and 7B). Vicariance is expected to be the leading factor in the high level of structuring observed in clade F. The bifurcating pattern we observed in this clade favors the key role of forest fragmentation as an

agent of isolation for forest elephant populations. As the four haplogroups nested within clade F occupy ranges with little overlap, it adds impetus to the theory of forest refuges in central Africa (Crowe and Crowe, 1982; Hamilton, 1982). Reciprocally, the homogeneity of clade S and its long internal branch advocate for its recent foundation and expansion throughout Africa (Eggert et al., 2002; Nyakaana et al., 2002). If true, it might also account for the lack of specimens of the *cyclotis* morphotype with a S haplotype.

In all cases, the five haplogroups found within *L. africana* clearly constitute a level to deal with in conservation planning, rather than the species or subspecies themselves. By deciphering the structure and remoteness of the four haplogroups in equatorial Africa (all but haplogroup 3; Fig. 7B), in conjunction with their critical demography (Barnes et al., 1999), we stress that better planning conservation in this area is a matter of urgency.

Thus, considering that the African elephants belong to a single species is not in any case a way of denying the importance of preserving subordinate entities. It indicates that the relevant level to focus on, in conservation biology, is the variability below the species level, rather than any index of species richness.

Acknowledgments

First and foremost I am grateful to Prof. Pascal Tassy and Prof. Jeheskel Shoshani for critical readings and assistance in the processing of this manuscript. Prof. Tim Crowe and an anonymous reviewer of a former version of this manuscript are thanked for their accurate critiques. I thank Prof. Wim van Neer and Wim Wendelen from the Musée Royal d'Afrique Centrale (Tervuren, Belgium), for sampling possibility on the MRAC collection. I am also grateful to Prof. Daniel Robineau, Francis Renoult, Prof. Philippe Tranier and Jacques Cuisin for the sampling facilities in the collections of the MNHN (Paris, France). All zoological gardens cited, and Dan Koehl, are thanked for their help. Ludovic Momont and Prof. Pierre Pfeffer are thanked for sampling the Lope and Zakouma carcasses. This work was supported by the Service de Systématique Moléculaire of the MNHN. I thank Prof. Simon Tillier for funding and Annie Tillier for assistance.

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Appendix

Primer designation	Primer sequence
Long fragment	
L16026 E	5'-GCCCTAACACAGTCAAGCAAC-3'
H00007 E	5'-CATTAACGAGATGGGGTAGAA-3'
L00023 +	5'-GCAAGGTACTGAAAATACCTAGACGA-3'
L00151 +	5'-CTAAATCATCGCTGATCAAAGAGAG-3'
H00457 +	5'-AGTCACTTTTCGTAGGCTATTTTGTC-3'
L00521 +	5'-GCCCTAAACTTTGATAGCTACCTTT-3'
H00699 +	5'-GAAGATGGTGGTATATGGACTGAATT-3'
H01075 +	5'-TCTTCTGGGTGTAGGCCAGATGCTTT-3'
Long fragment	
L14096*	5'-GCTTGATATGAAAAACCATCGTT-3'
L14147 E*	5'-ATGACCCACAYYCGAAAATCTCA-3'
L14283 E*	5'-TAACAGGATTATTCCTAGCCA-3'
H14349 E*	5'-TGGGATATAGATGAAAATGCA-3'
L14421 E*	5'-TCTGCCATACACACACATTTGGA-3'
H14452 E*	5'-GATGTTCCGTCCAATGTGTG-3'
L14639 E*	5'-TGAGGAGGCTTTTCRGTAGATAA-3'
H14769 E*	5'-GAATTGTTTGAGCCTGTTTCGTG-3'
L14899 E*	5'-AGACCCTGACCACTACATACC-3'
H14946 E*	5'-TGTAGGGGRGTATTTAGTGG-3'
L14981 E*	5'-GCCATCCTACGATCTGTACCA-3'
H15038 E*	5'-TTGATAGGAGTAGGGCTAGGA-3'
L15151 E*	5'-TACATGAATTGGCAGTCAACC-3'
H15283 E*	5'-TTACTTAATGAGGTAGTTTTTCG-3'
L15384 E	5'-CCCAAACTGAAATTCCTTCTT-3'
L15526 E§	5'-CGTGCATCACATTATTTACCC-3'
H15593 E§	5'-GAATATGACTTGACACATTAGTTA-3'
L15750 E§	5'-TACCTACCTCCGAGAACTA-3'
H15769 E§	5'-TGGTTTCTCGGAGGTAGGTA-3'
H16127 E§	5'-TTATGTCTCCGAGCATTGAC-3'

The letters L and H refer to the light and heavy strands of mitochondrial genome, respectively. Associated numbering refers to 5' end position in the complete mitochondrial sequence of *Loxodonta africana* (Hauf et al., 1999). Primers marked with a "+" are taken from Noro et al. (1998), those with a "*" are from Debruyne et al. (2003a) and those with a "§" are from Debruyne et al. (2003b). Other primers are new.