GENETIC VARIATION OF THE FOREST ELEPHANT

Loxodonta africana cyclotis

ACROSS CENTRAL AFRICA

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I dedicate this thesis to my lovely children, Line and Franck-Olivier, my sisters: Louise, Irène, Isabelle and my late sister Yvonne; to my brothers: Freddy, Richard, William, Christian, Hughes and Gilles; to my mother Georgeline and my father John for their love, support and encouragement; finally to all my nieces and nephews.

Akewa m'polo!

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SUMMARY

The first comprehensive genetic study of central African forest elephants (CAEs) is presented here based on mitochondrial DNA (mtDNA) and nuclear microsatellite loci. MtDNA analysis revealed low genetic divergence between most groups. Haplotype distribution was not correlated with geographical localities, indicating high levels of gene flow. Two divergent haplogroups, illustrated by a bimodal distribution of pairwise differences in the control region, implies that secondary contact and ongoing introgression has occurred between populations expanding from at least two putative glacial refugia. Similarly, microsatellite analysis revealed low genetic differentiation among sites, suggesting high levels of gene flow as well as regional admixture with two genetically-based clusters inferred from Bayesian analyses. It is important to note through, that although both mtDNA and microsatellites identified two groups or genetic clusters, assignment of individuals to these clusters was not consistent across genomes possibly a result of differential admixture in nuclear and mitochondrial DNA No correlation was found between genetic and geographical distances for both genomes.

Previous phylogenetic analyses, using either on genetic or morphological characters, were based on a very limited number of forest elephant samples. A large-scale re-assessment of mitochondrial DNA diversity in CAEs compared to published data on both forest and savannah forms revealed a complex phylogeographic history for African elephants, and an evolutionary trajectory more complex than prevailing two-taxon models have assumed. Mitochondrial control region and Cytochrome b sequences were analysed for CAEs and compared to other African elephant data. CAE populations fell into at least two lineages with West African elephants (both forest and savannah) sharing their mitochondrial history almost exclusively with Central African forest elephants. Extant African elephant populations therefore seem to have originated from multiple refugia lineages that have subsequently undergone introgression. Thus, the complex phylogeographic history of African elephants does not support a simple two-taxon model and management strategies incorporating the two-taxa model could be misinformed until further data give clarifies the origins of elephant populations throughout Africa.

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CHAPTER 1

General Introduction

1.1 Evolution of Proboscidea

Living elephants comprise the only remaining family (*Elephantidae*) in the Order Proboscidea, so named because of their most distinguishing feature, the proboscis-like trunk, although the term pachyderm meaning "thick skin" is also used to describe elephants. The ancestors of modern elephants first emerged 60 million years ago in the Paleocene and from this time to the end of the Pleistocene, 10,000 years ago, the Proboscideans underwent a spectacular radiation and occupied extreme environments, from swamps, tundra, boreal forests, deserts, to savannas, tropical rainforest and from sea level to high elevations (Sukumar, 2003). Their fossils have been found all over Africa, Eurasia and the Americas (Spinage, 1994), providing evidence of a remarkable, flourishing evolution (Kingdon, 1979). This radiation was accompanied by large morphological changes including gigantism and dwarfism, and a change in dentition, which was driven by climate change and the consequent variation in vegetation. As a result, adaptations in anatomy and physiology of the ancestral proboscideans were as diverse as the range of habitats they occupied (Kingdon, 1979; Sukumar, 2003). Today, only two major lineages of elephants exist, the African elephant (*Loxodonta sp*) and the Asian elephant (*Elephas sp*).

In the past, a larger number of proboscideans existed, including the mammoths, stegodons, phiomia and deinotheria. The very first proboscidean-like creature was called *Phosphatherium* whose fossil was discovered in phosphate deposits in Morocco, appearing 60 million years ago during the Paleocene (Gheerbrant *et al.*, 1996). By the early Eocene (55 million years ago), the global climate had become warmer and tropical

forests expanded into the polar Arctic and Antarctic. Most of the mammalian orders known today emerged during this period (for example primitive deer, the earliest horse and lemur-like creatures). Between the early and the middle Eocene, several proboscideans prospered (Figure 1.1), such as *Moeritherium,* a hippopotamus-like creature with incipient tusks living in marshy habitat (Sukumar, 2003). Towards the end of the Eocene, the global climate became drier, creating new types of vegetation. During this period the proboscideans began a general increase in body size (from *Moeritherium*) and developed prominent ridged teeth leading to the emergence of an elephant-like animal named *Barytherium* (Sukumar, 2003). The Oligocene Epoch (36 million years ago) was more climatically stable and marked by seasons. During this time, the *Paleomastodon* emerged, which from fossil records appears to have been two metres tall and was an inhabitant of forest and open woodland. *Paleomastodon* gave rise to two proboscidean branches: the mammutids and the gomphotheres (Spinage, 1994). The latter of which, the gomphotheres, are the sister-groups of modern elephants (Sukumar, 2003). The Miocene (24 million years ago), mostly characterised by a warm and dry climate, was a time of substantial geological changes with the creation of the seas such as the Mediterranean, the formation of mountain ranges and the establishment of climatic patterns with an emphasis of contrast between hot and cold in higher and lower latitudes.

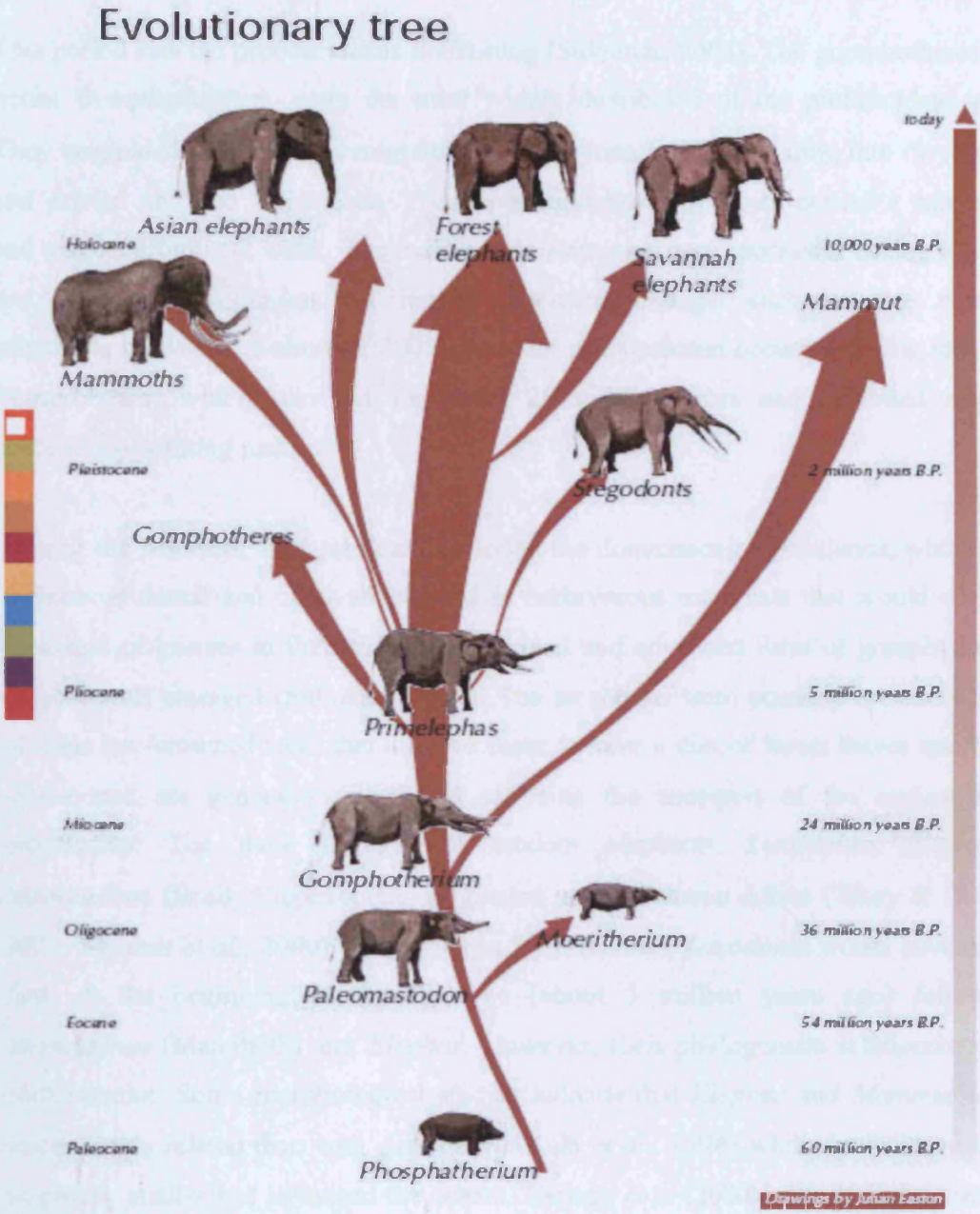

Figure 1.1. Tree representing the evolution of diverse forms of extinct and living proboscidean (Blake, 2007).

This period saw the proboscideans flourishing (Sukumar, 2003). The gomphotheres, of the genus *Gomphotherium*, were the most widely distributed of the proboscidean groups. They originated in Africa and migrated to all continents, differentiating into diverse forms and existed until the Pleistocene. Their upper and lower lips both carried a pair of tusks and a short prominent trunk. The mammutids were also very successful during this period and fossil records suggest that their dentition had changed such that they were well adapted to browsing (Sukumar, 2003). Another proboscidean occurred during this period, *Deinotherium,* which survived for about 20 million years and exhibited a pair of descending-pointing tusks.

During the Miocene, increasing aridity led to the dominance of grasslands, which in turn influenced dental and other adaptations in herbivorous mammals that would enable the inclusion of grasses in their diet and a derived and advanced form of gomphothere, the stegodontids emerged (Sukumar, 2003). The stegodons were possibly browsers because of their low-crowned teeth that allowed them to have a diet of forest leaves and bamboo shoots and are generally recognised as being the ancestors of the earliest African elephantids. The three lineages of modern elephants, *Loxodonta*, *Elephas* and *Mammuthus* (family Elephantidae) originated in sub-Saharan Africa (Tassy & Debruyne, 2001; Thomas *et al.*, 2000). According to fossil records, *Loxodonta* would have diverged first, at the beginning of the Pliocene (about 5 million years ago) followed by *Mammuthus* (Mammoth) and *Elephas*. However, their phylogenetic relationship is still controversial. Some morphological studies indicate that *Elephas* and *Mammuthus* were more closely related than with *Loxodonta* (Kalb *et al.*, 1996) while further investigation in genetic studies had advanced the debate. Thomas *et al* (2000) supported the mammoth-African elephant clade in common with Tassy & Debruyne (2001). In Thomas *et al* (2000)'s study they did not find sufficient statistical support to reject the *Mammuthus – Elephas* clade , while Rohland *et al* (2007), with a complete mitochondrial genome of the *Mammut americanum*, show that the ancestors of African elephants diverged from the mammoth-Elephas clade approximately 7.6 million years ago. In the same study, African savannah and forest elephants were shown to have diverged approximately four million

years ago. But at this time *Loxodonta* was well differentiated in Africa with the first recognizable species, *Loxodonta adaurora* (Sukumar, 2003). While the Pliocene saw the three modern genera flourishing, the Pleistocene (less than 1 million years ago) was the period of their greatest divergence (Kingdon, 1997). Pleistocene was also a time that saw the extinction of the gomphotheres and mammutids (Sukumar, 2003).

During the Pleistocene (about 2 million years ago) the world was characterised by alternating glacial and interglacial phases: intense periods of cold interrupted by warmer episodes. This epoch led to the migration of mammals and consequently to their evolution (Sukuma r, 2003). While the genus *Loxodonta* never left the African continent, *Elephas* migrated to Asia and eventually disappeared in Eurasia and Africa 20,000 years ago. *Mammuthus* went extinct at the end of the Pleistocene and the beginning of the Holocene (approximately 10,000 years ago) with some populations surviving until very recently (Sukumar, 2003). During the Holocene, forests expanded in the tropics with warmer climate and high rainfall. *Loxodonta* by this time was very widespread throughout Africa and was present from the lower Nile area throughout the North Africa boarding the Mediterranean (Sukumar, 2003).

1.2 The Taxonomy of modern elephant: *Loxodonta*

Taxonomy in pachyderms has been and is still a controversial subject based on fossil remains and morphological differentiation. The taxonomy of modern *Loxodonta* remains rather unclear. In terms of nomenclature, Linné in 1758 classified Asian and African elephants in a single genus, *Elephas* , while later in 1797, the German naturalist Blumenbach distinguished two subspecies of elephant *Elephas africanus* and *Elephas asiaticus* based on differences in tooth patterns (Spinage, 1994). The following year (1798) Cuvier reconsidered Blumenbach's distinction finding sufficient differences in the dental patterns that he created the new genus *Loxodonta* for the African elephant (Spinage, 1994). Meanwhile, Illiger in 1811 created *Proboscidea* to name all elephant families in a single Order. Elephants were successfully adapted to a variety of ecological

conditions, developing numerous traits in body size, ear, tusk and skull shape, skin texture and colour. Subsequently, 25 subspecies of elephants have been described (Kingdon, 1997).

However, Matschie (1900) reduced the number of the so-called subspecies of African elephants considerably when he divided them into four, based on geographical location, ear and skull morphology. The subspecies he described were (1) *Loxodonta africana africana* (Blumenbach, 1797) from the Cape, (2) *L. a. knochenhauri* (Matschie, 1900) from Tanganyika, (3) *L. a. oxyotis* (Matschie, 1900) from eastern Sudan and (4) *L. a. cyclotis* (Matschie, 1900) from southern Cameroon. This view persisted until the 1940s when two subspecies of the African elephants were generally recognized: *L. a. africana*, the larger bush or savannah elephant, and *L. a. cyclotis*, the smaller forest elephant. Much debate has occurred about the taxonomy of the forest elephant leading to a proposed reclassification as a new species based on morphological characters (Grubb *et al.*, 2000) and genetics (Roca *et al.*, 2001) (discussed in more detail in Chapter 4). There are also cranial and social differences (Grubb *et al.*, 2000; White *et al.*, 1993): savannah elephants have a social organisation consisting of one or more related adult females and their offspring while the mother-offspring unit is the most frequent family unit for forest elephants (White *et al.*1993). Their diet and habitat have also been compared. Elephants in east and southern Africa, for instance, have a diet dominated by grasses in open grassland areas while their forest counterparts at the Lopé National Park have a diet including diverse items such as leaves, bark and fruit (White *et al.*1993). However the two forms do interbreed in places where their habitats come into contact (Kingdon, 1997; Spinage, 1994). The African Elephant Specialist Group believes that premature allocation into more than one species may leave hybrids in an uncertain taxonomic and conservation status (Blanc *et al.*, 2003). Therefore, the World Conservation Union (former International Union for Conservation of Nature), IUCN, currently recognizes *Loxodonta africana* as a single species encompassing both forest and savannah populations (African Elephant Specialist Group, 2004). Based on the findings from this study and IUCN one-species recognition, only *L. a. cyclotis* and *L. a. africana* will be used to name forest and savannah forms, respectively.

1.3 Morphological differences

1.3.1 African elephant vs Asian elephant

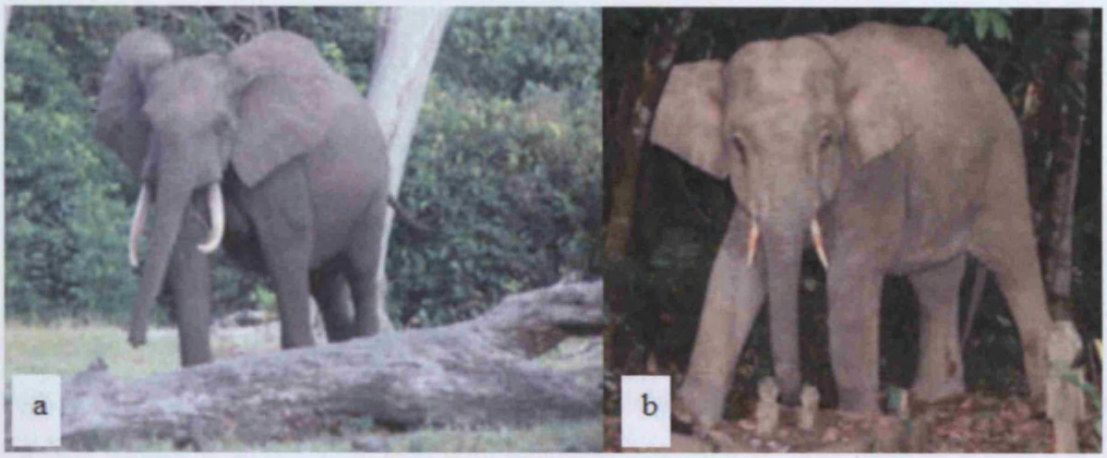

Figure 1.2. Morphological differences between a) African elephant (forest type) and b) Asian elephant. Note ears shape and size differences (proportionally to the head), also forehead differences (photos: B. Goossens)

Two genera remain today: *Loxodonta* and *Elephas*, the African and the Asian elephant respectively. The most obvious difference between African and Asian elephants is the size of their ears (Figure 1.2 above). However there are other morphological differences, some are listed below:

African elephant (savannah type: Grubb *et al.* 2000; Kingdon, 1997)	Asian elephant (Shoshani & Eisenberg, 1982)
Sub-Sahara region of Africa	Southeast of Asia
Larger ears	Smaller ears
Bulls can weigh up to 7 tonnes and reach 4 m at the shoulder	Large bulls weigh 5.4 tonnes and are 3.20 m at the shoulder
Females weigh up to 3.5 tonnes and reach 3.4m at the shoulder height	Females average weight is 2.7 tonnes and average height is 2.24 m

Tusks in both sexes	Tusks mostly in males
Two finger-like on the trunk tip	One finger-like on the trunk tip
"Floppy" trunk	Trunk more rigid
4 nail-like on forefoot and 3 on rear foot	5 nail-like on forefoot and 4 on rear foot
Concave back	Convex back

Three subspecies of Asian elephants were recognized (Shoshani & Eisenberg, 1982):

- *Elephas maximus maximus* from Sri Lanka,
- *E. m. indicus* from the Asian mainland,
- *E. m. sumatrensis* from Sumatra.

Recently, genetic data on Bornean elephants support their recognition as a unique subspecies: *Elephas maximus borneensis* (Fernando *et al.*, 2003b). Two subspecies of the African elephants were recognized until recently:

- *Loxodonta africana africana*, the larger bush or savannah elephant,
- *L. a. cyclotis*, the smaller forest elephant.

1.3.2 African forest elephants Vs African savannah elephants

The African forest elephant is distinguished from its savannah counterpart by its small size, 2.4 to 3.0 m tall at the shoulder in the male and 1.8 to 2.4 m in the female, and a weight of 2.0 to 4.0 tonnes (Grubb *et al.*, 2000; *Kingdon*, 1979; Spinage, 1994). It has characteristic rounded and small ears, unlike the "map of Africa" shape of the savannah elephant, and almost straight, downwardly pointing tusks while its counterpart has upwardly curved tusks (see Figure 1.3). Its body is more compact and nearly straight compare to the more slender body of its counterpart with a concave back. The savannah elephant has a higher carriage of the head, which is low in the forest elephant. Grubb *et al.*, (2000) portrayed differences at the skull level with a more flared rostrum in savannah than in forest elephants. Forest and savannah elephants are also distinguishable in their behaviour and ecology. The former occurs in moist semi-deciduous and rainforest, and it is mostly a browser and frugivore rather than the grazer and browsing elephant found in

arid woodland and savannah (Grubb *et al.*, 2000). The forest elephant has a nuclear family of 2 to 4 individuals, while savannah elephant family groups can extend up to 14.

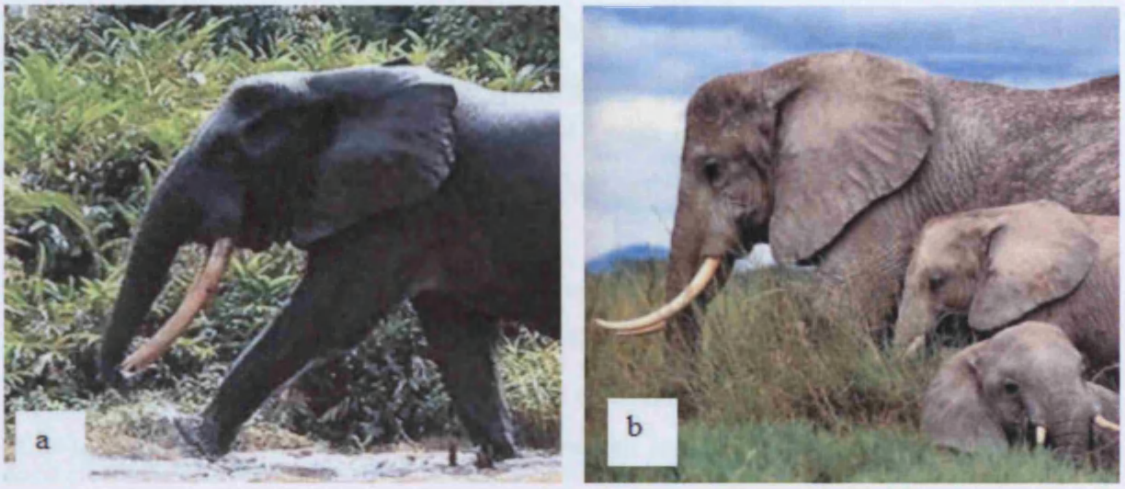

Figure 1.3. Morphological differences between a) African forest elephant and b) African savannah elephant. Note mainly, the ears and tusks shape dissimilarities (Photos: a) from M. Cazemajor; b) from Google)

1.3.3 West African elephants

Based on morphological features, Frade (1955) separated forest and bush elephants into two different species. However elephants from west Africa could not be determined as an individual could have some traits from both types forest and bush elephant. These elephants are taxonomically indeterminate (Frade, 1955) and have been described as having an intermediate morphology (Groves, 2000). Alternatively, Eggert *et al.* (2002) proposed the west African elephant as a third taxonomic group as their study revealed that west African populations are genetically distinct from other forest and savannah elephants.

1.3.4 The distribution of *Loxodonta*

During the Holocene, *Loxodonta* was widespread throughout Africa, including North Africa (Spinage, 1994; Sukumar, 2003). Today, *Loxodonta* is found in sub-Saharan Africa ranging from west African coastal regions, through the equatorial rainforests of the Congo Basin and savannah woodlands of South and East Africa, mountains of East Africa, to semi-desert in Namibia and Mali (Spinage, 1994).

Illegal hunting for ivory has massively reduced the number of African elephants over the past three decades. From the five to 10 million estimated in 1930 (Stuart and Stuart, 1997), only 600,000 are estimated to have remained by 1992 (Spinage, 1994; Stiles, 2004). Elephants in West Africa have suffered an intensive slaughter for their ivory for many centuries, during which international trade was well established, supplying North Africa, Europe and North America with ivory (Barnes, 1999). The intense hunting, rapid growth of the human population and consequent loss of habitat and these factors combined, led the elephants of West Africa in an decline from which they have never recovered (Blake, 2007). Today, elephants are found in fragmented zones with isolated populations, which are vulnerable to poaching and general human disturbance such as accessibility of remote forests by roads, railways, navigable rivers, plus forest destruction and fragmentation (Barnes, 1999; Blake, 2007). Consequently, African elephant populations became fragmented and its distribution is mainly concentrated in Central, East and northern parts of Southern Africa (Figure 1.4).

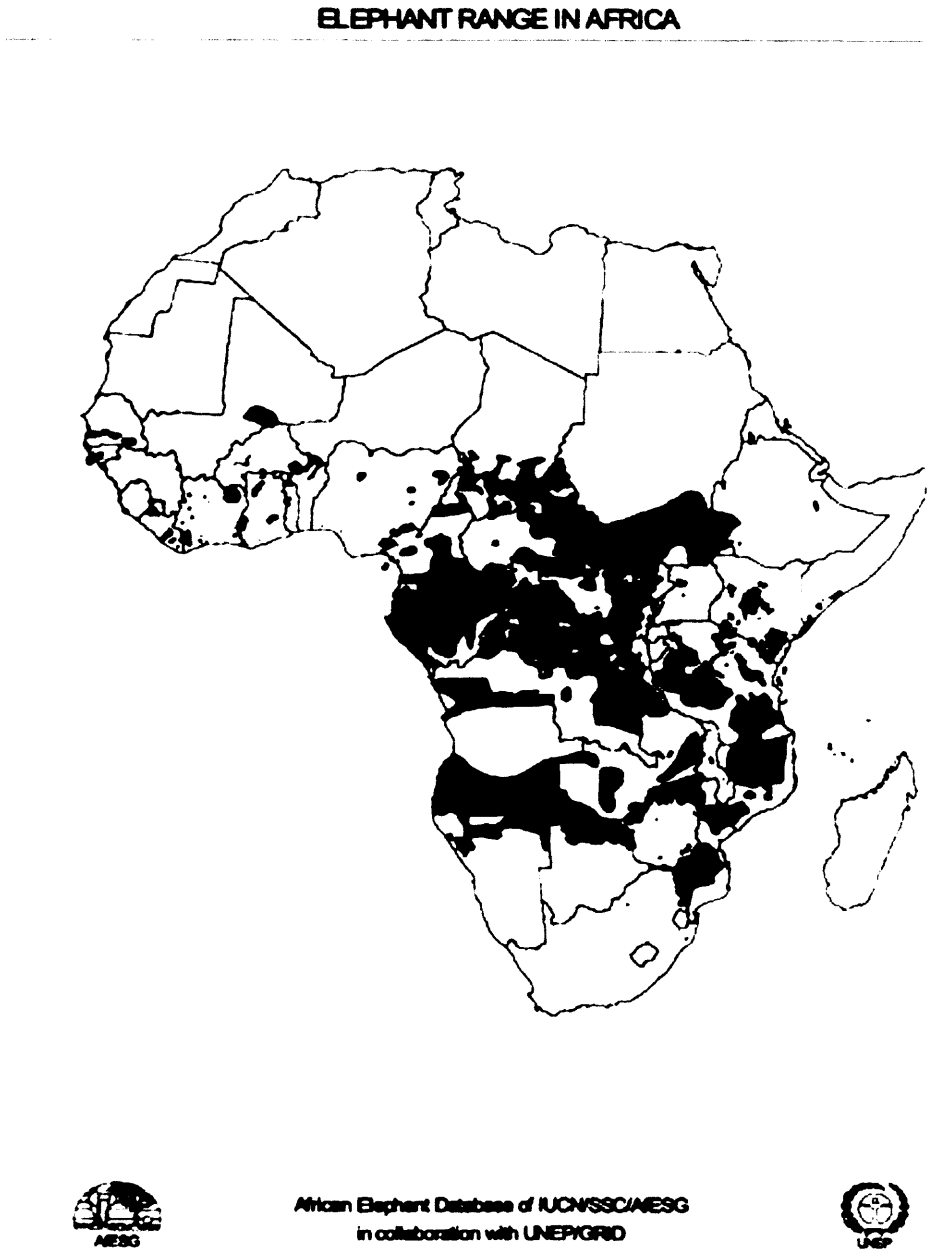

Figure 1.4: Map of the distribution of African elephants (from African Elephant Database IUCN/SSC/AfESG in collaboration with UNEP/GRID.

1.4 The central African forest elephant: *L. africana cyclotis*

Found in the tropical lowland forest of central Africa, the forest elephant, unlike its counterpart of the savannah, has been the subject of relatively few studies because visibility is often restricted either at ground level or overlapping canopies (Tutin *et al.*, 1997). Consequently, elephant population structure, social organization and behavioural information are based mainly on data obtained from savannah elephants. Forest elephant population studies have been based on tracks (Blake *et al.*, 2007; Morgan, 2007), dung analysis (Barnes *et al.*, 1997; Eggert *et al.*, 2003; Fay, 1991; Walsh & White, 1999; White, 1994), feeding ecology (Morgan & Lee, 2007; White *et al.*, 1993b), and satellite tracking (Barnes *et al.*, 1997; Blake, 2007; Blake *et al.*, 2001) to determine their movements and range. Forest elephant populations, particularly in Central Africa are difficult to monitor because of a crucial lack of infrastructure, staff and funding (Walsh & White, 1999).

Direct observations are extremely difficult for individual identification, behavioural studies, and habituation (Turkalo & Fay, 1996; Turkalo & Fay, 2001). Researchers of populations in African savannahs (Archie *et al.*, 2007; Archie *et al.*, 2006; Lee & Moss, 1986; Moss, 2001) and Asian dry forests (Fernando & Lande, 2000) do not encounter these types of issues. However the discovery of clearings in the African forest habitat, called *bais*, which are often salt-licks or serve as other sources of nutrients, has made field observations less difficult (Momont, 2007; Turkalo & Fay, 1996). Other natural open habitats such as the mosaic forest-savannah zones found at the Lopé National Park in Gabon (Momont, 2007; White *et al.*, 1993b), or the coastal habitat at Loango National Park, Gabon where free ranging forest elephants have been observed (Morgan & Lee, 2003) are also used for observational studies.

In general, indirect study methods are used for census, observation and genetic studies of elephant populations occurring in dense woodlands and forests. Mark-recapture methods have been suggested (Morley & Van Aarde, 2007) as a reliable estimator of population size for elephants in habitat with low visibility, while Wood *et al.* (2005) proposed an

accurate and precise census technique using a seismic sensors (geophone) to record footfalls of elephants. Most genetic studies rely on non-invasive sampling methods (Goossens *et al.*, 2003) for which field-workers do not need to see individual animals but rather collect their remains. Many genetic studies on diverse species with the aim to investigate genetic structure, population size or geographic distribution were based on dung (Fernando *et al.*, 2000; Goossens *et al.*, 2000; Okello *et al.*, 2005b; Zhan *et al.*, 2006), hair (Anthony *et al.*, 2007a; Clifford *et al.*, 2002; Goossens *et al.*, 2005; Jeffery *et al.*, 2007; Jensen-Seaman & Kidd, 2001), feathers (Segelbacher, 2002; Taberlet & Bouvet, 1991); with museum samples, phylogeographic studies can even be done without going to the field (Debruyne, 2005; Moodley & Bruford, 2007).

1.4.1 Distribution

Since the Pleistocene, *L. a. cylotis* has been tied to the humid forests of Central Africa (Sukumar, 2003) in spite of the numerous cyclical phases of forest contraction and expansion typical of the period. In fact it is known from the fossil record (Kingdon, 1979) that the *Elephas recki* lineage dominated a wide range of the African continent, except tropical forest areas, throughout the entire Pleistocene period. Therefore, *Loxodonta* was probably restricted to the forest and retained adaptive features (e.g. small size and small family units). Forest elephant habitat in central Africa covers almost 2 million km^2 and it was believed to be a vast and remote refuge for the 172,400 elephants estimated to exist in the late 1980s (Blake, 2007). The Democratic Republic of Congo (DRC) and Gabon were estimated to be home to approximately 64,000 and 60,000 elephants, respectively. In total one third of Africa's elephants can be found into the dense forest of the Congo Basin in central Africa, which ranges from Cameroon, Gabon, Equatorial-Guinea, Congo, Central African Republic, and DRC, and almost all elephants from those areas are forest populations (Blake, 2007).

1.4.2 Ecology– Habitat / Diet

Forest elephants are often documented to be generalist feeders because they consume a wide variety of plants. Their feeding behaviour depends largely on the seasonal availability of food. Forest elephants, for instance, in Lopé National Park (Gabon) have a diet comprising of, at least, 307 separate items, most of which are leaves and bark (White *et al.*, 1993). Fruit is also an important part of the diet in tropical forest habitats. At least 72 species of fruits are consumed at Lopé (White *et al.*, 1993) and elephants need to move in small groups to be able to access patchily available resources. This contrasts with the diet of eastern and southern savannah elephants where grasses dominate.

The diet in a population may be influenced by the specific needs of younger versus older individuals and by females versus males (Momont, 2007). In the coastal habitat at Loango NP (Gabon), elephants consumed fruits of at least 49 species (Morgan & Lee, 2007). Studies by Barnes *et al.* (1991) and Olivier(1978) showed that both African and Asian elephants have a preference for secondary forests. Elephants are attracted to this type of forest by the greater diversity of food plants, which grow faster and have less toxins and tannins (Barnes *et al.*, 1991). The study by Barnes *et al.* (1991) also found higher concentrations of elephants in marshes during the dry season. In Lopé National Park (Gabon) where both forest-savannah mosaic and savannah marsh habitat can be found, females with their dependant offspring have been shown to have a high preference for savannah habitat (Momont, 2007).

In addition, high densities of elephants in Lopé regularly used marantaceae forests to feed on herbaceous plants (White *et al.*, 1993) and forests where the tree *Sacoglottis gabonensis* is the dominant species during fruiting (Momont, 2007; White, 1994). Gallery forests and woodlands are also used for feeding on leaves and small shrubs. Momont's (2007) study suggested that elephants at Lopé strategically use their diverse habitat according to their food needs with seasonal shifts. He mentioned that at Ivindo National Park, elephants move between their core area in mature forest and the clearings to feed on mineral salts (Turkalo & Fay, 1996; White *et al.*, 1993). Hence, elephant movements are

driven by food availability in different types of habitat and by seasonal fluctuations. They structure their habitats by creating network of paths (Turkalo & Fay, 1996) which are used regularly for long distance migrations and for foraging (Vanleeuwe & Gautier-Hion, 1998).

1.4.3 Reproduction

A long-term and complete study of elephant life history could take over six decades as elephants are long-lived mammals with a relatively long period before sexual maturity and a slow rate of reproduction (Moss, 2001). Elephants, as with most mammals, are polygynous: males mate with several females (Sukumar, 2003). Puberty in the male occurs (both Asian and African) between eight and 15 years of age while sexual maturity occurs between two to three years later (Sukumar, 2003). Sexual maturity in the bull elephant dictates its social behaviour. An adult bull secretes a fluid from its temporal gland and may constantly trickle urine. This phenomenon is called *musth* and has been well documented in Asian elephants since ancient times (Sukumar 2003). The adult male in musth lives a period of intense aggression toward other males and has a sexual interest in oestrous females. Asian and African elephants in musth express the same variable behaviours, postures, vocalizations and urine excretion (Sukumar, 2003). There is at least one bull in musth at any given time of the year (Sukumar, 2003). Musth, as well as tusks, may act as sexual characters that influence female choice of mates (Sukumar, 2003). In the African female elephant, puberty is attained somewhere between 9-18 years of age, with 12 years being the average (Moss, 2001) while in the Asian elephant puberty in the cow is estimated to occur between 16 and 18 years (Spinage, 1994).

The interval between birth and the next conception varies from nine months to about four years in the African savannah elephant (Moss, 2001) and is between 3.5 and 4 years in forest elephants at Dzanga clearing in CAR (Turkalo & Fay, 2001). Pregnancy lasts between 20 and 22 months and elephants usually bear a single young. Weaning is a very gradual process, which begins during the first year of life. The African cow has a

reproductive life until the age of 52 or a total period of 40 years (Spinage, 1994) with a maximum female lifetime of 65 years (Moss, 2001).

1.4.4 Social structure

The elephant is considered to have one of the most advanced mammalian social organizations (Sukumar, 2003). The size of elephant groups (close spatial associations of members of a population) is a product of social evolution, habitat features, resource availability and dispersion, seasonality, and levels of human disturbance (Sukumar, 2003). The basic unit of the elephant social structure is the mother-offspring association. This is the most common family unit for forest elephants at Lopé NP and Dzanga clearing (Turkalo & Fay, 2001; White *et al.*, 1993a). Males are generally solitary. The mean group size in forest elephants is 2.7 to 3.1, excluding solitary individuals (Dudley & Mensah-Ntiamoah, 1992; Merz, 1986; Morgan, 2007; Turkalo & Fay, 1996; Turkalo & Fay, 2001; White *et al.*, 1993b), which is generally smaller than those recorded in savannah populations (Douglas-Hamilton, 1972; Rugiero, 1989). In Amboseli National Park, the mean group size was 15.1 during a drought year when food was scarce and 45.9 in a rainy year with abundant food (Moss, 1988) This can be characteristic of a fission-fusion social system. In other mammals, members of the same group form frequently changing subgroups, for instance in wild communities of chimpanzees (Lehmann & Boesch, 2004) and orang-utans (Van Schaik, 1999). The major benefit to grouping is principally social with mating opportunities, protection and socialization of infants (Van Schaik, 1999). In savannah areas elephants tend to aggregate when fresh grass is abundant (Moss, 1988; Rugiero, 1989; Western & Lindsay, 1984) unlike forest elephants which do not aggregate even when their preferred food is available and abundant (Turkalo & Fay, 1996). Further, sub-adults and juveniles of both sexes have been observed roaming without familial ties (Turkalo & Fay, 2001).

Most African populations show a higher number of adult females than males, which may be due to selective human hunting. However in the Dzanga population in Central African Republic, Turkalo and Fay (2001) observed that the number of females and males visiting

clearings were almost equal (733:665), which may indicate that both sex are equally hunted.

The social group allows the development of social interactions between young calves and between calves and older elephants. These interactions can be friendly or unfriendly, particularly when involving males associated temporarily with a group, and tend to initiate aggressive behaviour toward young elephants (Turkalo & Fay, 1996). In Dzanga clearings, certain young males rejoin their maternal groups occasionally and do not remain consistently in any group. While they are in the clearing they associate temporarily for few minutes to a day (Turkalo & Fay, 1996). The interactions between individuals include a wide repertoire of informative acts, which are used to communicate (Sukumar, 2003). These include physical, visual, auditory, and chemical signals. Studies in east and southern Africa have revealed coordinated movements of elephant groups and the maintenance of contact using infrasonic calls over distances up to 5 km (Poole *et al.*, 1988). Minerals in *bais* (clearings) attract elephants but possibly social activity is also a major attraction (Turkalo & Fay, 1996). Young elephants can learn about interaction activities, females have a better chance to be inseminated by prime bulls, and males can establish a dominance hierarchy. This social behaviour, in general, is expressed in a familiar area that is determined by the movement of the matriarch. In savannah elephants, it has been demonstrated that the oldest female or matriarch, which leads the family units, is the repository of enhanced discriminatory abilities of the whole group (McComb *et al.*, 2001), which may derive to a higher fitness benefits for female groups, an access to resources, a lower risk of predation/conflict with humans by avoiding unprotected areas, and a lower expenditure of energy during the dry season (Wittemyer *et al.*, 2007). This social discrimination could harm the family group, as matriarchs are mainly the target of hunters or poachers because of their large size (McComb *et al.*, 2001). Given that in forest, there is no predation beside humans (Turkalo & Fay, 2001), food is patchy though abundant in fruiting season, forest elephants are more optimally spread in small numbers in order to avoid food competition (Turkalo & Fay, 2001).

1.4.5 Movements

The ranging behaviour of elephants is strongly influenced by their need for water and forage of a certain type and quality. In habitats with low and medium-rainfall, from desert through to semi-arid savannahs and woodlands to deciduous forests, the home-range of elephants shrinks in the dry season and increases during the wet season (Sukumar, 2003). Knowledge of elephant movement in tropical rain forests is very limited. The study in Dzanga NP revealed that in the Dzanga Sangha-Nouabale Ndoki complex, elephants migrate from the north-west to the south-east in an annual cycle (Blake *et al.*, 2001). One female African forest elephant was tracked by GPS telemetry in the rain forest of the Dzanga-Sangha region of CAR and Congo, and ranged over 880 km^2 within a year. In Waza National Park (Cameroon), Tchamba *et al* (1995) estimated a mean range of 785 km^2 for resident females and 2,775 km^2 for migrant females. A tracked mother with her infant migrated 2000 km back and forth across the Ndoki forest from Dzanga bai (Central African Republic) to Goualougo Triangle (Congo), whereas a small female in Ivindo National Park had a home range of 52 km^2 (Blake, 2007). However both elephants walked around 6.5 km per day, a comparable distance to that (7.2 km) recorded in a study by Momont (2007) of elephants in Lopé.

Migration appears to be correlated with diet (Turkalo and Fay, 1996; White, 1994) and there are regular tracks as opposed to evidence of random movements in the forest (Vanleeuwe & Gautier-Hion, 1998). A similar regular migration was observed in savannah elephant populations in northern Kenya (Thouless, 1995). Their movements were associated with rainfall between dry and wet seasons. At Odzala, Nouabale-Ndoki and Dzanga-Sangha National Parks in Congo and CAR, long distance elephant movements, were associated with visits to forest clearings for mineral deposits and Marantaceae forests for herbaceous plants as well as fruit (Blake & Inkamba-Nkulu, 2004; Turkalo & Fay, 1996; Vanleeuwe & Gautier-Hion, 1998). Turkalo & Fay (1996) indicated that forest elephant males may be more mobile and migrate from greater distances than females, and probably form the migratory segment of the population and that bull elephants can create their own "home range" when they disperse. Tracked

elephants, at both Lopé and Langoué bai (Ivindo National Park, Gabon), ranged on average 445 km^2 and 615 km^2, respectively (Momont, 2007), much lower than the ranges mentioned above. Momont summarized all previous observations in ranging behaviour but because each study used different methods, a statistical comparison was not made.

1.4.6 Poaching and habitat loss

Elephant populations declined in the 1970s and 1980s because of poaching for ivory (Blake *et al.*, 2007; Stiles, 2004) and approximately 700,000 were killed for ivory trade around the world (Douglas-Hamilton, 1989). The status ofsavannah elephant populations is apparently stable or increasing with generally low poaching rates in Eastern, Southern and western Africa (Blanc *et al.*, 2003). On the other hand, the status of forest elephants in Central Africa is poorly known because of the difficulties to monitor and the lack of logistic necessary for reliable population surveys (Walsh & White, 1999). Barnes *et al* (1995)'s survey shows Gabon to have one of the largest elephant populations on the continent with an estimate of 61,800 ± 20,200 elephants, although there are significant factors, which currently threaten the integrity of those populations. Human population growth, industrial logging, road and infrastructure expansion, all increase hunting pressure (Naughton-Treves & Weber, 2001; Wilkie & Laporte, 2001). Some studies show that roads had significant negative impact on forest elephants as the level of road avoidance increases with hunting pressure (Blake *et al.*, 2007; Laurance *et al.*, 2006). Elephant poaching was the heaviest in Central and Eastern Africa between 1979-2002 (Stiles, 2004) while Southern African elephant populations were growing. Despite the 1989 CITES ban of the African elephant ivory trade, elephant populations continued to decline in some countries such as the Central African Republic, Democratic Republic of Congo and Sudan (Blake *et al.*, 2007; Blanc *et al.*, 2003; Fay & Agnagna, 1991), showing that other poaching determinant factors exist, such as the lack of law enforcement, political stability and good governance (Blake *et al.*, 2007; Stiles, 2004).

Forest cover in Central Africa is close to 2 million km^2 (Blake, 2007) and the Congo Basin was believed to be a vast and inaccessible refuge for elephants compared to West

Africa where elephants have been persecuted for their ivory for centuries. West African elephants were accessible to humans thanks to roads and railways, which led to their eradication following hunting, forest destruction and fragmentation, and human population expansion (Blake, 2007). In Central Africa the situation seems to be the opposite with a larger number of elephants inside a remote vast and dense forest, and a low human population size (Blake, 2007), though little is known on the status of central African forest elephants (Barnes *et al.*, 1991; Blake *et al.*, 2007). This is in contrast to its savannah counterpart, which has been studied intensively for almost three decades (Barnes *et al.*, 1991). In 2003-2004, a regional survey was carried out by the Monitoring of the Illegal Killing of Elephants (MIKE) Programme with the goal of providing information needed for elephants. The results of the programme showed that forest elephant numbers and range have drastically declined around the last twenty years (Blake, 2007). In 1999, a 2000 km continuous survey called the Megatransect, passed through six protected areas from Northern Congo to the Gabonese coast. One of the aims of the Megatransect was to provide information on diversity and abundance of large mammals (Fay, 1999). The Megatransect survey revealed that elephants were more abundant inside protected areas than in the surrounding forest and also showed a positive relationship between abundance and increasing distance from the roads (Fay, 1999). Barnes (1991) found the same relationship in Gabon as did Stromayer & Ekobo (1992) in Southeastern Cameroon, and they proposed that present-day elephant distribution is mostly governed by the distribution of human activities not by the vegetation.

1.5 Justification of the study

After many years of neglect the forest elephant has recently regained the spotlight after a series of genetic studies advocated species status (Roca *et al.*, 2001), rather than being a subspecies of African elephant. Currently the IUCN does not recognize this species status, and still lists the forest elephant as *Loxodonta africana cyclotis*. Morphologically, socially and ecologically, *L. a. cyclotis*, is very distinct from its savannah relative. Cranial, ear and tusk morphology, in addition to overall body size and weight (Grubb *et*

al., 2000), differentiate these two taxa, as do group size, diet and habitat, yet most of our conceptions of elephants are based on the larger, more spectacular and more accessible savannah elephant of the plains of eastern and southern Africa (Kingdon, 1997). Urgent calls for more extensive genetic studies to resolve the taxonomic status of forest elephant types have recently been made (IUCN, 2002).

The status of the forest elephant appears critical, as both habitat destruction and poaching seriously threaten its existence. Countries with low population densities and / or extensive forest cover become key habitat areas for the forest elephant, such as Gabon (White *et al.*, 1993b), Republic of Congo (Fay & Agnagna, 1991), the Democratic Republic of Congo (Alers *et al.*, 1992) and the Central African Repub lic (Fay, 1981). The dense vegetation of the tropical rain forest renders visual contact with forest-dwelling mammals difficult and unpredictable. The discovery of forest clearings (Turkalo & Fay, 2001) has rendered the forest elephant visible and revolutionized our understanding of forest elephant socio-ecology. It plays an important role in determining the structure and species composition of tropical forests (White *et al.*, 1993b). The loss of the elephant from tropical forests would therefore have profound effects on this ecosystem.

In order to improve conservation efforts, we need an improved understanding of their social organization, migration patterns, and the genetic variability within and between central African populations, which can only be brought about through the application of non-invasive genetic techniques. Genetic studies on *L. a. cyclotis* will also reveal important information on the evolution of elephants in Africa, since it appears that the ancestral type resided in forests and that adaptation to drier habitats is relatively recent (Kingdon, 1997). Given the current interest in forest elephants as a potentially new species, and the lack of information on basic ecology and demographics of populations, and the fact that non-invasive genotyping has made considerable progress recently in producing reliable methods for generating phylogenetic and phylogeographic data, it was timely to initiate a study examining the genetic variability within and between populations of the forest elephant across varying ecological settings within their tropical rainforest habitat in Central Africa.

1.6 Genetics

There are a variety of molecular markers, which were used to study population and evolutionary genetics of a wide range of organisms, incorporating also conservation genetic studies of endangered species (Anthony *et al.*, 2007b; Bruford *et al.*, 1996; Fernando *et al.*, 2003b; Goossens *et al.*, 2005; Taberlet, 1996; Zhan *et al.*, 2006).

1.6.1 Choice of molecular marker

Polymerase Chain Reaction using non-invasive samples.

The invention of the Polymerase Chain Reaction (PCR) revolutionized molecular biology and the entire field of population biology allowing researchers to amplify very small quantities and any desired fragment of DNA from almost any biological source including dung (Fernando *et al.*, 2000; Johnson *et al.*, 2007; Kohn & Wayne, 1997; Zhan *et al.*, 2006; Zhan *et al.*, 2007), hairs (Anthony *et al.*, 2007b; Clifford *et al.*, 2004; Jeffery *et al.*, 2007), plants (Born *et al.*, 2006; Muloko-Ntoutoume *et al.*, 2000), feathers (Segelbacher, 2002; Taberlet & Bouvet, 1991), museum material (Moodley & Bruford, 2007; Yang *et al.*, 1996) and even fossils up to several thousands of years old (Cooper & Drummond, 2004; Cooper & Poinar, 2001; Gilbert & Wilson, 2004; Thomas *et al.*, 2000).

PCR also comes with several disadvantages including mis-incorporation of nucleotides, recombination among the amplification products, failure of PCR reactions for many reasons and contamination issues resulting in interpretive errors. Some PCR problems occur due to the small quantities of DNA used during genetic typing of ancient samples, forensic samples, museum specimens, hair, and faecal samples of free ranging animals. These non-invasive samples, usually from species of conservation concern, generate (i) the possibility of not detecting alleles in individuals and (ii) the problem of PCR-generating false alleles (Taberlet *et al.*, 1996). As PCR is powerful enough to amplify

small target DNAs millions of times, it is essential to avoid degraded or contaminated template. More and more experimental procedures have been developed to produce reliable genotyping results (Fernando *et al.*, 2003a; Goossens *et al.*, 2000; Goossens & Waits, 1998; Morin *et al.*, 2007; Taberlet *et al.*, 1996).

Mitochondrial DNA (mtDNA)

Genetic markers such as mtDNA have been widely utilized in phylogeography since the late 1970s, and since the advent of molecular techniques genetic analysis has become more feasible, cheaper, and less time consuming. Animal cells contain several hundred mitochondria each comprising a circular DNA molecule of 15-20 kilobases (kb) in length and composed of 37 genes coding for 22 tRNAs, 2 rRNAs, and 13 mRNAs, a "Control Region" (CR) or D-loop of about 1 kb, which initiates replication and transcription (Avise, 1994). The entire mtDNA genome is involved in the coding function without introns, large families of repetitive DNA and pseudogenes.

Animal mtDNA is maternally inherited (from mother to offspring) in most species but several exceptions to strict maternal inheritance are known, for example marine mussels (*Mytilus*) where "paternal leakage" is common (Avise, 1994). In addition, mtDNA does not recombine and is passed from mother to offspring as a single entity therefore making it especially useful as a genetic marker.

Gene arrangement is generally stable in mtDNA but some variation can appear that distinguishes higher animal taxa. Mitochondrial DNA normally evolves rapidly at the sequence level, and control region has a high mutation rate and is highly variable, allowing us to specifically trace female lineages, or migration patterns and also to distinguish taxa (Frankham *et al.*, 2003).

The biology of the mitochondrion differs substantially from the nuclear genome and this affects the pattern and process of its evolution. For example, the mitochondrial genome is about only 0.00055% of the total human genome in size so it is untenable to infer general

patterns from a small particular fraction of the genome (Ballard & Whithlock, 2004). The mitochondrial and nuclear genomes have other differences such as the ploidy, mode of inheritance, degree of recombination, number of introns, effective population size, and mutation rate (Scheffler, 1999). Ballard and Whithlock (2004) assert that the lack of recombination in mtDNA means that the entire molecule has a single history determined by mutation and selection, and that can infer only one part of the true story of the species since mtDNA is a haploid genome and is usually maternally inherited. In the last three decades, mtDNA was the main tool for inferring the evolutionary and demographic past of both populations and species. However, in recent years, researchers in molecular ecology and phylogeography have demonstrated an increasing awareness that this single molecule alone will not always be sufficient to answer the many interesting questions asked of it (Ballard & Whithlock, 2004; Moodley *et al.*, 2008). Many studies have combined both mitochondrial and nuclear microsatellite DNA markers to assess genetic diversity and population genetic structure of wild African species such as the plains zebra (*Equus quagga*) (Lorenzen *et al.*, 2008), the African malaria vector, *Anopheles arabiensis* (Temu & Yan, 2005), and also wild fish species in North American coast such as the striped bass (*Morone saxatilis*) (Brown *et al.*, 2005).

Nuclear integrations of mitochondrial DNA (Numts)

The nuclear genomes of most multicellular organisms contain integrated fragments of mtDNA (Zhang & Hewitt, 1996). Such insertions may be inadvertently amplified and mistaken for organelle DNA when mtDNA sequences from samples such as hair, are amplified by PCR. Several studies have reported the existence of Numts (Anthony *et al.*, 2007a; Clifford *et al.*, 2004; Clifford *et al.*, 2002; Garner & Ryder, 1996; Horai & Hayasaka, 1995). Greenwood and Pääbo (1999) reported that in Asian elephant, hairs amplify a majority of nuclear mtDNA sequences due possibly to a higher ratio of nuclear genomes to mitochondrial genomes in hair than in blood. Thus, the amplification of Numts may lead to misleading results when samples such as hair (perhaps other unusual tissues) are used. In the same paper, Greenwood and Pääbo (1999) advised the cloning of PCR products whenever direct sequencing yields ambiguous results (Eggert *et al.*, 2002).

However, to date no pseudogenes have been reported in genetic study of elephants based on mtDNA markers (Barriel *et al.*, 1999; Debruyne, 2005; Eggert *et al.*, 2002; Fernando *et al.*, 2003b; Nyakaana *et al.*, 2002; Roca *et al.*, 2005).

Microsatellite DNA

Microsatellites are sequences predominantly found in the nuclear genome. Microsatellite loci consists of a short tandem repeat (or STR) of mostly di-, tri-, or tetranucleotide units, which can be highly variable, giving numerous alleles at each locus within a population. Population variation is often much higher at STR loci than with mtDNA due to the high mutation rate of microsatellites (about 10^{-3} or 10^{-4} per locus per gamete and per generation). PCR primers allow the screening of genotypes at specific STR loci, by displaying the co-dominant alleles in simple electrophoretic systems. These alleles can be separated using electrophoresis on acrylamide gels according to size.

Microsatellites have advantages over other DNA markers as they combine high variability with biparental co-dominant inheritance (suitable for introgression studies, for example) and they can be typed following non-invasive sampling (Frankham *et al.*, 2003). They provide one of the most powerful and practical means currently available for analysing genetic diversity in threatened species. However it should be borne in mind that primers developed in some species may detect lower levels of variation among species that are not closely related (Primmer *et al.*, 1996).

1.7 An overview on African elephant genetic studies

The first genetic study carried out on elephants examined the molecular phylogeny of extant and extinct Elephantidae, including one forest elephant from Sierra Leone, using the cytochrome *b* mitochondrial gene (Barriel *et al.*, 1999). The analysis showed that the single *L. a. cyclotis* sample used was highly divergent from *L. a. africana*, but they did not conclude that they were separate species. Roca *et al.* (2001) then examined DNA

sequence variation in four nuclear genes (1732 base pairs) from 21 elephant populations (four forest and 17 savannah) and based on their data, estimated that forest and savannah elephants diverged approximately 2.63 (± 0.94) million years ago. Comstock *et al.*, (2002) examined 16 microsatellite loci and found a lower genetic diversity in savannah elephants compared to the forest elephants. As with Roca *et al.* (2001), they found evidence for hybridization in Garamba (a forest site in north-east of the Democratic Republic of Congo, DRC) where one elephant possessed intermediate genotypes from forest and savannah forms. Despite this possible "hybrid zone" they recognised the species-level distinctions between African taxa. One drawback of these studies was the absence of West African elephant samples, since Groves (2000) suggested that forest and savannah elephants found elsewhere on the continent coexist and can interbreed in this region of West Africa.

Frankham *et al.* (2003) defined the introgression as the mixture of alleles between species or sub-species. Introgression is particularly important for closely related sympatric taxa where hybridization is more likely and viable hybrids can be formed (Rokas *et al.*, 2003). Horizontal transfer of haplotypes through introgression is possible where reproductive barriers between lineages are incomplete. Rokas *et al.* (2003) called this transfer 'introgressive hybridization', because haplotypes from different species from, for example, a given glacial refuge are more similar than individuals of the same species across refuges.

Molecular studies using mtDNA including the mtDNA data from the study by Roca *et al.* (2005) point to a more complex evolutionary scenario for African elephants. Debruyne (2005) examined several thousand base pairs of mtDNA from wild born elephants from across Africa and although he also reported two highly divergent molecular lineages, these did not conform to the morphological delineations of *cyclotis* and *africana*. He interpreted these results as a consequence of incomplete isolation between forest and savannah African elephant populations, followed by recurrent and ongoing introgression between the two forms. Debruyne (2005) also performed morphometric analysis of museum elephant skulls, and found a continuum in the morphology of the two

morphotypes rather than two groups, suggesting that, despite historical events that promoted subdivision, these two forms freely interbreed wherever their ranges intersect.

Roca *et al.* (2005) obtained very similar mitochondrial results but explained the non-concordance between mitochondrial and nuclear markers as a result of "*cytonuclear genomic dissociation*" such that the mitochondrial tree did not reflect the species tree. The mtDNA results observed were proposed to have arisen due to episodes of backcrossing between successive generations of savannah males with forest females, leading to half of extant savannah elephants surveyed possessing 'forest' typical mitochondrial haplotypes but almost exclusively 'savannah' nuclear X and Y-chromosomal DNA. According to Roca *et al.* (2005), larger savannah males out-compete forest males when they come into contact, consequently forest males and hybrids would be reproductively disadvantaged and a dilution or limited spread of forest nuclear sequences in many savannah elephants. Based on this phenomenon of cytonuclear dissociation, Roca *et al.*, (2005) concluded that African forest and savannah elephants are distinct species separated by a hybrid zone.

Eggert *et al.* (2002) included samples from West Africa in their study time and found a more complex picture using mtDNA and nuclear microsatellites, and suggested that western savannah and forest elephants formed a potential third *Loxodonta* taxonomic unit.

All the above-mentioned studies are characterised by a pronounced lack of forest elephant sequences. The nuclear DNA studies of Roca *et al.* (2001, 2005), Comstock *et al.* (2002) and Wasser *et al.*, (2004) featured extremely limited sampling from central African forest elephant populations. Despite describing a narrow hybrid zone between the two elephant types only one population located in this zone (Garamba, DRC) was included and none from elsewhere in DRC or from West Africa were examined. Elsewhere, Debruyne (2005) included elephants from across the DRC in his study but was again limited by small sample sizes. Eggert *et al.* (2002) included samples of forest and savannah elephants from West Africa but subsequent analysis (Debruyne, 2005) with

more populations grouped these elephants with central forest counterparts, potentially undermining the conclusion of the genetic uniqueness of western elephants. To date, no study has addressed the partitioning of elephant genetic diversity on a large scale in the equatorial forests of Africa. Further, the potential effect of Pleistocene forest refugia, previously reported as having a major influence on large mammal (Anthony *et al.*, 2007b) distribution and range dynamics has yet to be addressed in African elephants.

1.8 Hypotheses and aims

Part of my study examines hypotheses to explain the evolutionary history of Central African forest elephants, and to determine their genetic structure and gene flow across their range of distribution. The forest elephant is a highly mobile animal that is widespread throughout tropical forests in central Africa (IUCN, 2005). It is physically able to disperse through wide ranges of habitats and migrate over long distances with no obstruction from ecological and geographical barriers. Further, this animal is assumed to live in strong matrilineal social groups characterised by female philopatry as with its savannah counterpart. Male-biased gene flow, widely recognised in mammals (Greenwood, 1980; Slatkin, 1985), would lead to homogenization of nuclear alleles while the maternally inherited mitochondrial marker should detect any population structure present (Nyakaana & Arctander, 1999).

Currently, little is known about forest elephant social behaviour. Our best knowledge of its social structure would lead us to predict that:

- Low levels of genetic differentiation, based on nuclear microsatellite markers, should be observed among populations since the extent of gene flow is related to the dispersal potential of individuals (Slatkin, 1987) which has been observed to be high in male forest elephants while
- Mitochondrial (maternally inherited) DNA should show greater genetic subdivision among populations because of more limited female dispersal.

- Alternatively, since females and their dependents can also potentially move over large distances (Blake, 2007) mitochondrial haplotype admixture is expected to be detected between adjacent and very distant populations and a limited relationship between geographical and genetic structure should be found.
- The distribution of mitochondrial lineages in forest elephants in the Congo Basin is expected to reflect climate-mediated forest fragmentation and concomitant allopatric divergence during the Pleistocene. Climate change is known to have influenced the evolutionary history of many African mammals such as gorillas (Anthony *et al.*, 2007b; Clifford *et al.*, 2004), hippopotamus (Okello *et al.*, 2005a) and many African bovids (Arctander *et al.*, 1999; Flagstad *et al.*, 2001; Nersting & Arctander, 2001; Van Hooft *et al.*, 2002). Hence, forest elephant is likely to exhibit a complex evolutionary and demographic history related to climatic variation.

My study used DNA obtained non-invasively from faeces of forest elephants from Central Africa. The overall aim of this research project was to assess the genetic structure of forest elephant populations by investigating genetic diversity and gene flow, using mitochondrial and microsatellite DNA markers (Chapters 3 and 5). Further, the status and phylogeography of the African elephant will be assessed across its range of distribution (Chapter 4 and published article in Appendix). Mitochondrial DNA has a greater ability to detect population genetic structure at large geographical scales while microsatellites are more useful in detecting admixture, introgression and dispersal. Mitochondrial and microsatellite data in combination can provide different levels of resolution and more valuable complementary information of the evolutionary history of the African elephant.

The mitochondrial genome and microsatellite loci used in this study were equivalent to those in previously published studies, allowing us to examine forest elephant sequences with the largest possible sample set within the largest geographic coverage.

Chapters in this study are all self-contained with their own reference lists. Tables and figures are inserted in each Chapter's text. All appendices are found at the end of the thesis.

Chapter 4 has been published in BMC Evolutionary Biology (Johnson *et al.*, 2007), and is attached at the end of the thesis.

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CHAPTER 2

Materials and Methods

2.0 Abstract

This chapter describes the sampling approach, laboratory procedures and general data analysis approaches in the thesis. Non-invasive samples, such as faeces have been shown to be suitable and reliable for population genetic studies, and were the only option for sampling African forest elephants in this study. Mitochondrial DNA (mtDNA) and microsatellites were selected as molecular markers to reveal the genetic structure of the forest elephant populations sampled. A 630 base pair (bp) fragment of mtDNA control region was amplified for the phylogeographic study, with twelve polymorphic microsatellite loci.

2.1 Study sites

This study included twelve sites across the Congo basin (see **Figure 2.1,**). Sites were located in Gabon, Republic of Congo and the Central African Republic (CAR). The study sites include those of the Lopé National Park and the Langoué saline bai in Gabon, Nouabale-Ndoki National Park in Congo and Dzanga-Sangha saline bai in CAR. Each location has a different predominant forest type (**Table 2.1**), from swamp forest to savannah-forest mosaic, including saline clearings surrounded by canopy forest where forest elephants congregate (**Figure 2.2**).

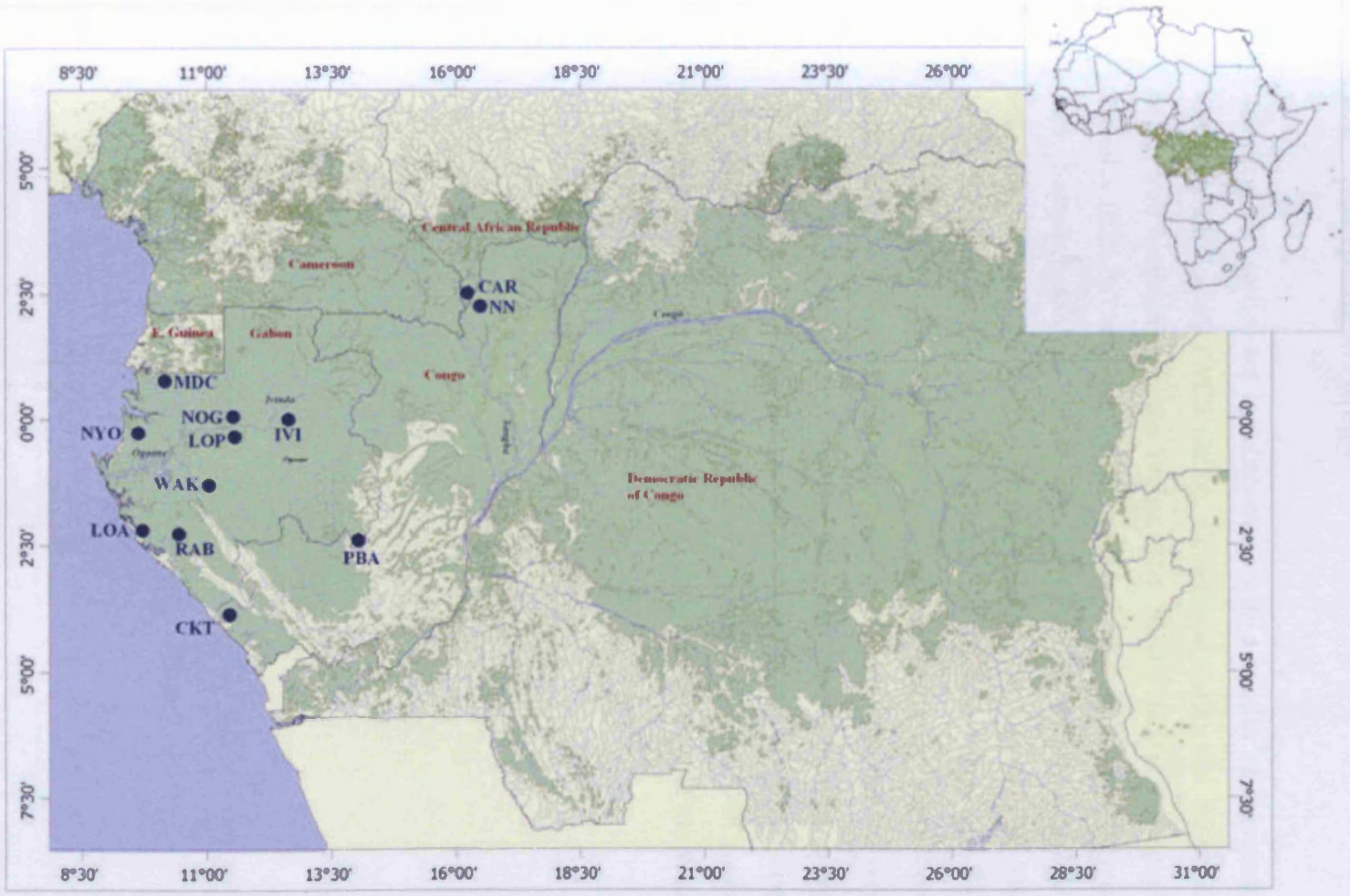

Figure 2.1. Study sites in west central Africa.

2.2 Sampling strategy

Sampling was carried out in collaboration with a number of organisations. The Wildlife Conservation Society (WCS Gabon, Congo, and CAR) was the principal field partner being well established in the region. The NGO "Habitat Ecologiq ue et Liberté des Primates (HELP)" was also a main partner in Congo in the Conkouati-Douli National Park. Sampling was carried out from mid-February to mid-July 2004. Each sampling team was supplied with tubes (about 30-50 per site) containing RNAlater (QIAGEN buffer for RNA preservation but suitable for DNA) or silica gel. The WCS office in Libreville, Gabon provided a convenient and important central point for sampling organisation as it was the meeting point of all collaborators and is a very important centre for conservation in central Africa with many different conservation organisations basing their offices there. CITES permits were not necessary for faecal samples, however the appropriate permits from the Centre of Documentation (CEDOC) were obtained when exporting samples abroad. Authorisations from the Ministry of the Water and Forestry in Gabon, CAR and Congo were obtained.

Table 2.1. Description of each site with habitat type (see Figure 1), and partner in charge with sampling in the locality. NP = National Park; CIRMF = Centre International de Recherche Medicale in Franceville (Gabon); WCS = Wildlife Conservation Society; CENAREST = Centre National de la Recherche Scientifique et Technologique (Gabon); HELP = Habitat Ecologique et Liberté des Primates (Congo), MEF = Ministère des Eaux et Forêts.

Site	Country	Habitat type	Partner	Code
Lopé, NP	Gabon	Forest-savannah mosaic	CIRMF, WCS	LOP
North of Ogouée River	Gabon	Closed canopy forest	CIRMF	NOG
Waka, NP	Gabon	Closed canopy forest	WCS	WAK
Ivindo, NP	Gabon	Saline clearing within forest	WCS	IVI
Monts de Cristal, NP	Gabon	Closed canopy forest	WCS	MDC
Rabi-Ndogo, NP	Gabon	Closed canopy forest	WCS	RAB
Nyonie	Gabon	Mangroves and lagoons, seasonally inundated and coastal forest	CENAREST, CIRMF	NYO
Loango/Mayumba NP	Gabon	Mangroves and lagoons, seasonally inundated and coastal forest	Project Loango, WCS, MEF	LOA
Plateaux Batéké, NP	Gabon	Degraded forest	CIRMF, WCS	PBA
Conkouati-Douli, NP	Congo	Seasonally flooded and swamp forest	HELP, WCS	CKT
Nouabalé-Ndoki NP	Congo	Lowland rainforest	WCS	NN
Dzanga-Sangha NP	CAR	Saline clearing within forest	WCS	CAR

Forest-savannah mosaic

Closed canopy forest

Saline clearing

Lowland forest inundated

Mangroves and lagoons, coastal

Figure 2.2. Different forest types where forest elephants congregate

2.2.1 Sample collection and training of field researchers.

It was essential to provide training on how to correctly collect elephant dung samples. A handout was prepared (see **Appendix 1**) after several samples were received from the field, which were over-filled and the medium was inadequate to preserve the sample which became mouldy or were incorrectly labelled and thus useless as the geographic origin was unknown. Practical training was given to field researchers in Conkouati-Douli National Park, Congo (23 April-2 May 2004), and in Lopé National Park, Gabon (20-23 June 2004) (Figure 2.3).

Figure 2.3: Mireille Johnson giving practical training to field research assistant.

2.2.2 Non-invasive sampling

It is essential that methods for DNA analysis are sensitive and specific enough to be able to detect host DNA in different types of sample (e.g. faeces, skin, muscle or blood). Field

workers cannot usually use blood-derived products for molecular analysis, as this requires direct intervention with the animal, which is neither ethical nor practical (Morin & Woodruff, 1996; Taberlet *et al.*, 1999). Molecular scatology has become the method of choice for most field studies (Kohn & Wayne, 1997), and much research has focused on improving the reliability of results generated from DNA analysis when the source material yields a small amount of DNA (Fernando *et al.*, 2003; Flagstad *et al.*, 1999; Gerloff *et al.*, 1995; Morin *et al.*, 2001a; Reed *et al.*, 1997). Field samples are subject to a variety of conditions, which degrade the small amounts of DNA present in the sample. Field collection techniques have focussed on preserving the sample and reducing the action of degrading enzymes before the sample reaches the laboratory (Frantzen *et al.*, 1998; Wasser *et al.*, 1997).

Using dung from elephants as a source of DNA should in theory provide sufficient material for analysis, as elephants defecate up to 20 times per day (White & Edwards, 2000) and fresh dung piles are relatively easy to locate, even in dense forest. The brown dots in Figure 2.4 are flies, which are good indicators to find hidden dung piles and were often used to help locate samples.

Figure 2.4. Collection of forest elephant dung sample in central African rainforest.
A fresh dung pile detected thanks to small flies (dots on the picture).

2.2.3 Dung sample preservation

Faecal collections commenced in 2002 and samples were available from early 2003. Faeces were first collected in a variety of different ways: into silica gel, 95% ethanol or RNAlater (Ambion). Samples considered fresh were between 0 to 24 hours old and were shiny (due to the presence of a mucus layer) when collected. An initial test was carried out to check the best preservation medium and the age of the dung (results not shown). We collected samples from the same dung pile and stored them in each medium (silica gel, ethanol and RNAlater) and also collected samples from dung piles of different ages from 5min to 200 hours in order to determine the best storage medium, the easiest method for field transportation and the optimal for quality of DNA extract. We stopped using ethanol because it easily leaks and evaporates in the field. Good quality of dung sample mainly depends of its age (< 24h). The best media we finally used for dung collection were silica gel and RNAlater. However, we used ethanol when both former were not available. Nyakaana *et al* (2002) and Eggert *et al* (2002) preserved their faecal and/or tissue samples in 25 % dimethylsulfoxide (DMSO) saturated with sodium chloride (Amos & Hoezel, 1991).

2.2.4 Extraction

Faecal, blood and tissue extraction facilities and techniques had already been established at CIRMF for primate species and protocols were developed at UGENET (Darwin Initiative: Conservation Biology and Genetics of Lowland Gorillas). Consequently no pilot study was needed. We used a laboratory dedicated to the extraction of faecal samples with a biological safety hood and another laboratory available for blood, tissue and hair extraction with the aim of avoiding cross-contamination between faecal samples and the other types of samples.

Faecal extraction

Faecal samples were extracted with the QIAmp® DNA Stool Mini Kit (QIAGEN, Germany, catalogue number 51504) following the manufacturer protocol's with samples stored in RNAlater buffer, samples on silica gel were dry and need a longer incubation time (>1h) in the lysis buffer until the sample was well soaked.

Before each extraction process, the spatially isolated laboratory was decontaminated: the bench was sterilized with bleach (10%) and equipment inside the hood was exposed to UV-light (>30 minutes). Each sample was extracted twice. One blank sample (negative control) was used per sample batch, and care was taken to avoid cross-contamination between different samples. Instead of eluting in 200 µl water as recommended by the QIAGEN protocol, the DNA was eluted in a final volume of 150 µl to concentrate the extracted DNA.

Blood and skin tissue extraction

Opportunistic blood/skin samples were collected during captures to attach radio-collars. DNA from blood and ear skin of forest elephants were sampled at Lopé National Park, Langoué saline bai and Iguela in Gabon. Two skin samples from Mpassa, Plateaux Batéké National Park (South-East) in Gabon were collected from corpses (a mother and her infant). Blood sample extractions were carried out using a standard phenol/chloroform protocol (Sambrook *et al.*, 1989) and DNA was eluted in 50 µl water. Skin samples were extracted with QIAamp® DNA Mini kit (QIAGEN) and eluted in 100 µl of sterile water (Sigma).

2.3 Molecular techniques

2.3.1 Mitochondrial DNA (control region and cytochrome b) sequencing

A 630 bp fragment of mtDNA was analysed, including the 3' end of the cytochrome *b* gene, threonine and proline transfer RNAs and 358 bp of the control region. This region was amplified using the primers MDL3 and MDL5 (see primer sequences **Table 2.2**) (Fernando *et al.*, 2000) from good quality DNA. As DNA extracted from non-invasive samples can be highly degraded, only short DNA fragments could be amplified reliably and primers **(Table 2.2)** AFDL1 and AFDL2 (400 bp from the 3'end of the cytochrome b gene through the 5' end of the control region), and AFDL3 and AFDL4 (377 bp from the 3' end of tRNA proline to the 5'end of the control region; Eggert *et al.* 2002) were employed. In addition only DNA from fresh dung piles (less than 24 hours) was attempted.

A 494 bp fragment of cytochrome *b* was also used in this study. This part of the gene was amplified with the primers L15024 and H15516 (Barriel *et al.* 1999).

Table 2.2: Primer sequences for PCR amplification and direct sequencing for control region and cytochrome *b* gene.

Primer	Sequence	Literature reference
MDL3	5'-CCCACAATTAATgggCCCggAgCg-3'	Fernando et al. 2000
MDL5	5'-TTACATgAATTggCAgCCAACCAg-3'	Fernando et al. 2000
AFDL1	5'-TTACACCATTATCggCCAAATAg-3'	Eggert et al. 2002
AFDL2	5'-TgACACATTgATTAAACAgTACTTgC-3'	Eggert et al. 2002
AFDL3	5'-CTTCTTAAACTATTCCCTgCAAgC-3'	Eggert et al. 2002
AFDL4	5'-gTTgATggTTTCTCggAggTAg-3'	Eggert et al. 2002
L15024	5'-TCTgCCTATACACACACATTggA-3'	Barriel et al. 1999
H15516	5'-TAgTTgTCAgggTCTCCTAgT-3'	Barriel et al. 1999

Amplification

PCR reactions were performed in a final volume of 25 µl containing 2 µl of DNA extract, 2 µl 100 mg/ml BSA, 2 µl reaction buffer, 1.25 mM of dNTP mix, 0.5 µl of 10 µM primers, 0.2 µl of Taq DNA polymerase (Invitrogen) and 14.55 µl of water. Amplifications for control region were carried out in a Perkin Elmer 9700 programmable DNA thermocycler as follows: a denaturation step for 4 min at 95°C followed by 40 cycles of 94°C denaturation for 45 sec, primer annealing at 63°C for 45 sec and 1 min of primer extension at 72 °C. For cytochrome *b*, amplifications were carried out following a denaturation step for 3 min at 94°C with 40 cycles of 94°C denaturation for 1 min, primer annealing at 55°C for 1 min and 2 min of extension at 72 °C. PCR products were visualized under UV light on a 1.5 % agarose gel stained with 0.5 µg/ml ethidium bromide. Extraction blanks and reaction blanks containing only PCR reagents were also included in order to control for potential contamination.

Sequencing

PCR products were purified using the Qiaquick PCR Product purification kit (Qiagen, Germany catalogue number 28104) following the manufacturer's instructions and were either cloned into the PCR2.1-TOPO vector (TOPO TA cloning kit, Invitrogen, catalogue number K4500-40) prior to sequencing with M13 Forward (-20) and M13 reverse primers or directly sequenced using the PCR primers. Thirty µl of purified PCR product was sent via DHL to Europe and later to South Korea to be sequenced commercially (Sequentia, France, and Macrogen, South Korea).

Several published elephant haplotypes of known geographic provenance were also included in the phylogenetic analyses. These sequences are available in Genbank under the accession numbers indicated in Appendices 2 and 3. A collaborator from Makerere University, Uganda, Silvester Nyakaana (SN) kindly provided 27 unpublished cytochrome *b* sequences (see Appendix 3).

Cloning

Selected PCR products were cloned to test for the presence of nuclear copies. As had been found by Eggert et al. (2002), nuclear integrations of mitochondrial sequences or *Numts* were suspected which could lead to erroneous interpretations of the data. *Numts* have already been described in the elephant (Greenwood & Paabo, 1999). Nyakaana *et al.* (2002) carried out RT-PCR in order to check for nuclear copies although they had no reason to suspect pseudogenes in their study. Phylogenetic analysis of the control region revealed 4 major groupings (described in Chapter 4). Two individuals from each mitochondrial group were selected and between five and 10 clones (depending on the cloning yield (45.5%)) were sequenced. If multiple (nuclear or heteroplasmic mitochondrial) copies were present, some distinct sequences among individual clones would be expected, but all clones were identical to the original sequence.

2.3.2 Microsatellite analysis

Screening

A battery of 37 microsatellite loci (Table 2.3) has been characterised for both African and Asian species (Archie *et al.*, 2003; Comstock *et al.*, 2000; Eggert *et al.*, 2000; Nyakaana & Arctander, 1998; Nyakaana *et al.*, 2005). They were all tested to see if they amplified robustly and were polymorphic with forest elephant faecal samples. Each microsatellite locus was amplified in order to optimise the PCR reaction, then to define the annealing temperature and the number of cycles appropriate for each locus. The screening process was carried out with a PCR reaction conducted in a 10 µl volume containing 5 µl of QIAGEN Multiplex PCR Master Mix (from QIAGEN® Multiplex PCR Kit), 1 µl of the 10X primer mix (0.2µM of each primer, forward and reverse), 2µl of DNA, 1µl of 0.5X Q-Solution (provided in the kit) and 1 µl of water. The amplification profile consisted of a denaturation step at 95°C for 15 min, followed by 35-45 cycles of 94°C denaturation for 30 sec; 1.5 min of primer annealing from 55 °C to 60°C and 1.5 min of primer extension at 72°C, depending of the primer. Control extraction blanks and PCR reaction controls for

which no DNA was added were included in each batch of amplifications. Five faecal DNA samples were chosen randomly to be screen with each locus. PCR products were run on a Spreadex® ready-to-use gel using an Elchrom™ SEA2000 electrophoresis system with M3 marker for Spreadex® gels. They were stained with ethidium bromide and visualised using UV light.

Table 2.3. Microsatellite primers selected from the literature and used for forest elephant screening.

Locus	Fluorescent dye	Size range (bp)	Author
FH1	HEX	81	Comstock *et al.*, 2000
FH19	6FAM	185	Comstock *et al.*, 2000
FH39	NED	242	Comstock *et al.*, 2000
FH40	6FAM	243	Comstock *et al.*, 2000
FH48	NED	178	Comstock *et al.*, 2000
FH60	6FAM	148	Comstock *et al.*, 2000
FH65	5TET	241	Comstock *et al.*, 2000
FH67	6FAM	97	Comstock *et al.*, 2000
FH71	NED	69	Comstock *et al.*, 2000
FH127	6FAM	150-174	Comstock *et al.*, 2002
FH153	NED		Comstock *et al.*, 2002
LA2	HEX	227-241	Eggert *et al.*, 2000
LA4	5TET	117-137	Eggert *et al.*, 2000
LA5	5TET	130-154	Eggert *et al.*, 2000
LA6	6FAM	158-214	Eggert *et al.*, 2000
LAFMS01	5TET	189-204	Nyakaana & Arctander, 1998
LAFMS02	HEX	134-154	Nyakaana & Arctander, 1998
LAFMS03	6FAM	140-150	Nyakaana & Arctander, 1998
LAFMS04	6FAM	143-159	Nyakaana & Arctander, 1998
LAFMS05	Unlabeled	160	Nyakaana & Arctander, 1998
LAFMS06	Unlabeled	138-156	Nyakaana *et al.*, 2005
LAFMS07	VIC	154-170	Nyakaana *et al.*, 2005

LAFMS08	Unlabeled	175-189	Nyakaana *et al.*, 2005
LAFMS09	Unlabeled	144-160	Nyakaana *et al.*, 2005
LAFMS10	Unlabeled	108-116	Nyakaana *et al.*, 2005
LAFMS11	Unlabeled	130-136	Nyakaana *et al.*, 2005
LAT05	VIC	255-307	Archie *et al.*, 2003
LAT06	Unlabeled	281-366	Archie *et al.*, 2003
LAT07	VIC	340-398	Archie *et al.*, 2003
LAT08	VIC	166-234	Archie *et al.*, 2003
LAT13	Unlabeled	234-262	Archie *et al.*, 2003
LAT16	Unlabeled	295-327	Archie *et al.*, 2003
LAT17	Unlabeled	323-355	Archie *et al.*, 2003
LAT18	Unlabeled	286-318	Archie *et al.*, 2003
LAT24	Unlabeled	211-231	Archie *et al.*, 2003
LAT25	6FAM	298-318	Archie *et al.*, 2003
LAT26	6FAM	352-392	Archie *et al.*, 2003

Multiplex constitution

Sixteen microsatellite loci, consisting of both di- and tetra-nucleotide repeats, were polymorphic and fulfilled the conditions of annealing temperature, number of cycles and size range in order to make four multiplexes designated M1, M2, M3 and M4 (Table 2.4). Each multiplex was assembled taking into account the allele size (from the original study) and the non-overlap of fragment sizes for loci labelled with the same fluorescent dyes. After the screening process, four tetra-nucleotide loci (LAT07, LAT25, LAT26, LAT05) were removed from the study because of difficulties in amplification. Okello *et al* (2005) in their study successfully amplified these tetra-nucleotides, though they observed a higher error rate in the tetra-nucleotide than di-nucleotide microsatellite loci.

Genotyping criteria

Specific conditions were followed for genotyping. PCR was carried out for each locus on each extraction at least twice. Given the initial number of samples (roughly 400 samples from eight populations), time and financial considerations prevented us from typing each individual as many time as recommended Taberlet *et al.*, (1996) when genotyping nuclear loci of very low DNA samples. Their study suggested three positive PCR to assign heterozygous individual and four additional positive PCR for homozygous and further experiments for individual with ambiguous results. Given the use of the QIAGEN multiplex kit, which significantly improves the PCR conditions and increases the yield of DNA amplified and the power of recent automated sequencers and efficient genotype software analysis the PCR success rate of microsatellite loci used in this study was high, with an average of 61% success per locus genotyped. Therefore alternative criteria were used instead of those recommended by (Taberlet *et al.*, 1996). An individual was typed as heterozygous if both alleles appeared at least twice within the four replicates, and a homozygous was typed if it appeared at least three times otherwise it was repeated one more time or eliminated. PCR products were run on an ABI 3740 XL (Applied Biosystems/Perkin-Elmer), automated genetic analyser with LIZ 500 standard size (Applied Biosystems/Perkin-Elmer) by a commercial company, Macrogen, in South Korea.

Table 2.4. Panel of microsatellite multiplexes with the fluorescent dye of each locus used for screening in this study. The size range was from the original study.

Multiplex	Locus	Repeat motif	Dye	° C x cycles	Allele size	Authors
M1	FH39	Di-nucleotide	NED	55 ° C x 38	242	Comstock *et al.* 2000
	FH67	Di-nucleotide	6-FAM		97	Comstock *et al.* 2000
	FH127	Di-nucleotide	6-FAM		340-398	Comstock *et al.* 2002
	LAT07	Tetra-nucleotide	VIC		298-318	Archie *et al* 2003
	LAT25	Tetra-nucleotide	6-FAM		69	Archie *et al* 2003
M2	FH71	Di-nucleotide	NED	58 ° C x 37	142	Comstock *et al.* 2000
	LAFMS03	Di-nucleotide	6-FAM		154-170	Nyakaana & Arctander, 1998
	LAMS07	Tetra-nucleotide	VIC		352-392	Nyakaana *et al.* 2005
	LAT26	Tetra-nucleotide	6-FAM		148	Archie *et al* 2003
M3	FH60	Di-nucleotide	6-FAM	60 ° C x 37	158-214	Comstock *et al.* 2000
	LA6	Di-nucleotide	NED		166-234	Eggert *et al.* 2000
	LAT08	Tetra-nucleotide	VIC		185	Archie *et al* 2003
M4	FH19	Di-nucleotide	RED	60 ° C x 40	243	Comstock *et al.* 2000
	FH40	Di-nucleotide	6-FAM		178	Comstock *et al.* 2000
	FH48	Di-nucleotide	NED		255-307	Comstock *et al.* 2000
	LAT05	Tetra-nucleotide	VIC			Archie *et al.* 2003

Dye colors: NED:yellow; 6-FAM: blue; VIC: green; RED: red

2.4 Data analysis

2.4.1 Mitochondrial DNA

Sequence alignment

Forward and reverse sequences for each individual and the consensus sequences for all individuals were aligned using SEQUENCHER (Gene Codes Corporation 1998, version 3.1.1) and rechecked by eye. All consensus sequences saved in a file were converted into NEXUS format and used for appropriated analyses.

Genetic diversity

Genetic diversity was estimated using haplotype (*h*) and nucleotide (*p*) diversity indices as implemented in ARLEQUIN ver. 3.11 (Excoffier *et al.*, 2005). Haplotype diversity is defined as the probability that two randomly chosen haplotypes in a sample are different (Nei, 1987), and nucleotide diversity is the probability that two randomly chosen homologous nucleotides are different (Nei, 1987).

Genetic differentiation and analysis of molecular variance (AMOVA)

Populations were defined according to their geographical locality. Genetic differentiation between pairs of localities was tested using the exact test using 10,000 Markov chain steps, as implemented in ARLEQUIN ver. 3.11, and this program was also employed for nested analysis of molecular variance (AMOVA) to test for patterns of population genetic structure (Weir & Cockerham, 1984). The correlation among haplotype distances is used as an *F*-statistic analog (Phi) at various hierarchical levels where the total variance is partitioned into covariance components due to inter-individual differences, and/or inter-population differences. In the case of a simple hierarchical genetic structure consisting of haploid individuals in populations (such as is generated using mitochondrial DNA), the implemented form of the algorithm produces a fixation index Phi_{ST}. In the case of a

hierarchical genetic structure with nested groups of populations, the significance of the fixation indices is tested using a non-parametric permutation approach described in (Excoffier *et al.*, 1992), consisting of permuting haplotypes within populations among groups (Phi_{ST}), permuting haplotypes among populations within groups (Phi_{SC}), and permuting populations among groups (Phi_{CT}).

Phylogenetic relationships

Hamming distances, which are the sum of nucleotide differences between two sequence types (Bandelt *et al.*, 1999), was used to derive a median joining network (MJN) with the program NETWORK V4.1.1.1. Haplotype networks more effectively portray the relationship among sequences for populations within species than maximum likelihood or maximum parsimony phylogenies which are the traditional methods developed to define interspecific relationships, leading to poor resolution at the population level (Posada & Crandall, 2001). Furthermore, networks allow multi-furcations and also permit geographic location to be used to infer topological positioning of haplotypes in a phylogeny (Crandall & Templeton, 1993).

However, a phylogenetic analysis was also carried out with MODELTEST 3.06 (Posada & Crandall, 1998) to determine the substitution model (or model of evolution) that best fitted the data according to a hierarchical likelihood ratio test. Sequences were analyzed by the neighbor-joining (Saitou & Nei, 1987) method implemented in PAUP 4.01b (Swofford, 1998) using the appropriate model. Node support was tested using 1000 bootstrap replicates. A phenogram was constructed using Neighbor-Joining (Saitou & Nei, 1987). A bootstrap consensus tree was inferred fom 1000 replicates (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed and the percentage of replicate trees in which associated taxa clustered together is shown next to the branches.

Analysis of population demography

Tests were performed to detect evidence of past demographic change in central African forest elephants. ARLEQUIN ver. 3.1 was used to perform a pairwise haplotype mismatch distribution in the total sample and for the haplogroups identified by the MJN analysis, comparing the distribution of the observed pairwise nucleotide site differences with the expected distribution in an expanding population (Rogers & Harpending, 1992). In a single origin, demographically expanding population, mismatches should follow a unimodal Poisson distribution, whereas in populations at demographic equilibrium or with sub-groups or genetic substructure, the distribution is expected to be multimodal. The mismatch distribution also allowed estimation of the time of the demographic expansion event by calculating the value of three parameters assuming that an initial female population at equilibrium with a size $\theta = \theta_0$, grows rapidly to a new size at which $\theta = \theta_1$, and this burst of growth is assumed to occur tau (τ) units of mutational time before the present (Rogers & Harpending, 1992). The goodness-of-fit was tested for the observed data to a simulated model of expansion with the sum of square deviations (SSD) and the Harpending's raggedness index *r* which takes larger values for multimodal distributions found in stationary population than for unimodal and smoother distributions typical of expanding populations (Rogers & Harpending, 1992). Population history was inferred using Tajima's D (Tajima, 1989) and Fu's F_S (Fu, 1997) tests of neutrality to examine whether all mutations are selectively neutral. A negative value of Tajima's *D* statistic reflects a relative excess of low-frequency polymorphisms (Tajima, 1989), and Fu's *Fs,* which is a powerful test for rejecting the hypothesis of neutrality of mutations (Fu, 1997), tends to be negative when there is an excess of recent mutations. Both tests can therefore be used to detect the signal of a demographic expansion, where low frequency mutations are expected.

2.4.2 Nuclear microsatellite loci

Several genetic studies of wild animals have relied on the use of non-invasive samples (Clifford *et al.*, 2004; Clifford *et al.*, 2002; Garner & Ryder, 1996; Morin *et al.*, 2001b).

Hence, faecal samples provide a reliable source of genomic DNA for population studies (Flagstad & Roed, 1999; Goossens & Waits, 1998; Wasser *et al.*, 1997). However, there is may be an impact on genotyping results due to genotyping errors with samples containing degraded and/or tiny amount of DNA (Morin *et al.*, 2001b; Wandeler *et al.*, 2003). Samples used in this study were obtained non-invasively (dung collection) and were therefore likely to contain low and/or degraded DNA quality. In this case, incorrect genotypes can be scored due to an allele failing to amplify (Miller & Waits, 2003), large allele drop out or short allele dominance (Wattier *et al.*, 1998) or slippage during PCR-amplification (Shinde *et al.*, 2003). Such genotyping errors must be detected prior to following population genetic analyses as they can cause bias in differentiation estimators such as F_{ST} and genetic distance (Chapuis & Estoup, 2007; Dakin & Avise, 2004). A variety of programs can help detecting genotyping errors such as MICRO-CHECKER 2.2.1 (Van Oosterhout *et al.*, 2004). This software was used in this study to identify genotyping errors (null alleles, large allele dropout, and scoring of stutter peaks).

Genetic diversity analysis

GENETIX 4.05 (Belkhir *et al.*, 1998) was used to perform all standard population genetic analyses: mean number of alleles per locus (A), allele frequencies differentiation between populations at each locus, gene diversity (H_e) and observed heterozygosity (H_o). Heterozygote deficiency was tested, as compared to Hardy-Weinberg equilibrium for each locus. Deviation from Hardy-Weinberg equilibrium were tested calculating Weir & Cockerham's estimate of F_{IS} (Weir & Cockerham, 1984) for each locus and also globally, using GENETIX with 1000 permutations. Significant positive values of F_{IS} indicate heterozygote deficiency, and significant negative values indicate heterozygote excess. Genotypic linkage disequilibrium was performed for each pair of loci per population and across all populations using ARLEQUIN 3.1.1.

Genetic differentiation between populations was determined by estimating Wright's F_{ST} statistics (Weir & Cockerham, 1984; Wright, 1951), using GENETIX, and pairwise estimates of gene flow between populations were calculated using the same program. To

test for correlations between genetic and geographical distances (isolation by distance) a Mantel test was performed using ARLEQUIN. F_{ST} was used as the genetic distance and linear distances (km) measured from a map between population locations were used as geographic distances. Values of the correlation (r) and probability (p) were obtained after 10000 permutations.

Analysis of molecular variance

We examined the hierarchical genetic structuring based on an analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992), as executed in ARLEQUIN. AMOVA measures the partitioning of variance at different levels of population subdivision (individuals into populations and populations into groups), giving rise to an analogue of F-statistics called Φ-statistics. The different genetic variance components (within populations and among populations) were estimated for the whole sample.

Population structure and genetic admixture analysis

The genetic structure of the forest elephant population was investigated using a clustering method based on Bayesian model: STRUCTURE version 2.2 (Falush *et al.*, 2003; Falush *et al.*, 2007; Pritchard *et al.*, 2000). The number of populations (K) is treated as an unknown parameter processed by the Markov Chain Monte Carlo (MCMC) computations. STRUCTURE was used as it is the standard reference software to infer population structure and assign individuals to source clusters (or jointly to two or more clusters in cases of admixture) using multilocus genotype data. Both, no-admixture and admixture models assume there is Hardy-Weinberg equilibrium and linkage equilibrium, but in this study the admixture model was considered as it is more likely that in practice each individual may have recent ancestor in more than one population (Falush *et al.*, 2003). Unlike Pritchard *et al.* (2000) who assumed a model with independent allele frequencies in different populations, Falush *et al.* (2003) recommended a correlated allele frequencies model which would be more accurate and may improve performance on

cases with subtle population structure. This method also includes studying hyb rid zones, identifying migrants and admixed individuals. STRUCTURE's procedure consists in running several MCMC with different values for K populations in order to cluster individuals into populations and estimate, for the admixture model, the proportion of membership in each population for each individual. Several runs for each K, from $K = 1$ to 10, were performed in order to verify the consistency of the results. The mean posterior probability, which is the mean value of the log likelihood of the data at each step of the MCMC, was calculated for each K over its runs and was also used to identify the true number of populations K using the maximum value of the mean likelihood.

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CHAPTER 3

Population genetic structure of forest elephants in west central Africa based on mitochondrial DNA control region variation

3.0 Abstract

The genetic diversity and structure of the African forest elephant was investigated for the first time in 12 populations from Gabon, Congo and the Central African Republic using mitochondrial DNA control region sequences. Our results reveal relatively low nucleotide diversity (0.013 ± 0.007) and high haplotype diversity (0.95) in forest elephant populations. Despite the sometimes large geographical distances between sampled populations, very low genetic divergence was observed between most groups. Haplotypes were distributed with little restriction to geographical localities, indicating high levels of gene flow. Two divergent haplogroups, illustrated by a bimodal distribution of pairwise differences in the control region, implies that secondary contact and ongoing introgression has occurred between populations expanding from at least two putative refugia formed whe n the central African forest belt retracted during the last glacial period in Central Africa.

3.1 Introduction

During the late Quaternary, cyclical climatic fluctuations are believed to have had a substantial impact on the distribution and range dynamics of many African taxa (Maley, 1996). The arid climate experienced at this time led to the retraction of tropical forest into a few favourable regions, forming refugia and fostering allopatric divergence between

isolated populations of the fauna and flora associated with these isolated regions (Grubb, 2001). Unlike certain forest-dwelling species such as gorillas, that are restricted to closed canopy forest and do not occur in forest-savannah mosaic habitats (Tutin *et al.*, 1997; Yu *et al.*, 2004), forest elephants seem to be a less restricted and more dispersive species, with a wide range of associated habitats including flooded forest, swamps, savannah, forest/savannah mosaic, gallery forest and closed canopy forest (Momont, 2007; Morgan & Lee, 2007; Tutin *et al.*, 1997; White, 1994).

Very little has been reported in the literature about forest elephant movements, social behaviour and structure until the last decade when researchers started using Global Positioning System (GPS) telemetry to track the movements of individuals (Blake *et al.*, 2001), and discovered forest clearings ,known as bais (Turkalo & Fay, 1996a) which allow direct observations and studies on social behaviour. While GPS telemetry, today, gives a high level of detail on daily and seasonal movements of forest elephants (Blake *et al.*, 2001), genetic studies have been almost entirely lacking and are needed to support and complement such ecological data.

Migrations in forest elephant appear to be correlated with diet (Turkalo & Fay, 1996; White, 1994) since elephants can move over long distance to visit forest clearings for mineral deposits, Marantaceae forests for herbaceous food and fruit found in mature forest (Blake & Inkamba-Nkulu, 2004; Turkalo & Fay, 1996b; Vanleeuwe & Gautier-Hion, 1998). These movements provide regular tracks in the forest(Vanleeuwe & Gautier-Hion, 1998) . Similar regular migrations have been observed in savannah elephant populations, northern Kenya (Thouless, 1995) where their movements are associated with rainfall between dry and wet season ranges. Forest elephant bulls, in Dzanga-Sangha National Park, are more mobile than females and migrate further (Turkalo & Fay, 1996b), however Blake *et al.* (2001) reported a collared female in Noubale-Ndoki National Park (NNNP, Congo) crossing an international border from NNNP to Dzanga-Sangha National Park (DSNP, south Central African Republic) moving a straight line distance of 60 km. They suggested that the movements observed may not be seasonal migration but short-term displacements within a range. Larger movements

may occur over regional areas between Central and Eastern Africa and between Central and West Africa (Blanc *et al.*, 2007) . In addition, cross-border movements are known to occur between Congo, Gabon, Cameroon, and Equatorial Guinea (Blanc *et al.*, 2007). Some studies shown that savannah elephants are not confined in designated protected areas (Douglas-Hamilton *et al.*, 2005; Thouless, 1995) but they move through unprotected corridors between their favourite core zones (Douglas-Hamilton *et al.*, 2005). The same movement patterns have been observed with forest elephants in Cameroon (Nzooh *et al.*, 2005). Home range or migratory movements in savannah elephants are determined by the availability of resources (Douglas-Hamilton *et al.*, 2005; Thouless, 1995), which are as sparse in forest (for fruiting trees) as they are in many savannah habitats (for primarily water sources) (Blake *et al.*, 2001). Water is not a range limit factor in the forest, unlike in the savannah where large seasonal fluctuations occur. Thus, forest elephants have the ability to cover long distances in response to geographic distribution of resources over a large spatial scale (White, 1994). However, poaching for meat and ivory and habitat loss may disturb these continuous movements, though it is difficult to determine the impact that these threats may be having on forest elephant populations (Blanc *et al.*, 2007) because of the difficulties of monitoring in forest and the lack of infrastructure necessary to monitor elephant populations (Walsh & White, 1999). Human activities (poaching, logging, oil concessions, villages and roads) have been found to strongly influence elephant dispersal patterns (Barnes *et al.*, 1991; Blake *et al.*, 2007; Buij *et al.*, 2007; Laurance *et al.*, 2006). Blake *et al.* (2007) showed that the probability of elephant presence increased with distance to roads whereas human signs declined. Consequently, these factors might also influence social and population structure of elephants.

To date, no molecular studies have assessed the patterns of population genetic structure for forest elephants in central Africa. Analysis of mtDNA has provided important insights into understanding genetic diversity and population structure in African savannah elephants (Charif *et al.*, 2005; Muwanika *et al.*, 2003; Nyakaana & Arctander, 1999; Nyakaana *et al.*, 2002), Asian elephants (Fernando *et al.*, 2000), and several other key African mammal species such as wildebeest (Arctander *et al.*, 1999); gorillas (Anthony

et al., 2007; Clifford *et al.*, 2004), hartebeest (Flagstad *et al.*, 2001) and bushbuck (Moodley & Bruford, 2007).

Nyakaana *et al* (1999) revealed how poaching and habitat loss have strongly affected the genetic diversity of elephant populations in Uganda using mitochondrial and nuclear loci. They found a significant genetic differentiation between their three remaining populations, which are presently restricted in protected areas suggesting limited gene flow. However, they have reported high levels of heterozygosity and negative local inbreeding (F_{IS}) values. They explained this result in terms of male-biased gene flow, and a social organisation where most matings involved females and unrelated males, since males at sexual maturity are expelled from their natal group. The same study also revealed a low level of nucleotide diversity (1.4 %) compared to other large mammals in East Africa. In 2002, Nyakaana *et al.* studied the population structure of the African savannah elephant in a regional scale using the same molecular markers. They observed 2.0 % nucleotide diversity and 85 % haplotype diversity, and found a significant genetic differentiation between populations within and among regions. The nucleotide diversity was lower than other large African mammals such as Grant's gazelle and buffalo which have 10.9% and 5.0%, respectively (Arctander *et al.*, 1996; Simonsen *et al.*, 1998). They reported that Pleistocene refugia could explain the observed regional genetic subdivision as a result to population divergence in allopatry with recent admixture following population expansion. The evolution of many other large African mammals has also been influenced by climatic change during the Pleistocene (Anthony *et al.*, 2007; Arctander *et al.*, 1999; Clifford *et al.*, 2004; Flagstad *et al.*, 2001; Muwanika *et al.*, 2003; Okello *et al.*, 2005).

Bottlenecks or declines in population size are known to affect present day genetic diversity. Intense poaching pressure in Ugandan parks has been reported to affect the genetic diversity of several species (Muwanika *et al.*, 2003). Molecular markers have proved to be valuable in describing extreme reductions in nucleotide diversity using mitochondrial DNA. They also have depicted a social behaviour breakdown in populations, which have suffered stress led by severe poaching in the past (Nyakaana *et*

al., 2001). In the light of factors such as migration, habitat loss or social stress, and Pleistocene climate change, which are known to govern the social and population structure in several well studied large African mammal populations, including savannah elephants, I could ask the question, what we would expect to discover in forest African elephants populations?

(i) Since these elephants can move very long distances, high levels of gene flow between different sampled sites and local admixture would be expected.

(ii) The contraction and expansion of the forest in the Congo Basin during the Pleistocene has likely played an important role in the evolutionary history of African forest elephant populations, and the genetic signature of these events are expected to be detected using mtDNA, such as evidence for recent population expansions from refugial areas within the region.

In this study, the geographical distribution of genetic variation within forest elephants in central Africa was examined to shed light on the population genetic structure and evolutionary history of this group.

3.2 Materials and Methods

3.2.1 Samples

Tissue and faecal samples were collected from 12 sites in west central Africa (Table 3.1; Figure 3.1, and see Appendix 4 for sample details). The geographic locations are: Gabon: LOP, Lopé National Park (NP); LOA, Loango NP; RAB, Rabi-Ndogo NP; WAK, Waka NP; IVI, Ivindo NP; PBA, Plateaux Batéké NP; MDC, Monts de Crystal NP; NOG, North of Ogooué River in Lopé NP area; NYO, Nyonié, north Wonga-Wongué Reserve; Republic of Congo: CKT, Conkouati-Douli NP and NN, Nouabalé-Ndoki NP; CAR,

Dzanga-Sangha NP in Central African Republic.

Samples were stored in RNAlater (Ambion RNA*later*® and Qiagen RNA later™), 100% ethanol or silica gel, and DNA was extracted from faecal, blood and tissue samples using the QIAamp DNA stool mini kit (Qiagen, Hilden, Germany, catalogue #51504), and the Dneasy Blood & Tissue kit (Qiagen, Hilden, Germany, catalogue # 69504), respectively following the manufacturer's protocol.

Table 3.1. List of sample sites, site code, site number, country of origin and the number of samples analysed per site. Sample details are shown in Appendix 4.

Site	Site code	Site No.	Country	No. of samples
Lopé	LOP	1	Gabon	11
Ivindo	IVI	2	Gabon	16
Loango	LOA	3	Gabon	8
Monts de Cristal	MDC	4	Gabon	2
Waka	WAK	5	Gabon	5
Plateaux Batéké	PBA	6	Gabon	11
Rabi	RAB	7	Gabon	8
Nyonié	NYO	8	Gabon	1
North Ogooué	NOG	9	Gabon	8
Dzanga-sangha	CAR	10	CAR	16
Conkouati	CKT	11	Congo	1
Nouabalé-Ndoki	NN	12	Congo	9

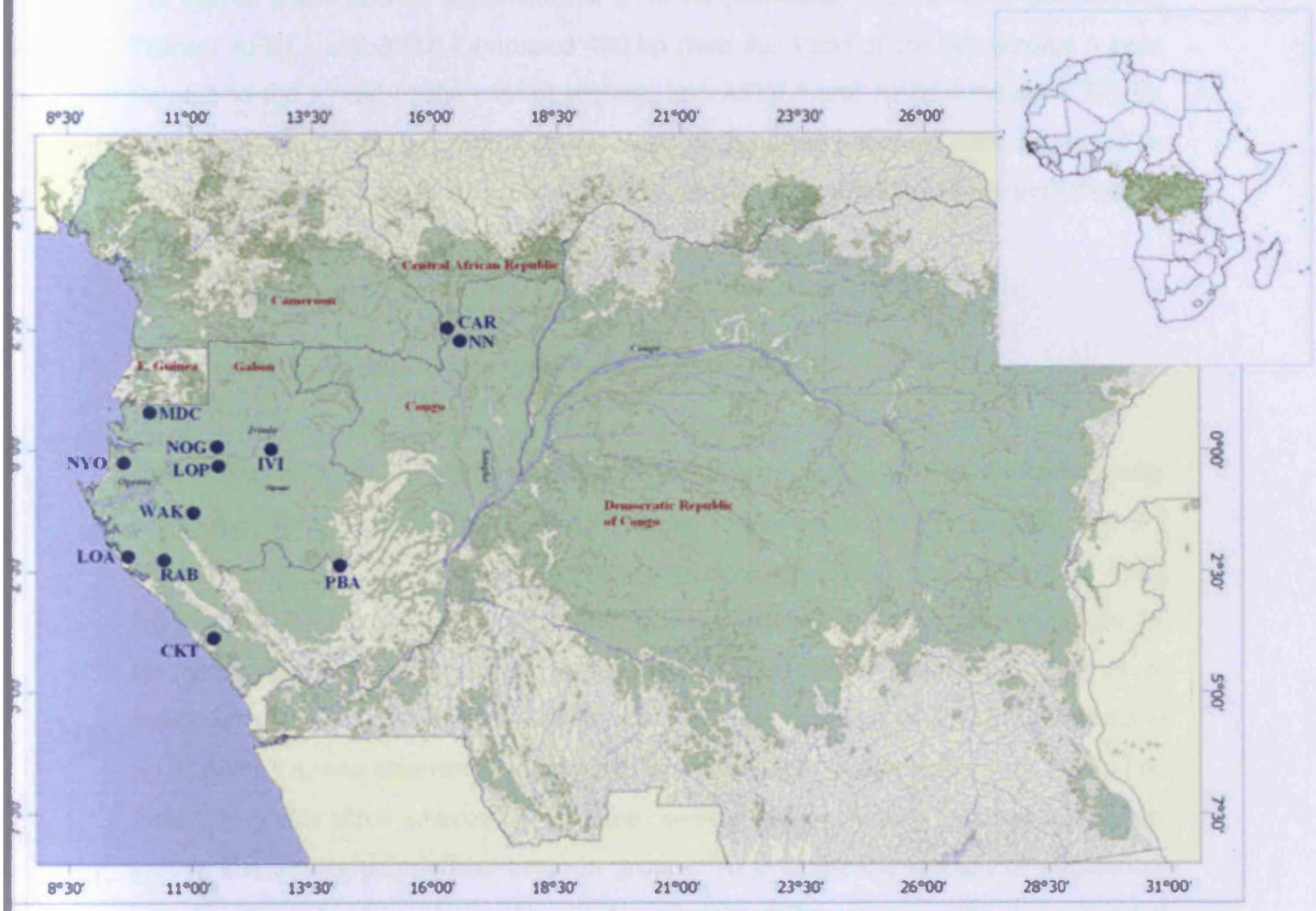


Figure 3.1. Map of Congo basin showing the sampling areas. The geographic locations are described as: LOP, Lopé National Park (NP); LOA, Loango NP; RAB, Rabi-Ndogo NP; WAK, Waka NP; IVI, Ivindo NP; PBA, Plateaux Batéké NP; MDC, Monts de Crystal NP; NOG, North of Ogooué River in Lopé NP area; NYO, Nyonié, north Wonga-Wongué Reserve; CKT, Conkouati-Douli NP; NN, Nouabalé-Ndoki NP; and CAR, Dzanga-Sangha NP.

3.2.2 Laboratory procedures

The control region section was amplified in 96 samples using primers MDL3 and MDL5. Primers AFDL1 and AFDL2 (situated 400 bp from the 3'end of the cytochrome *b* gene through to the 5' end of the control region), and AFDL3 and AFDL4 (situated 377 bp from the 3' end of proline tRNA to the 5'end of the control region) were employed to gain overlapping sequence for some degraded samples. Amplifications were performed as described in Chapter 2.

3.2.3 Analysis of genetic diversity and differentiation

Genetic diversity for the total sample and individual populations was estimated using haplotype (h) and nucleotide (p) diversities as implemented in ARLEQUIN ver. 3.1 (Excoffier *et al.*, 2005). A median joining network (MJN) was estimated using NETWORK 4.1.1.1 (Bandelt *et al.*, 1999). Genetic differentiation among populations for the entire data set was analysed, by implementing a simple hierarchical analysis of molecular variance (AMOVA, Excoffier *et al.*, 2005, as executed in ARLEQUIN version 3.1.). AMOVA was also tested to confirm the subdivided groups defined by MJN. The hierarchy yields three sources of variation: among groups, among populations within groups and among populations between groups. To evaluate the amount of population genetic structure, we estimated pairwise genetic differentiation (Phi_{ST}) among all populations studied based on the number of differences observed between haplotypes, using ARLEQUIN. The statistical significance of Phi-statistics was estimated using 1000 permutations.

A phylogenetic analysis was carried out using HKY 85 + G + I (Hasegawa *et al.*, 1985), the best-fit model selected by MODELTEST 3.06 (Posada & Crandall, 1998) with an assumed proportion of invariable sites (I) of 0.93 and a shape parameter of the gamma distribution of 0.58. The program ARLEQUIN 3.1 was used to implement a Mantel test of the correlation between Phi_{ST} and geographic distance by permutation (Smouse *et al.*, 1986), for the entire data set and the subdivided groups defined by MJN. The significance

of the observed correlation between these two distances matrices was assessed using 1000 permutations. Linear geographic distances were measured between the defined populations since forest elephants are not known to be constrained in their movements by elevation or rivers.

3.2.4 Analysis of population demography

ARLEQUIN was used to compute mismatch distributions (Rogers & Harpending, 1992) based on a sudden population expansion model for the total sample and the main groups of forest elephants defined by MJN. Demographic expansion parameters θ_0, θ_1 (size of population before and after population growth) and τ (expansion time) were also estimated (Rogers & Harpending, 1992). The same program was used to test for the selective neutrality with Tajima's D (Tajima, 1989) and Fu's Fs (Fu, 1997). Details are in Chapter 2.

3.3 Results

3.3.1 Sequences and haplotype analysis

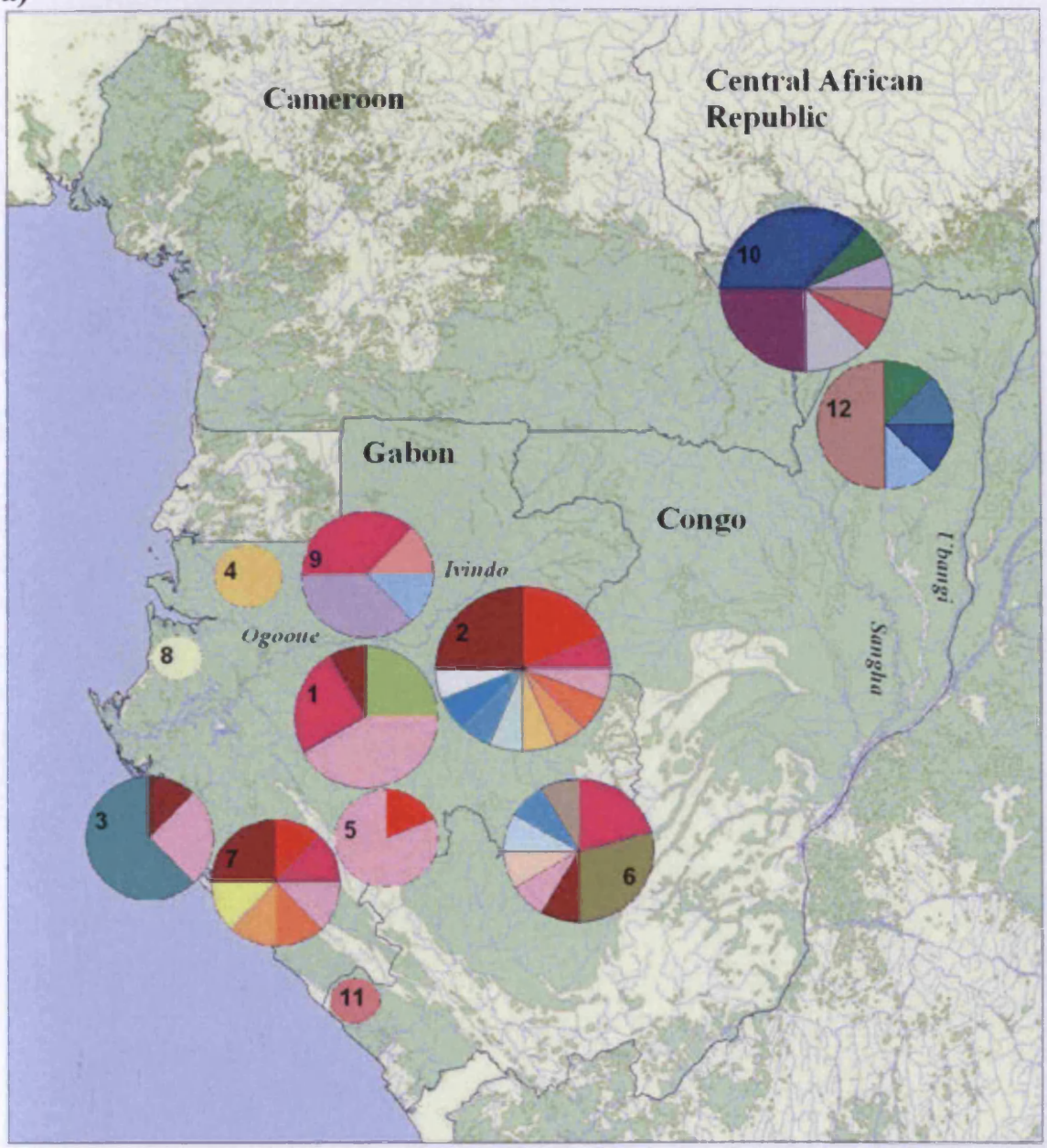
Twenty-four polymorphic sites, comprising 21 transitions, three transversions, and no insertions/deletions were observed. Thirty-two haplotypes (Table 3.2; Figure 3.2 a) were defined of which 34% (H01 to H07 and H20, H22, H25, H27) were shared between more than one population and the remaining 66% (H08 to H19 and H21, H23, H24, H26, H28 to H30 and H32) were observed only once. PBA (site 6) possessed the highest number of unique haplotypes (H08, H09, H24, H28, H30), followed by IVI, (H16, H18, H19, H32) and CAR (H13, H21, H23, H29). Three, four and 13 haplotypes were locality specific in Congo, CAR, and Gabon respectively. Two haplotypes (H20, H27) were shared between NN and CAR localities, and only NOG from Gabon (site 9) shared two haplotypes, H22, H25, with CAR and NN, respectively. Haplotype H04 had the highest frequency (13.5%)

and occurred in 50% (six) of the populations studied. Thirty-four percent of the haplotypes only occurred in IVI (site 2; n = 16) with 21 % in CAR (site 10; n = 16) and they shared no haplotypes. Haplotype H01 was widely distributed in Gabon but not in CAR and Congo.

Table 3.2. Description of the haplotypes distribution between sampled populations.

Haplotype	LOP	IVI	LOA	MDC	WAK	PBA	RAB	NYO	NOG	CAR	CKT	NN	**Total**
H01	1	4	1	.	.	1	2	.	.	.	.	.	**9**
H02	.	3	.	.	1	.	1	.	.	.	.	.	**5**
H03	3	1	.	.	.	2	1	.	3	.	.	.	**10**
H04	4	1	2	.	4	1	1	.	.	.	.	.	**13**
H05	.	1	.	.	.	.	1	.	.		.	.	**2**
H06	.	1	.	.	.	.	1	.	.	.	.	.	**2**
H07	.	1	.	2	.	.	.	.	.	.	.	.	**3**
H08	.	.	.	.	.	1	.	.	.	.	.	.	**1**
H09	.	.	.	.	.	3	.	.	.	.	.	.	**3**
H10	3	.	.	.	.	.	.	.	.	.	.	.	**3**
H11	.	.	.	.	.	.	1	.	.	.	.	.	**1**
H12	.	.	.	.	.	.	.	1	.	.	.	.	**1**
H13	.	.	.	.	.	.	.	.	.	1	.	.	**1**
H14	.	.	5	.	.	.	.	.	.	.	.	.	**5**
H15	.	.	.	.	.	.	.	.	.	.	.	1	**1**
H16	.	1	.	.	.	.	.	.	.	.	.	.	**1**
H17	.	.	.	.	.	.	.	.	.	.	.	1	**1**
H18	.	1	.	.	.	.	.	.	.	.	.	.	**1**
H19	.	1	.	.	.	.	.	.	.	.	.	.	**1**
H20	.	.	.	.	.	.	.	.	.	6	.	1	**7**
H21	.	.	.	.	.	.	.	.	.	4	.	.	**4**
H22	.	.	.	.	.	.	.	.	3	1	.	.	**4**
H23	.	.	.	.	.	.	.	.	.	2	.	.	**2**
H24	.	.	.	.	.	1	.	.	.	.	.	.	**1**
H25	.	.	.	.	.	.	.	.	1	.	.	1	**2**
H26	.	.	.	.	.	.	.	.	1	.	.	.	**1**
H27	.	.	.	.	.	.	.	.	.	1	.	5	**6**
H28	.	.	.	.	.	1	.	.	.	.	.	.	**1**
H29	.	.	.	.	.	.	.	.	.	1	.	.	**1**
H30	.	.	.	.	.	1	.	.	.	.	.	.	**1**
H31	.	.	.	.	.	.	.	.	.	.	1	.	**1**
H32	.	1	.	.	.	.	.	.	.	.	.	.	**1**
Total	**11**	**16**	**8**	**2**	**5**	**11**	**8**	**1**	**8**	**16**	**1**	**9**	**96**
Different	**4**	**11**	**3**	**1**	**2**	**8**	**7**	**1**	**4**	**7**	**1**	**5**	
Unique	**1**	**4**	**1**	**0**	**0**	**5**	**1**	**1**	**1**	**4**	**1**	**2**	

a)


Cameroon
Central African Republic
Gabon
Congo
Ivindo
Ogooue
Ubangi
Sangha
1
2
3
4
5
6
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10
11
12

b)

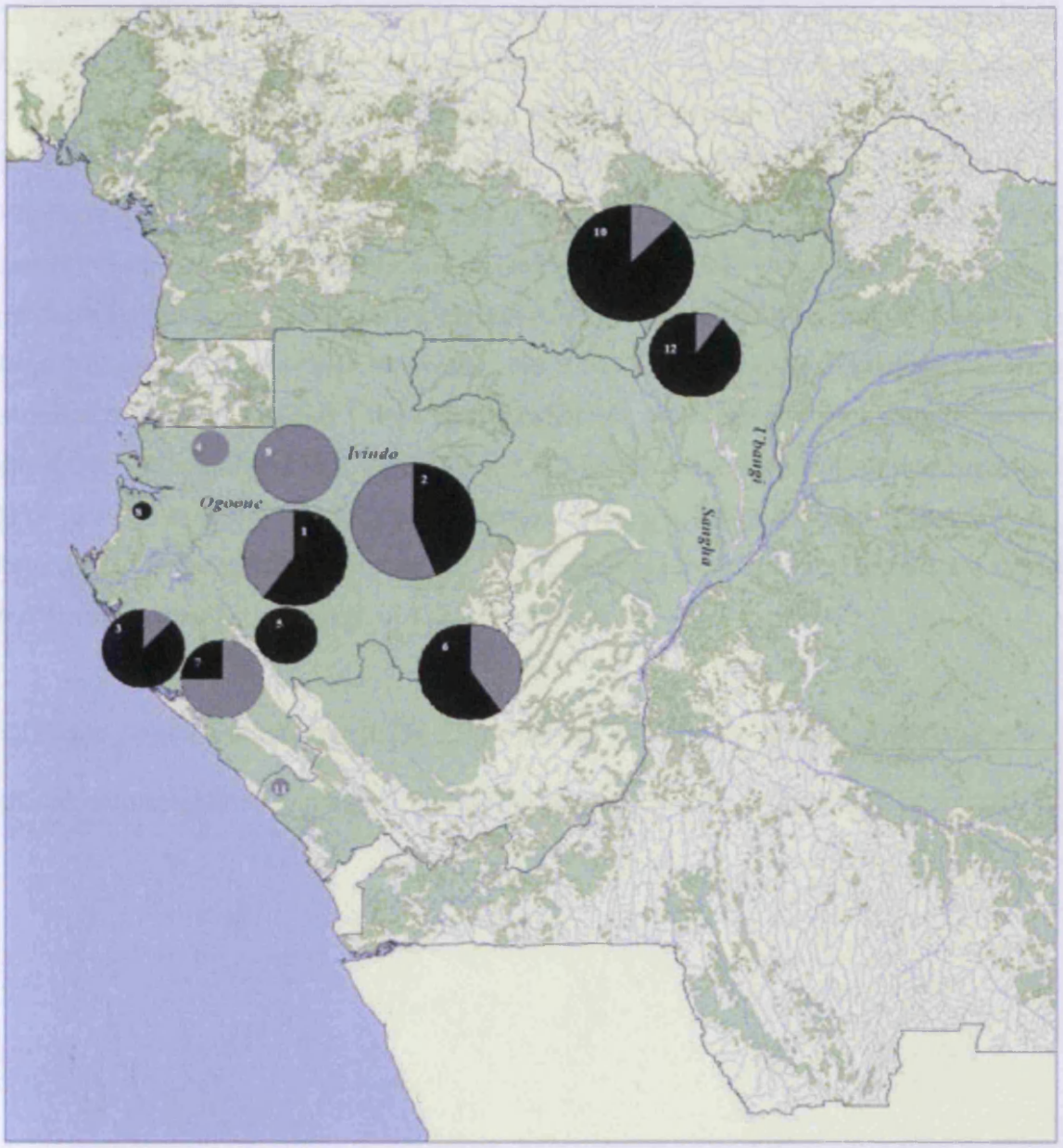


Figure 3.2. Geographic distribution of 32 haplotypes (a) representing by different colours and detailed in Table 1; and (b) the distribution of both haplogroups A (in black) and B (in grey). The size circle is proportional to the number of sequences at each site and number inside each circle is the site number defined in Table 1.

The median joining network (Figure 3.3) generated in this study revealed two main haplogroups (A and B) but no major groupings consistent with a defined geographical location. Haplogroup A (n = 53) and Haplogroup B (n = 43) were geographically overlapping and spanned from northeastern Congo and southern CAR to almost all sites in Gabon, except haplotypes from MDC, NOG and CKT that only occurred in Haplogroup B (see also Figures 3.2 a and b. Sites 4, 9 and 11 and haplotypes from WAK and NYO did not possess haplogroup B (see also Figure 3.2, sites 5 and 8). Fifty-eight percent of all the individuals in Haplogroup A were sampled at the Gabon sites and 42 % from NN and CAR localities. However, only 9 % of individuals in Haplogroup B were sampled in Congo and CAR sites with the remaining 91 % mainly being sampled in IVI and LOP National Parks in Gabon (Table 3.2; Figures 3.2 a and b). Note that haplotype H31 (n = 1) sampled in CKT, south-western Congo (Conkouati-Douli National Park) belonged to Haplogroup B and grouped with haplotypes from Gabon. Figure 3.2 b shows the distribution of both haplogroups as described above.

Phylogenetic reconstruction with the 32 haplotypes used for the network revealed 2 main groups with neighbour-joining (NJ) bootstrap support of 96 % (Figure 3.4).

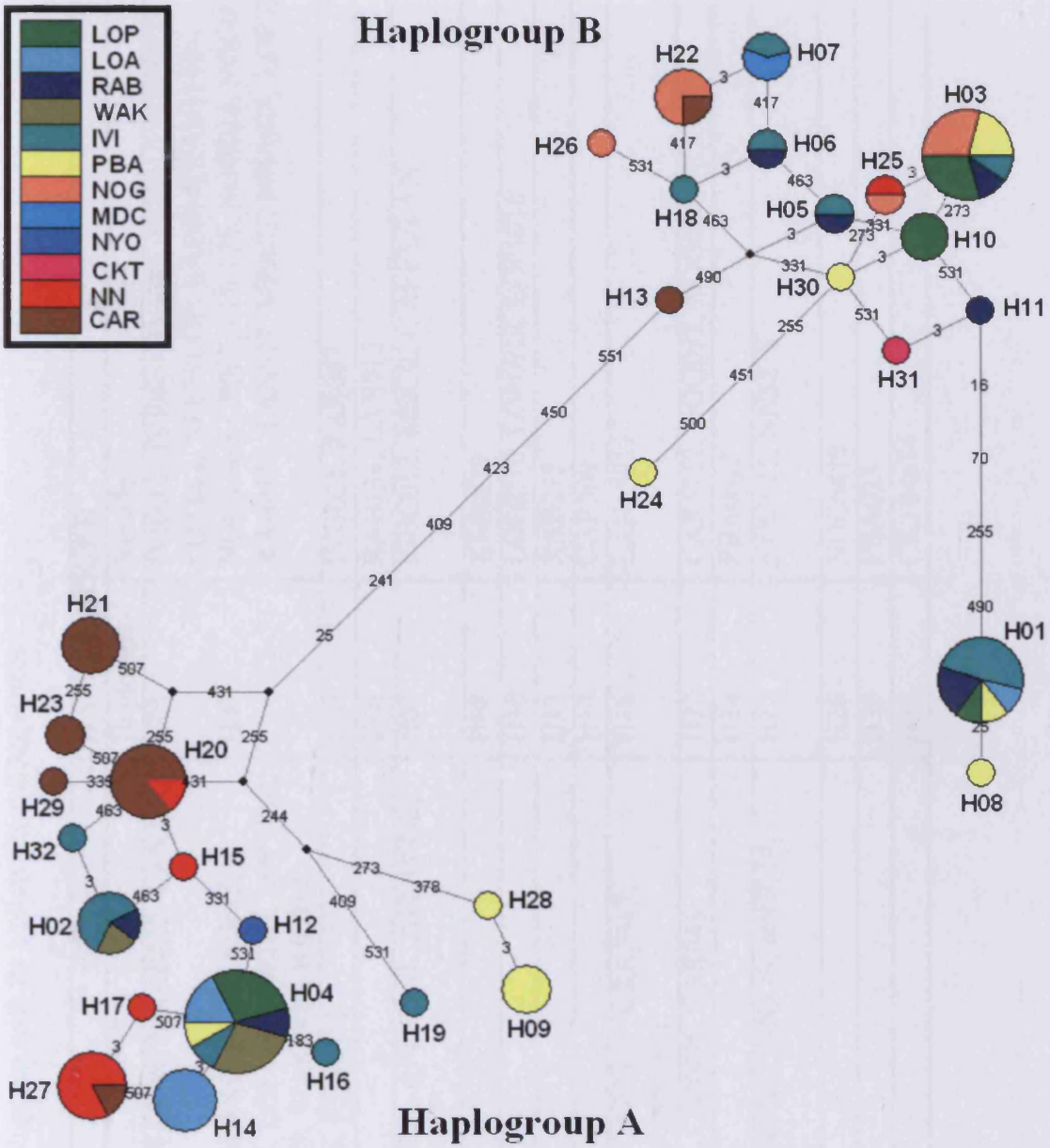


Figure 3.3. Median joining network of forest elephant sequences. Each circle represents a haplotype and its size is proportional to the haplotype frequency. Black dots are median vectors of unsampled or extinct ancestral sequences. Numbers indicates the nucleotide sites that have undergone substitutions. Each colour represents the sampled site where the haplotype has occurred

Table 3.3. Description of each haplogroup with its haplotypes and sequences.

HAPLOGROUP A		**HAPLOGROUP B**	
Haplotype	Sample	Haplotype	Sample
H02	IVI05a6, IVI05b8, WAK0817, RAB032, LAN209	H01	IVI06b2, IVI06c4, IVI088, IVI0910, KES0721, LOA062, AFE79LOP, RAB044, RAB1118
H04	IVI043, RAB0215, WAK0512, WAK0613, WAK0715, LOP146, LOP154, LOP167, LOP1810, LOA0310, MPA0319, WAK0410, IGL032	H03	NOG014, LOP067, LOP175, NOG026, NOG038, RAB0113, LAN027, MPA01, MPA028, LOP51a14
H09	KES0211, KES 0314, KES0415	H05	IVI1011, RAB067
H12	NYO0310	H06	RAB131, LAN015
H14	AFE85IGL, AFE86IGL, AFE87IGL, AFE88IGL, AFE89IGL	H07	MDC012, MDC024, AFE82LAN
H15	NN0713	H08	KES0819
H16	IVI1012	H10	LOP0710, LOP0914, LOP1016
H17	NN059	H11	RAB275
H19	LAN16014	H13	CAR309
H20	CAR274, CAR297, CAR3111, CAR3417, CAR4210, CAR5813, NN2911	H18	LAN15911
H21	CAR3315, CAR381, CAR405, CAR441	H22	CAR5712, NOG053, NOG066, NOG078
H23	CAR3214, CAR394	H24	PBA0612
H27	CAR3622, NN232, NN267, NN279, NN3014, NN3116	H25	NOG025, NN3218
H28	PBA0714	H26	NOG0810
H29	CAR3519	H30	PBA023
H32	LAN1566	H31	CKT04a14

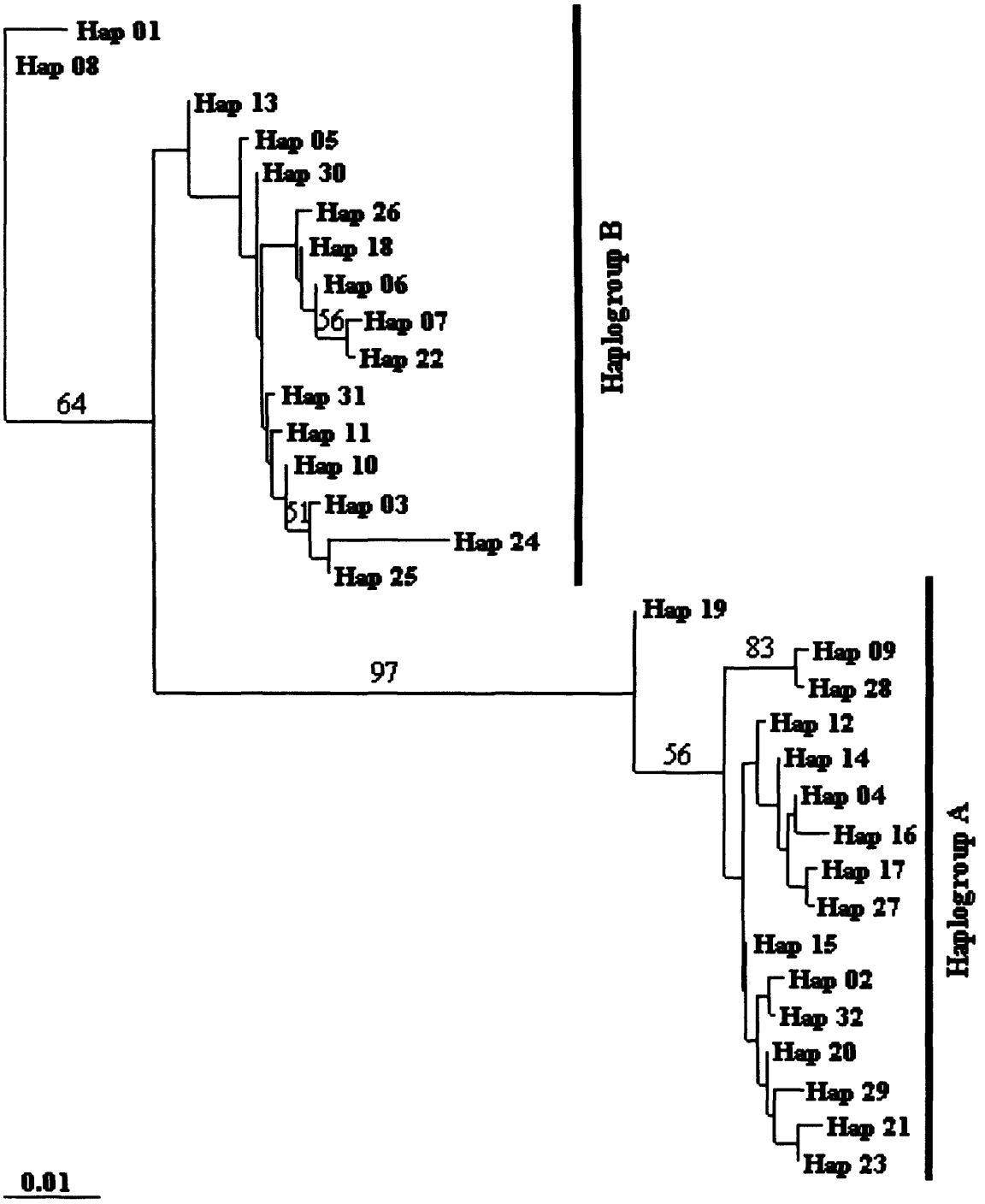


Figure 3.4. Neighbour-joining bootstrap consensus tree of forest elephant mitochondrial HVR1 haplotypes. Numbers above tree branches represent the percentage of bootstrap replicates for that branch. Estimates of bootstrap support are based on 1000 replicates and the tree is unrooted. Haplogroups A and B are indicated and matched to MJN in Figure 3.

3.3.2 Genetic diversity and population structure

The estimated haplotype diversity in the total sample was 95%, and ranged within populations from 40% (WAK) to 96% (RAB). Nucleotide diversity in the total sample was 0.013 (± 0.007), with the highest value in Gabon (0.013 ± 0.007) whereas CAR and NN possessed values of 0.006 (± 0.003) and 0.007 (± 0.005), respectively. Within populations, nucleotide diversity varied considerably from zero in MDC and NYO, 0.005 (± 0.003) in LOA to 0.015 (± 0.009) in PBA (Table 3.4), which was lower than has been observed in savannah elephants (2 %; Nyakaana *et al*, 2002), Asian elephants (1.8 %; Fernando *et al.*, 2000), and in several African mammals examined to date in Clifford *et al.* (2004) for the western lowland gorillas (6.2 %), in Nersting & Arctander (2001) for impala (3.6 %) and kudu (3.2 %), in Muwanika *et al.* (2003) for the common warthog (1.5 %), the savannah buffalo (4.7 %) and the common hippopotamus (1.84 %), and in Moodley & Bruford (2007) with the African bushbuck where both subspecies *Tragelaphus scriptus scriptus* and *T. s. sylvaticus* possessed nucleotide diversities of 3.5 % and 6.2 %, respectively.

The analysis of molecular variance showed a significant differentiation among populations in the total sample $Phi_{ST} = 0.33$; $P < 0.005$, with the majority of the variance, 66.97%, being partitioned within populations. AMOVA was also tested for the two haplogroups defined from MJN and NJ (Haplogroups A and B). The test revealed a highly significant subdivision among populations in both samples ($Phi_{ST} = 0.792$, $P < 0.005$), among populations within groups ($Phi_{SC} = 0.335$, $P < 0.005$) and, as expected, among defined haplogroups ($Phi_{CT} = 0.687$, $P < 0.005$; see Table 3.5).

Pairwise population differentiation tests revealed varying levels of subdivisions, with Phi_{ST} ranging from – 0.23; $P > 0.05$ (RAB-CKT) to 1.00; $P > 0.05$ (NYO – MDC). There was significant population differentiation in 48.5 % of the pairwise comparisons, where Phi_{ST} values were at the $P < 0.05$ significance level (see details in Table 3.5).

Table 3.4. Summary statistics for the control region variation in forest elephant populations with five or more samples in Gabon, CAR, NN, and for haplogroups A and B, and the entire sample.

	LOP	IVI	LOA	WAK	PBA	RAB	NOG	CAR	NN	Haplogroup A	Haplogroup B	Total
n	11	16	8	5	11	8	8	16	9	53	43	96
A	4	11	3	2	8	7	4	7	5	16	16	32
H	78	93	61	40	93	96	79	82	72	89.6	89.4	94.7
p	1.1	1.4	0.5	0.2	1.5	1.2	0.5	0.6	0.7	0.5	0.4	1.3

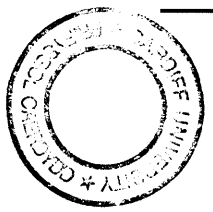
n = sample size; A = number of haplotypes in each population; H = percent haplotypes diversity; p = percent nucleotide diversity.

Table 3.5. Analysis of molecular variance (AMOVA) based on mitochondrial haplotypes.

	Total sample			Two groups		
	% of variance	F-statistics	*P*-value	% of variance	F-statistics	*P*-value
Among populations	33.03	0.330	$P < 0.005$			
Within populations	66.97	-	-			
Among populations				20.83	$Phi_{ST} = 0.792$	$P < 0.005$
Among populations within groups				10.49	$Phi_{SC} = 0.335$	$P < 0.005$
Among groups				68.68	$Phi_{CT} = 0.687$	$P < 0.005$

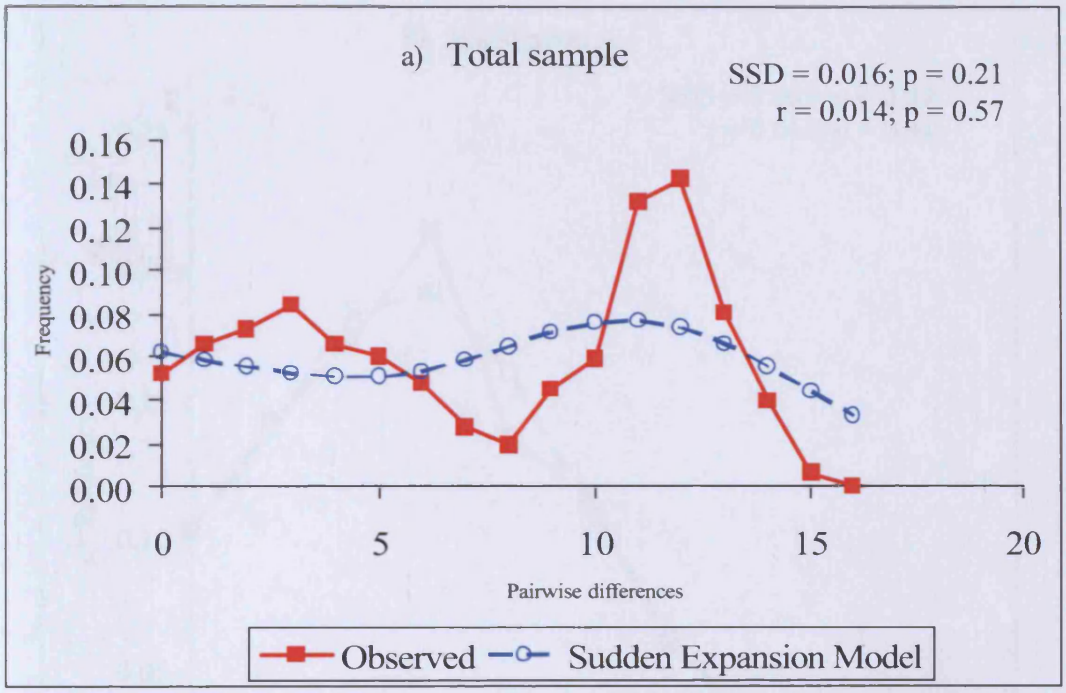
Table 3.6. Pairwise F_{ST} values between forest elephant population in Central Africa, based on variation in mitochondrial DNA. The level of significance shown as * denotes significance at $P = 0.005$.

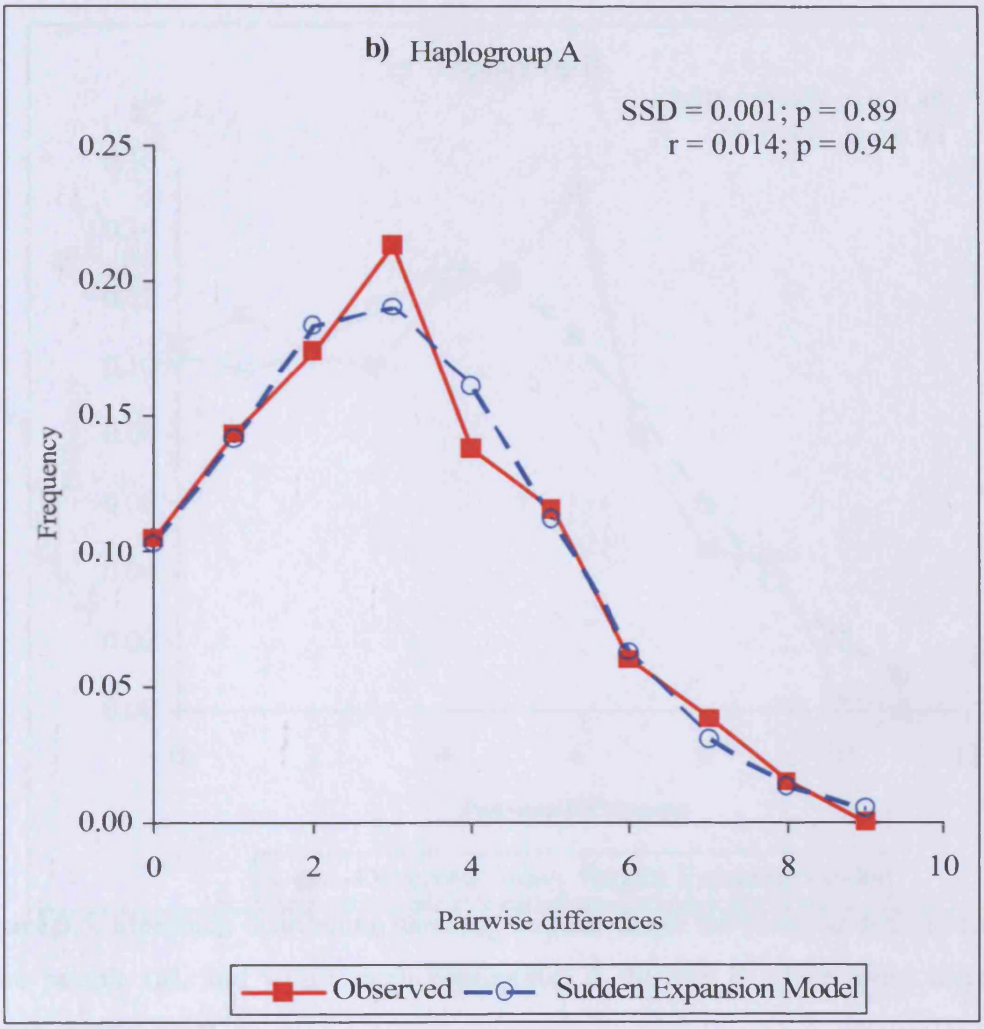
	LOP	IVI	LOA	MDC	WAK	PBA	RAB	NYO	NOG	CAR	CKT	NN
LOP		76	328	204	152	352	288	248	16	580	400	620
IVI	0.034		388	268	196	304	348	328	80	512	416	552
LOA	0.365*	0.253*		352	200	488	50	258	340	902	256	940
MDC	0.357	0.206	0.819*		284	556	344	122	192	692	528	740
WAK	0.397*	0.253*	0.117	0.923*		324	156	260	168	704	256	740
PBA	0.029	0.029	0.294*	0.298	0.312*		436	572	368	588	330	604
RAB	-0.052	-0.040	0.424*	0.194	0.452*	0.027		124	192	856	220	896
NYO	0.070	-0.107	-0.113	1.000	0.000	-0.122	0.152		248	796	528	844
NOG	0.298*	0.271*	0.749*	0.212	0.809*	0.289*	0.214*	0.739		572	416	612
CAR	0.478*	0.313*	0.381*	0.731*	0.425*	0.361*	0.496*	0.134	0.692*		872	52
CKT	-0.100	-0.042	0.681	1.000	0.887	-0.057	-0.234	1.000	0.029	0.665		900
NN	0.368*	0.264*	0.078	0.740*	0.197*	0.284*	0.419*	-0.161	0.695*	0.174*	0.581	



3.3.3 Population demography

The mismatch distribution showed a bimodal pattern when the total sample was analysed (Figure 3.5a), reflecting the existence of two differentiated haplogroups. When forest elephants were subdivided into the two groups corresponding to Haplogroups A and B, as defined by the network, the patterns observed were unimodal, and visually, they fitted well with their corresponding distributions expected under a sudden expansion model (see Figures 5b an 5c). Furthermore, the model parameters θ_0 and θ_1 calculated for Haplogroup A and B separately, showed values expected under a model of rapid growth in both cases (see Table 3.7). Tests for the goodness-of-fit of the observed data supportedthe expansion model as showed SSD value for both Haplogroup A and Haplogroup B (SSD = 0.001, P = 0.90; and SSD = 0.003, P = 0.89, respectively; see Figures 3.5b and 5c. The small raggedness index (r) value supported a smooth distribution and a sudden expansion hypothesis (Table 3.7). The neutrality test supported the expansion model for the total sample with a negative value (Fs = -6.68; P = 0.006), and for both haplogroups A and B with negative Fs values, although it was not significant for Haplogroup B (P = 0.08) (Table 3.7).


a) Total sample
SSD = 0.016; p = 0.21
r = 0.014; p = 0.57
Frequency
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0
5
10
15
20
Pairwise differences
Observed
Sudden Expansion Model


b) Haplogroup A
SSD = 0.001; p = 0.89
r = 0.014; p = 0.94
0.25
0.20
0.15
0.10
0.05
0.00
Frequency
0
2
4
6
8
10
Pairwise differences
Observed
Sudden Expansion Model

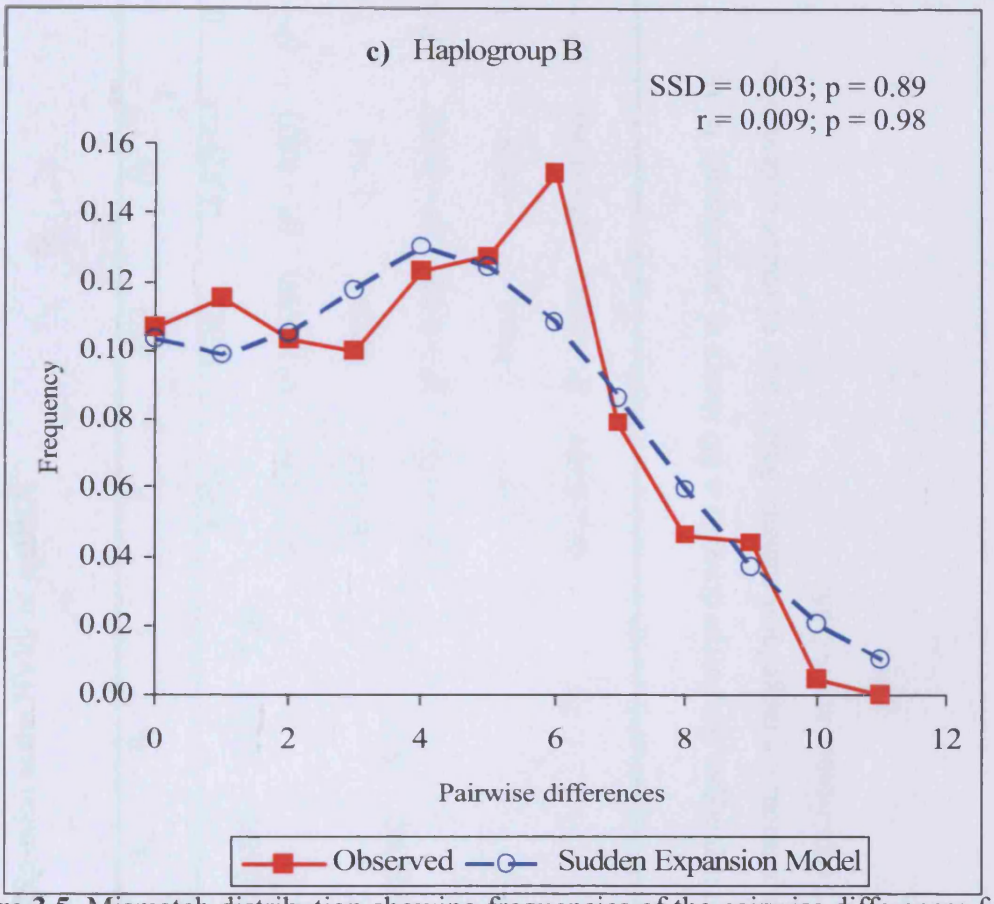


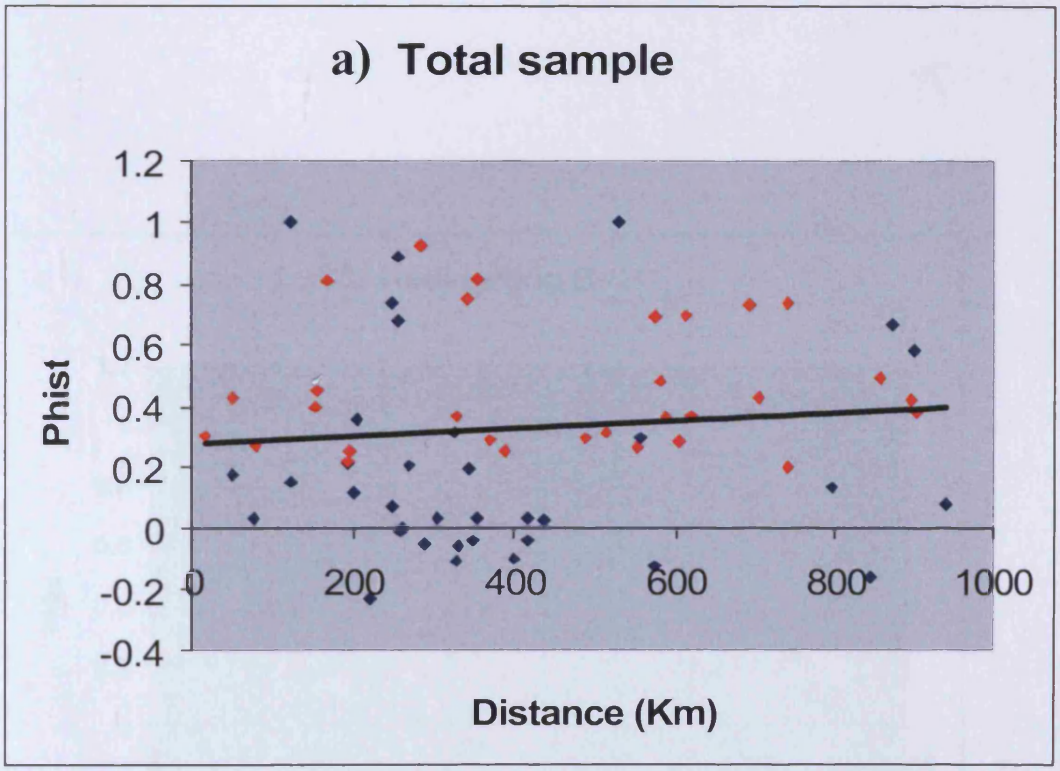
Figure 3.5. Mismatch distribution showing frequencies of the pairwise differences for the entire sample (**a**), and within each haplogroup A (**b**) and B (**c**) of forest elephants in Central Africa.

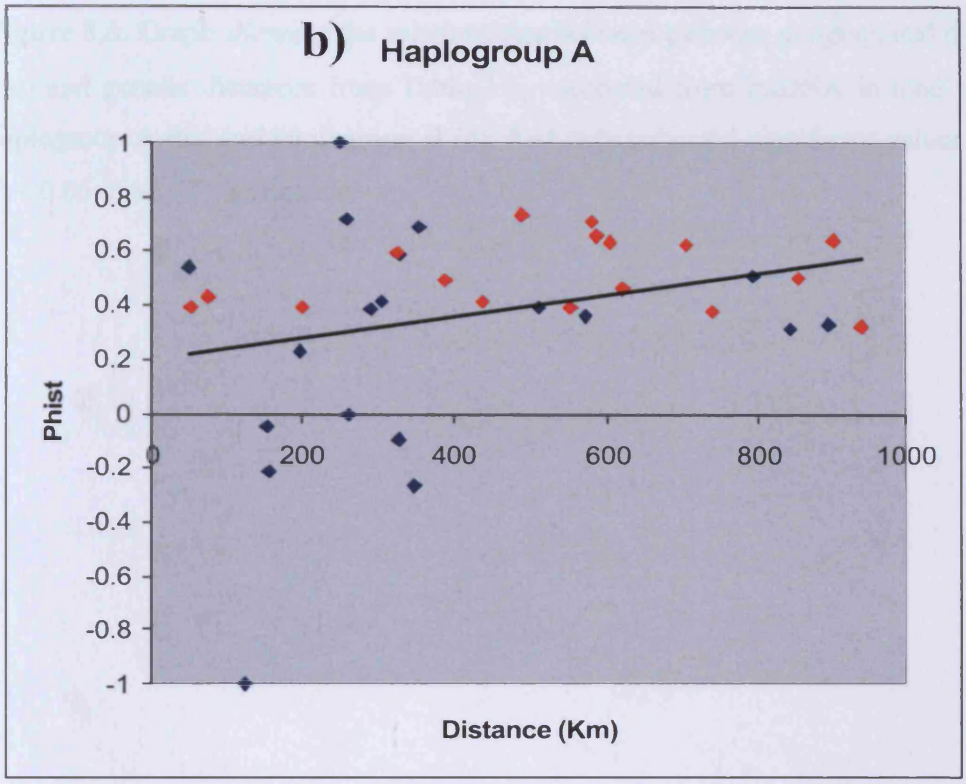
Table 3.7. Summary statistics for population expansion at the HVR1 of mtDNA.

	n	*h*	θ_0	θ_1	τ	*r*	*SSD*	*Fs*	*D*
Haplogroup A	53	16 (0.896 ± 0.000)	0.000	11.03	3.54	0.014 (*P* = 0.94)	0.001 (*P* = 0.90)	-4.829* (*P* = 0.03)	0.137 (*P* = 0.60)
Haplogroup B	43	16 (0.894 ± 0.000)	0.000	8.93	5.80	0.0094 (*P* = 0.98)	0.003 (*P* = 0.89)	-3.743 (*P* = 0.08)	1.045 (*P* = 0.87)
Total sample	96	32				0.015 (*P* = 0.57)	0.016 (*P* = 0.21)	-6.679 (*P* = 0.06)	1.80 (*P* < 0.005)

Note: *n* = number of sequences; *h* = number of haplotypes (haplotype diversity ± SD shown in parentheses); θ_0, θ_1 and τ are the parameters of the demographic expansion; *r* = raggedness statistic; *SSD* = sum of square of deviation; *Fs* = Fu's statistic and *D* = Tajima's statistic. * significance at $p < 0.05$.

A Mantel test, performed to check for isolation by distance, using Phi_{ST}, revealed, for the total sample, a correlation coefficient equal to 0.10, which was not significantly different from zero (P = 0.31) indicating no relationship between geographical and genetic distances. For example, the genetic differentiation between WAK and MDC was 0.923 (P = 0.036) with 284 km separating both sites, while the most geographically isolated populations, LOA and NN, were not significantly (P = 0.127) differentiated genetically (Table 3.6). When Haplogroup A and Haplogroup B were tested separately, the analyses revealed that the correlation between genetic and geographic distances was 0.29 (P = 0.093) for Haplogroup A and 0.25 (P = 0.088) for Haplogroup B. Both correlations were not significantly different from zero. These results are consistent with the distributions from Figure 3.6 a, b and c.




b) Haplogroup A
Phist
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
0
200
400
600
800
1000
Distance (Km)

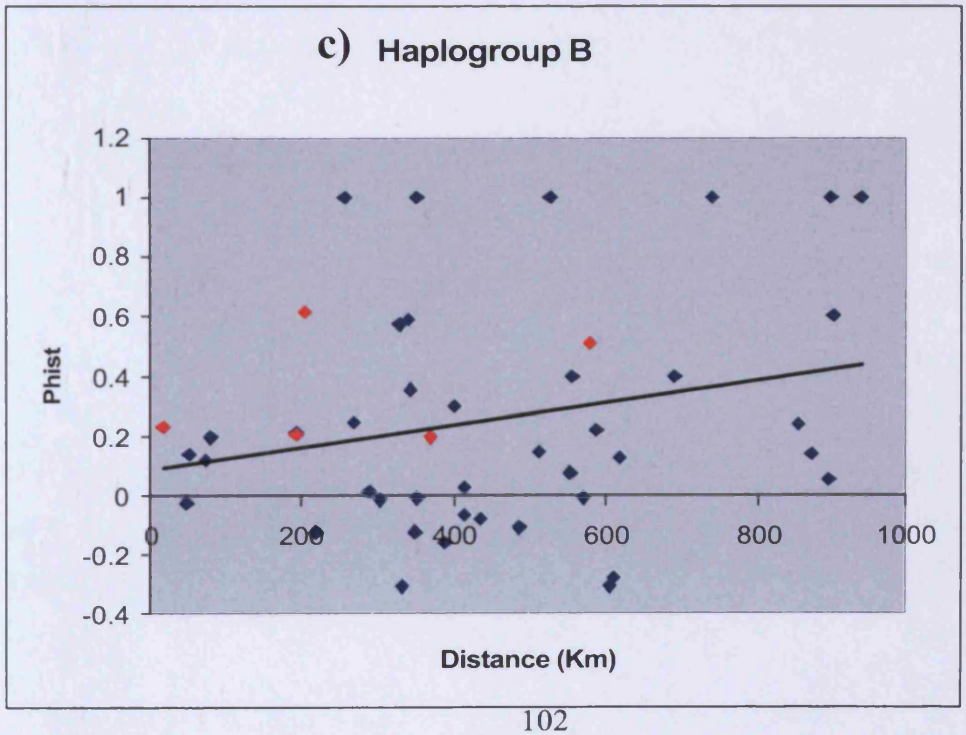

c) Haplogroup B
Phist
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
0
200
400
600
800
1000
Distance (Km)

Figure 3.6. Graph showing the relationships between pairwise geographical distances (in km) and genetic distances from Table 3.6, estimated from mtDNA in total sample (a), haplogroup A (b), and haplogroup B (c). Red dots indicated significant values of Phist at $P < 0.05$ level of significance.

3.4 Discussion

This study presents the first extensive analysis of genetic structure (featuring mitochondrial DNA or any other marker) among forest elephant populations on a regional scale in central Africa. The multiple expansions and contractions to refugia that the African tropical forest has experienced during the Pleistocene may have promoted divergence between fragmented populations. In this study, haplotype distributions and haplotype relationships within and among populations (Figures 3.2 a, b, 3 and 4), AMOVA analysis (Table 3.5), mismatch distribution (Figures 3.5a, b, c), and an absence of differentiation by distance (Figure 3.6a, b and c), suggest the existence of two main haplogroups, but portrayed a lack of broad geographical structure

In Ugandan elephant populations (Nyakaana & Arctander, 1999), two strong divergent clades (A and B) were defined but the phylogenetic relationships of some haplotypes did not coincide with their geographic distribution. A similar contrast has been observed in western lowland gorillas, where some genetic exchange between adjacent phylogroups was detected despite their very strong geographical sub-structuring (Anthony *et al.*, 2007; Clifford *et al.*, 2004). Likewise, at the subspecies level within African bushbuck, very strong genetic structure was inferred using ecoregion biogeographic history despite some connectivity between these ecoregions (Moodley & Bruford, 2007). In forest elephants, some haplotypes were geographically widespread, while related haplotypes were localized and in addition genetic exchange was not limited to the closest populations given that CAR and NN shared haplotypes with NOG, which was the nearest sampled population between CAR/Congo and Gabon. For example, H01, H03 and H04, the highest frequency haplotypes in six of nine Gabonese populations (LOP, IVI, LOA, WAK, PBA and RAB) were not found in CAR or Congo. Haplotypes H20 and H27 appeared in CAR/Congo but not in Gabonese populations. Two haplotypes, H22 and H25, were however shared between the two zones. This pattern suggests both retention of shared ancestral haplotypes, and recent maternal gene flow (Avise *et al.*, 1987). Perhaps surprisingly, given recent results for other African herbivores, both nucleotide and haplotype diversities observed for forest elephants are low, and this is especially clear

when compared to savannah elephant populations as shown in the studies of Nyakaana *et al.* (2002) for African elephants, and to Asian elephants in Fernando *et al.* (2000).

This analysis mostly shows that the majority of genetic variation is partitioned within populations. Significant genetic subdivision between populations and groups of populations has been reported in several African large mammals and particularly in savannah elephants where variation was partitioned between populations in Uganda, East Africa (Nyakaana & Arctander, 1999; Nyakaana *et al.*, 2002). This subdivision was correlated with a limited female dispersal between the three studied populations, while at a regional scale Nyakaana *et al.* (2002) reported a significant genetic differentiation between populations within regions and also among regions. They have discussed the mitochondrial control region DNA results as an allopatric divergence in refugia accompanied by a recent population admixture following a recent population expansion because they observed shared haplotypes between eastern and southern African savannah elephants. Several species of African ungulates have shown similar patterns (Arctander *et al.*, 1999; Birungi & Arctander, 2000; Flagstad *et al.*, 2001; Lorenzen *et al.*, 2007; Van Hooft *et al.*, 2002). In western lowland gorillas (Anthony *et al.*, 2007; Clifford *et al.*, 2004), substantial proportion of the total molecular variance was attributable to differences among the main haplogroups of the control region in a subspecies which occurs in the same geographic area as forest elephants in west central Africa.

Findings from this study also suggested significant pairwise genetic differentiation observed between populations, which may be due to female philopatry, since African savannah elephants often display strong natal and breeding-site fidelity (Georgiadis *et al.*, 1994). However, a female with her infant has been observed, by GPS telemetry, to migrate 2000 km back and forth across the Ndoki forest in central Africa (Blake, 2007). Social organisation involving dispersal or migration (Hoelzel, 1998); reproductive isolation (Brown *et al.*, 2007); diversity of natural ecosystems (Moodley & Bruford, 2007) and more substantially past isolation events during climatic fluctuations of the African Quaternary (Anthony *et al.*, 2007; Arctander *et al.*, 1999; Flagstad *et al.*, 2001) may strongly influence the genetic structure of populations, sub-species or species. In

African savannah elephants, a significant genetic subdivision of the mitochondrial control region sequences between populations in Uganda has been revealed, suggesting limited female dispersal among populations (Nyakaana & Arctander, 1999). In this study, a significant subdivision was observed between close populations such as LOP and NOG ($P = 0.014$) separated only by 16 km, CAR and NN ($P = 0.024$) separated by 52 km, and no significant differentiation between LOP and IVI ($P = 0.178$) separated by 76 km, though LOP is separated from NOG and IVI by the Ogooué River and the Sangha River separates NOG from CAR and NN. Figure 2 shows clearly lineage haplotypes exchange among most of the populations in Gabon and some genetic exchange between CAR and NN. The Ogooué and Sangha Rivers would probably have a little effect on the genetic diversity of central African forest elephant populations. This latter result conflicts largely with other taxa studied to date in Central Africa. The above rivers have played a role in genetic differentiation between western lowland gorilla haplogroups (Anthony *et al.*, 2007); the Ogooué River was also responsible of the divergence of mandrill populations in Cameroon and northern Gabon from those in southern Gabon (Telfer *et al.*, 2003). Other rivers such as the Congo, Ubangi, Ivindo and Sanaga have been reported to influence the genetic structure of bonobos (*Pan paniscus*) (Eriksson *et al.*, 2004), chimpanzees (*Pan troglodytes troglodytes*), and western lowland gorillas (Anthony *et al.*, 2007). However, forest elephants have been observed crossing the Ogooué River at Lopé National Park (Momont, 2007). An elephant likely could cross a river during the dry season when the depth of waters is low, and far from the estuary. Rivers can also change size and shape over time. Consequently, the influence of rivers as barriers to gene flow is likely limited as our data show significant differentiation between populations separate by a river such as NOG and LOP, NOG and WAK, though LOP and IVI are not significantly differentiated. Despite the NOG-LOP, NOG-CAR and NOG-NN subdivision, they still share haplotypes, indicating the Ogooué and Sangha rivers could constitute incomplete barriers to dispersal as shown with gorillas (Anthony *et al.*, 2007).

Elephants are highly mobile and their movements are not random but are driven according to diverse factors including human activities (Barnes *et al.*, 1991), which could limit their dispersal. In this study, Mantel test results indicated a lack of correlation

between genetic and geographic distances, which was also observed in savannah elephants in Uganda (Nyakaana & Arctander, 1999) as they likely had historical migrants or common ancestral populations. Forest elephants have the ability to live in different ecological habitats, from the coastal ecosystem with inundated forest and mangroves through forest/savannah mosaic and mineral clearings, to closed canopy forest. This ecological plasticity could be responsible of the lack of geographical structuring among haplotypes observed in the phylogenetic tree and the median joining network, despite significant frequency differentiation between populations. Similar patterns were shown in the common hippopotamus (*Hippopotamus amphibius*) populations in eastern and southern Africa (Okello *et al.*, 2005).

In the light of the results obtained with the sequences used here, alternative explanations for the history of African elephants become evident and suggest that forest elephants have been affected by cyclical climatic changes that occurred over the last 2.6 million years such as the colder drier periods experienced during Pleistocene. These periods are believed to have led to the repeated retraction of forest cover into refugial zones followed by re-expansion, fostering allopatric divergence between isolated populations (Van Hooft *et al.*, 2002), and by periods of secondary contact during climatic amelioration. In Europe, Pleistocene ice ages have inferred as likely engineers of the genetic structure of populations and species (Hewitt, 1996). However, the absence of phylogeographic patterning is expected in species with high migration rates (Hofreiter *et al.*, 2004) such as the European wolf (Hofreiter *et al.*, 2004; Vila *et al.*, 1999) which shows little partitioning of haplotypes on continental or regional scalesIn this study, the extensive distribution of some haplotypes (H04 for example within haplogroup A) shows a likely history of past bottleneck followed by a recent population expansion. The forest elephant's distribution range is therefore likely to have become centred around (but not exclusive to, given this taxon's ecological flexibility) such refugia on several occasions. The dataset presented here shows the first indications of at least two different refugia in the central African region harbouring distinct elephant populations that diverged allopatrically. If this was the case, forest elephants possessing distinct mitochondrial genotypes are likely to have come into contact relatively rapidly after the end of the last

glaciation (12,000 years BP), when the forests re-expanded. Such a scenario might explain not only the two haplogroups present in forest elephants but also the lower nucleotide diversity that characterises elephant populations found in forest habitat. Clifford *et al.* (2004) and Anthony *et al.* (2007) made similar suggestions about complex evolutionary histories within lowland gorillas located in the same area than this study zones (Gabon, CAR and Congo). They suggested a fluvial refuge in the restricted southern CAR and adjacent Congo and Anthony *et al.* (2007) suggested refugial origins in the Monts de Cristal in northwestern Gabon and adjacent Equatorial Guinea, and in the Massifs du Chaillu and Monts Doudou in southern Gabon. Muloko-Ntoutoume *et al* (2000)'s study also suggested the same refugia locations for Okoumé (*Aucoumea klaineana*) an endemic tree species in Gabon. Roca *et al.* (2001) and Nyakaana *et al.* (2002) depicted similar results in their study on the African savannah elephant, based on nuclear genes and mitochondrial control region, respectively. Lowland gorillas and forest elephants, two inhabitants of the dense equatorial forest in the Congo basin, would have experienced the same complex demographic history during the Pleistocene, giving the patterns observed today.

This scenario might also explain the high microsatellite diversity reported for forest elephants (Comstock *et al.*, 2002). If several populations diverged in isolation, accumulating different microsatellite profiles, and subsequently became sympatric as the forest expanded, the large single population that today comprises two central African forest elephant lineages might be expected to harbour higher microsatellite diversity.
The mitochondrial data presented in this study advances our understanding of the population genetic structure and evolutionary history of African elephants inhabitant the rainforest of central Africa. Phylogenetic analysis with MJN and NJ, and the bimodal pattern of mismatch distribution indicated that changes in the distribution of forest vegetation during the Pleistocene could have formed two main refugia (with the two haplogroups observed), and fostered allopatric divergence between isolated populations of forest elephants. A reduction of population size (bottleneck) likely happened at this time, decreasing the nucleotide diversity, but a population expansion brought the isolated populations into secondary contact increasing haplotype diversity. The lack of geographic

structure, showed by Figures 3.2 (a and b) and Mantel test (Figures 3.6 a, b and c), might be due to the absence of geographic barriers, although this can not be total since some local population structure was observed, which could indicate either a female philopatry or a recent negative impact of human activities with mainly poaching and habitat destruction, isolating some populations.

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CHAPTER 4

Complex phylogeographic history of African elephants does not support a two-taxon model

4.0 Abstract

Previous phylogenetic analyses of African elephants have included a very limited number of forest elephant samples. A large-scale assessment of mitochondrial DNA diversity in forest elephant populations reveals a more complex evolutionary history in African elephants as a whole than current two-taxon models assume. The hypervariable region 1 of the mitochondrial control region was analysed for 71 central African forest elephants and the mitochondrial Cytochrome *b* gene from 28 samples and these sequences were compared to other African elephant data. The central African forest elephant populations fell into at least two lineages and west African elephants (both forest and savannah) shared their mitochondrial history almost exclusively with central African forest elephants. Central African forest populations also showed lower genetic diversity than those in savannahs, indicative of a recent population expansion.

Our data do not support the separation of African elephants into two different taxa. The evolutionary status of African elephants seems more complex, with a combination of multiple refugial lineages and recurrent hybridization among them rendering a simple forest/savannah elephant split inapplicable to modern African elephant populations.

4.1 Introduction

The taxonomic status of the African elephant (*Loxodonta africana*) has been debated since the turn of the 20th century (Matschie, 1900) and up to 22 subspecies have been described (Krumbiegel, 1950). However, modern taxonomy refers to two types, with their names reflecting the habitat in which they are found, namely the larger savannah (*Loxodonta africana africana*) (Blumenbach 1797) and the smaller forest elephants (*Loxodonta africana cyclotis*) (Matschie 1900). It has become increasingly established in the literature that forest and savannah elephants are distinct species (*L. africana and L. cyclotis)* (Barriel *et al.*, 1999; Comstock *et al.*, 2002; Roca *et al.*, 2005; Roca *et al.*, 2001; Wasser *et al.*, 2004), with recent publications considering their datasets in the light of this concept. The most persuasive genetic basis for a two-taxon model originates from a series of studies exploring patterns of differentiation at nuclear loci, culminating in a study using male inherited *Y-chr,* and bi-parentally inherited *X-chr* sequences (Roca *et al.*, 2005) that concluded "*there was a deep and almost complete separation between African forest and African savannah elephants*". In this study, divergent nuc lear DNA sequences segregated with either forest or savannah elephant morphological types. There were, however, a number of exceptions, including a forest elephant from Garamba in the Democratic Republic of Congo (DRC, where forest and savannah populations are sympatric) that had nuclear sequences typical of savannah elephants and two savannah elephants from Cameroon (at the limit of the forest-savannah transition zone) that had nuclear sequences typical of forest elephants (Roca *et al.*, 2005). The study estimated the divergence between the savannah and forest elephants to be three million years. The two-taxon argument has also been used to explain data from two nuclear microsatellite DNA (Comstock *et al.*, 2002; Wasser *et al.*, 2004) and one morphological study (Groves, 2000; Grubb *et al.*, 2000). However, recently Debruyne (2005) performed a morphometric analysis of museum elephant skulls, and found evidence for a continuum between two morphotypes, suggesting that, despite historical separation that promoted subdivision, these two forms interbreed wherever their ranges intersect.

In fact, molecular studies using mitochondrial (mt) DNA (Debruyne, 2005; Eggert *et al.*, 2002) including data from the study by Roca *et al* (Roca *et al.*, 2005) point to a more complex scenario for African elephants. Debruyne (2005) examined several thousand base pairs of mtDNA from elephants across Africa and although he also reported two highly divergent molecular clades, these did not conform to the morphological delineations of *cyclotis* and *africana.* He interpreted these results as a consequence of incomplete isolation between forest and savannah African elephant populations, followed by recurrent and ongoing introgression between the two forms. Roca *et al.* (2005) obtained very similar mitochondrial results but explained the non-concordance between mitochondrial and nuclear markers as a result of cytonuclear genomic disassociation such that the mitochondrial tree did not reflect the species tree. The mtDNA results observed were explained as having arisen during episodes of backcrossing between successive generations of savannah males with forest females, leading to half of extant savannah elephants surveyed possessing 'forest' typical mitochondrial haplotypes but almost exclusively 'savannah' nuclear X and Y-chromosomal DNA. Eggert *et al.* (2002),in addition to Nyakaana et al.'s (2002) mitochondrial sequences included samples from west Africa and found a more complex picture using mtDNA and nuclear microsatellites, suggesting that western savannah and forest elephants formed a potential third *Loxodonta* taxonomic unit.

The above-mentioned studies largely share a pronounced lack of forest elephant data. The nuclear DNA studies (Eggert *et al.*, 2002; Roca *et al.*, 2001) featured extremely limited sampling from central African forest elephants. Despite describing a narrow hybrid zone between the two elephant types, only one population located in this zone (Garamba, (DRC)) was included and none from elsewhere in DRC or from west central Africa were examined. Elsewhere, Debruyne (2005) included elephants from across the DRC in his study but was again limited by small sample sizes. The study by Eggert *et al.* (2002) was limited by the inclusion of only two populations of Central African forest elephants, both from the edge of the forest range in Cameroon which may conceivably have influenced their conclusion of the genetic uniqueness of forest and western elephants. To date, no study has addressed the partitioning of elephant genetic diversity in the equatorial forests

of Africa. Further, the potential effect of Pleistocene forest refugia was partially addressed by Eggert et al. (2002) and also previously reported as having a major influence on large mammal distribution and range dynamics (Arctander *et al.*, 1999; Clifford *et al.*, 2004; Flagstad & Roed, 2003; Hewitt, 1996; Tosi *et al.*, 2005) has yet to be addressed in African elephants. The results from the most extensive sample of forest elephants are reported here, from the core of their range, and these results were compared with previously published DNA sequences for savannah elephants from east and southern Africa and populations from west Africa and DRC.

The phylogeographic history, population structure and past demography of African elephants were examined using patterns of molecular diversity for the mtDNA control region and cytochrome b sequences. Since mtDNA is maternally inherited, this marker provides a female-oriented view of population history and structure. The most variable mtDNA segment, the hypervariable region 1 (HVR1) of the control region was included since it has a high rate of nucleotide change, allowing recently diverged lineages to be distinguished (Douzery & Randi, 1997; Flagstad *et al.*, 2001; Van Hooft *et al.*, 2002). This segment is equivalent to data previously published by Eggert *et al.* (2002) and Debruyne (2005), allowing us to examine forest elephant sequences within the context of a sample set with the largest geographic coverage. Roca et al's (2005) mtDNA sequences were not used as he studied a different fragment (ND5 instead of control region).

4.2 Materials and Methods

4.2.1 Sampling and laboratory procedures

Elephant sequences from 66 sites across Africa were incorporated into the complete dataset (**Figure 4.1**). New forest elephant samples (HVR1 mtDNA: n = 71; Cyt *b* mtDNA: n=28) were obtained from 12 sites in the central African forest block (red dots, **Figure 4.1**). Sample storage and mtDNA amplification were described in Chapter 2.

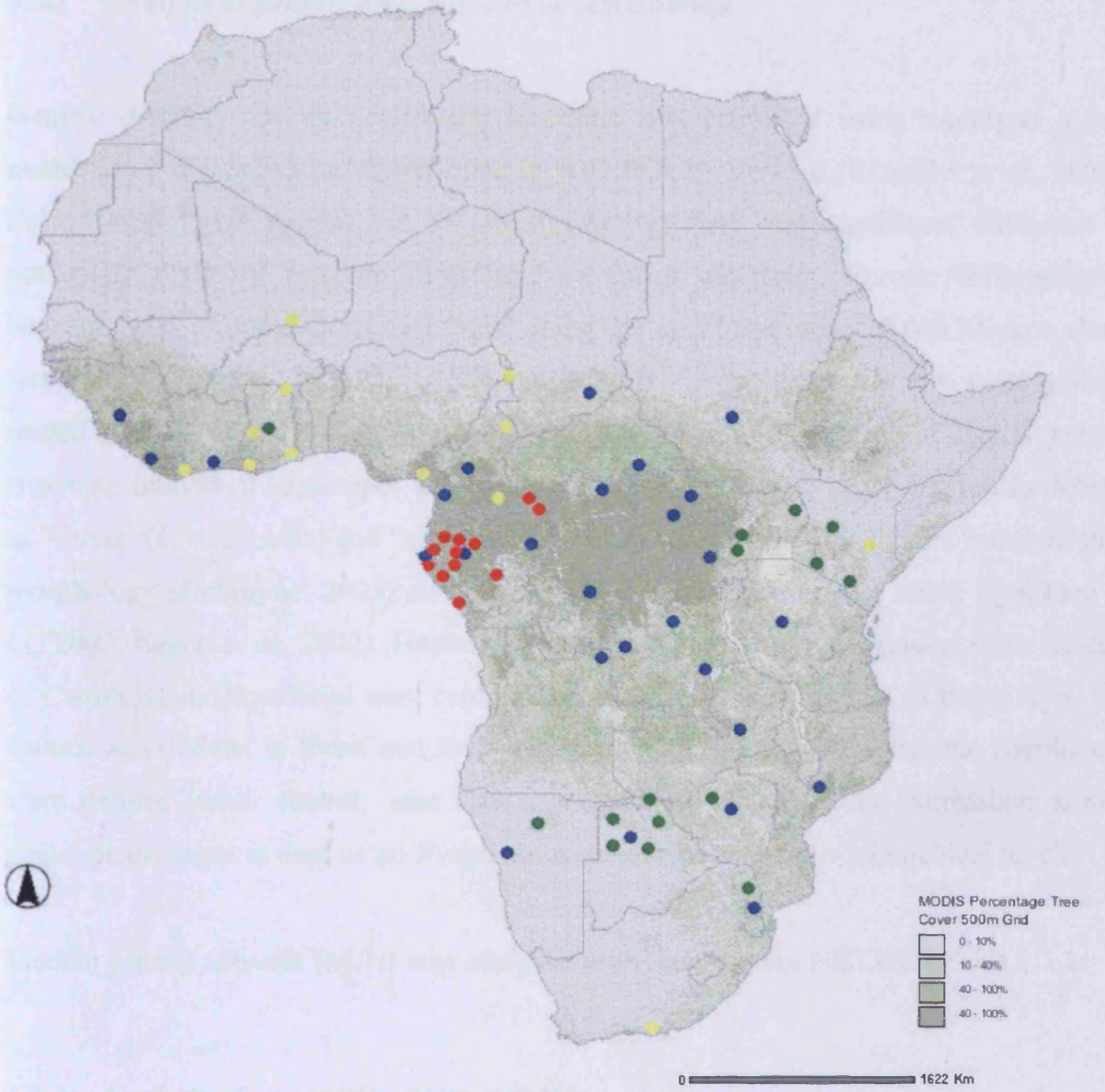

Figure 4.1. Map of Africa showing approximate sampling sites from previous mtDNA studies combined with those from this study. The green, yellow and blue dots are sampling sites from Nyakaana et al. (2002), Eggert *et al.* (2002) and Debruyne (2005), respectively. The red dots are the sites from this study.

4.2.2 Analysis of genetic diversity and differentiation

Genetic diversity for all geographic locations was estimated using haplotype *h* and nucleotide *p* diversities as implemented in ARLEQUIN ver. 3.0 (Excoffier *et al.*, 2005). Paired ttests were carried out to assess whether there was significant difference in nucleotide diversity between forest and savannah elephants. Genetic differentiation between pairs of populations was tested using the exact test using 10,000 Markov chain steps, as implemented in ARLEQUIN ver. 3.0, and this program was also employed for nested analysis of molecular variance (AMOVA) to test for patterns of spatial genetic structure. Individual haplotypes from genbank used in this study were previously defined as "forest" (*L. a. cyclotis*) and "savannah" (*L. a. Africana*) types by authors based on their morphology (Debruyne, 2005) and their original habitat (Debruyne, 2005; Nyakaana *et al.*, 2002; Eggert *et al.*, 2002). Haplotypes produced in this study originated from the core of Congo Basin (Equatorial west central African forest), then defined as forest type. The dataset was divided in forest and savannah groupings and then four regional populations were defined (west, central, east and south). Using AMOVA the correlation among genotype distances is used as an *F*-statistic analog (Phi) at various hierarchical levels.

Median joining network (MJN) was analysed with the program NETWORK V4.1.1.1.

4.2.3 Analysis of population demography

Analyses were carried out to detect evidence of past demographic change using the program ARLEQUIN ver.3.0. A pairwise mismatch distribution analysis was performed, comparing the distribution of the observed pairwise nucleotide site differences with the expected distribution in an expanding population (Rogers & Harpending, 1992). The goodness-of-fit of the observed data to a simulated model of expansion with the sum of square deviations (SSD) and the Harpending's raggedness index *r* were tested, using ARLEQUIN. Population history was also inferred using Fu's F_S test of neutrality (Fu, 1997).

4.3 Results

4.3.1 Central forest samples

We sequenced 316 bp of HVR1 of the control region from 71 samples and 396bp of the cytochrome b from 28 samples. No nuclear copies of mitochondrial DNA (*Numts*) were detected for either sequence.

4.3.2 Genetic diversity

For HVR1, we analysed 189 sequences from 66 sites across Africa in both forest and savannah elephants (**Figure 4.1**). Of these 189 sequences, 102 were from forest elephants (71 samples from the present study and 31 from Genbank) and 87 savannah elephants (all from Genbank). The combined dataset comprised eighty-eight haplotypes (33 and 51 from forest and savannah elephants, respectively) and four haplotypes found in both types. Of the 21 individual central African forest haplotypes identified in this study, 17 were novel (Genbank accessions EU096114 – EU096130). Mean nucleotide diversity (p) for HVR1 sequences for all African elephants was 0.030 (SD=0.015), while mean haplotype diversity (h) was 0.985 (SD=0.003). When haplotypes were divided into forest and savannah, based on prior designation, the forest population p was 0.022 (SD=0.11), significantly lower than for savannah elephants (0.034, SD=0.017; $p<0.001$). The mean haplotype diversity for forest and savannah populations was 0.960 (SD=0.007) and 0.986 (SD=0.004), respectively. The lowest nucleotide diversity of all groupings was for the new central African forest samples in this study (0.013, SD=0.007), while haplotype diversity was 0.947 (SD=0.009) (**Table 4.1a**).

For cytochrome *b*, 100 sequences were analysed, 28 from this study, 27 provided by SN and 45 from Genbank. Forty-four haplotypes were identified including three and 22 new forest and savannah elephant sequences, respectively (Genbank accessions EU115995 – EU116019; see the sequences in Appendix 3). Of the 44 haplotypes, 32 were found in

savannah elephants and 10 in forest elephants, with two haplotypes found in both. Mean p for cytochrome b was 0.023 (0.012) for all elephants. When forest and savannah elephants were subdivided, p was again significantly lower for forest populations (0.009, SD=0.005) than for savannah populations (0.026, SD=0.013; $p<0.001$) **(Table 4.1b)**. These results contrast with the study of Roca *et al.* (2005) who reported 15 haplotypes for 281 elephants at the mitochondrial ND5 locus and described low genetic diversity as being typical for savannah elephants.

Table 4.1 Summary statistics for (a) HVR1 variation in central forest sequences alone and with the combined data set; and (b) cytochrome *b* with the combined data set.

a)

Groupings	HVR1			
	Forest only (552 bp)	Combined short fragment (316 bp)		
		All African	Forest	Savannah
n	96	189	102	87
Hap	32	88	39	55
Ti/Tv	22/3	51/4	28/3	45/2
h	0.9474 (±0.0092)	0.9845 (±0.0026)	0.9604 (±0.0074)	0.9858 (±0.0042)
p	0.0135 (±0.0071)	0.0304 (±0.0155)	0.0221 (±0.0116)	0.0337 (±0.0172)

b)

Groupings	Cytochrome *b*		
	All African	Forest	Savannah
n	96	42	54
Hap	44	12	34
Ti/Tv	45/6	15/0	40/6
h	0.9254 (±0.0181)	0.7131 (±0.0725)	0.9448 (±0.0230)
p	0.0233 (±0.0120)	0.0089 (±0.0051)	0.0256 (±0.0132)

n = number of sequences, Hap = number of haplotypes, Ti/Tv = transition/transversion ratio, h = haplotype diversity and p = nucleotide diversity

4.3.3 Population structure

The median joining networks (MJN) for HVR1 and cytochrome *b* (**Figure 4.2** and **Figure 4.3**) exhibit patterns consistent with a complex demographic history. The HVR1 pattern is more complex (comprising four haplogroups - labelled HVR1 Haplogroup I, II, III and IV) than for cytochrome *b* (three haplogroups - labelled Cytb Haplogroup I, II and III). For the HVR1 region, the most obvious feature is that central African forest elephants (excluding those from DRC) fall into two separate groups (HVR1 Haplogroups I and II) with little geographic structuring, consisting of 19 (HVR1 Haplogroup I) and 20 (HVR1 Haplogroup II) haplotypes with variable frequencies. Only two forest elephants from DRC, share the same haplotype with other forest elephants in HVR1 Haplogroup II. The remaining seven DRC forest elephant haplotypes (all south-east of the Congo River) group with sequences in HVR1 Haplogroup III (which additionally comprises savannah elephants from eastern and southern Africa and one savannah elephant from Cameroon). The other striking feature is that for West African elephants, both forest and savannah types possess haplotypes found almost exclusively within the same haplogroup as central African forest elephants (HVR1 Haplo groups I and II). Twenty-five out of 26 individuals from west Africa are more closely related to central Forest elephants from Gabon, Congo and CAR. A single western savannah individual can be found in HVR1 Haplogroup IV which groups with savannah elepha nts from eastern, southern and central Africa. Analysis of Molecular Variance (AMOVA) of HVR1 sequences revealed a non-significant ($p=0.065$) genetic structure (18.62% variation among populations) when populations were grouped according to geographic distribution (west, central, east and southern Africa). As expected, Cytochrome *b* was less variable than HVR1. Unfortunately, direct comparison between patterns obtained from both regions is compromised due to a lack of equivalent individuals examined at both loci, specifically for savannah elephants. However the overall pattern when individuals from different populations were examined is consistent with the pattern obtained with HVR1, despite the resolution of only three haplogroups as opposed to four. Savannah elephant haplotypes fall into two distinct haplogroups (Cyt *b* Haplogroup II and III) as do forest elephant haplotypes (Cyt *b* Haplogroups I and II). Cytochrome *b* Haplogroup II, which is

divided into two haplogroups for HVR1, is characterised by a network structure in which forest and savannah elephant samples are not overlaid (see **Figure 4.3**). Again all western elephants, both forest and savannah, cluster with central African forest elephants (Cyt *b* Haplogroup I).

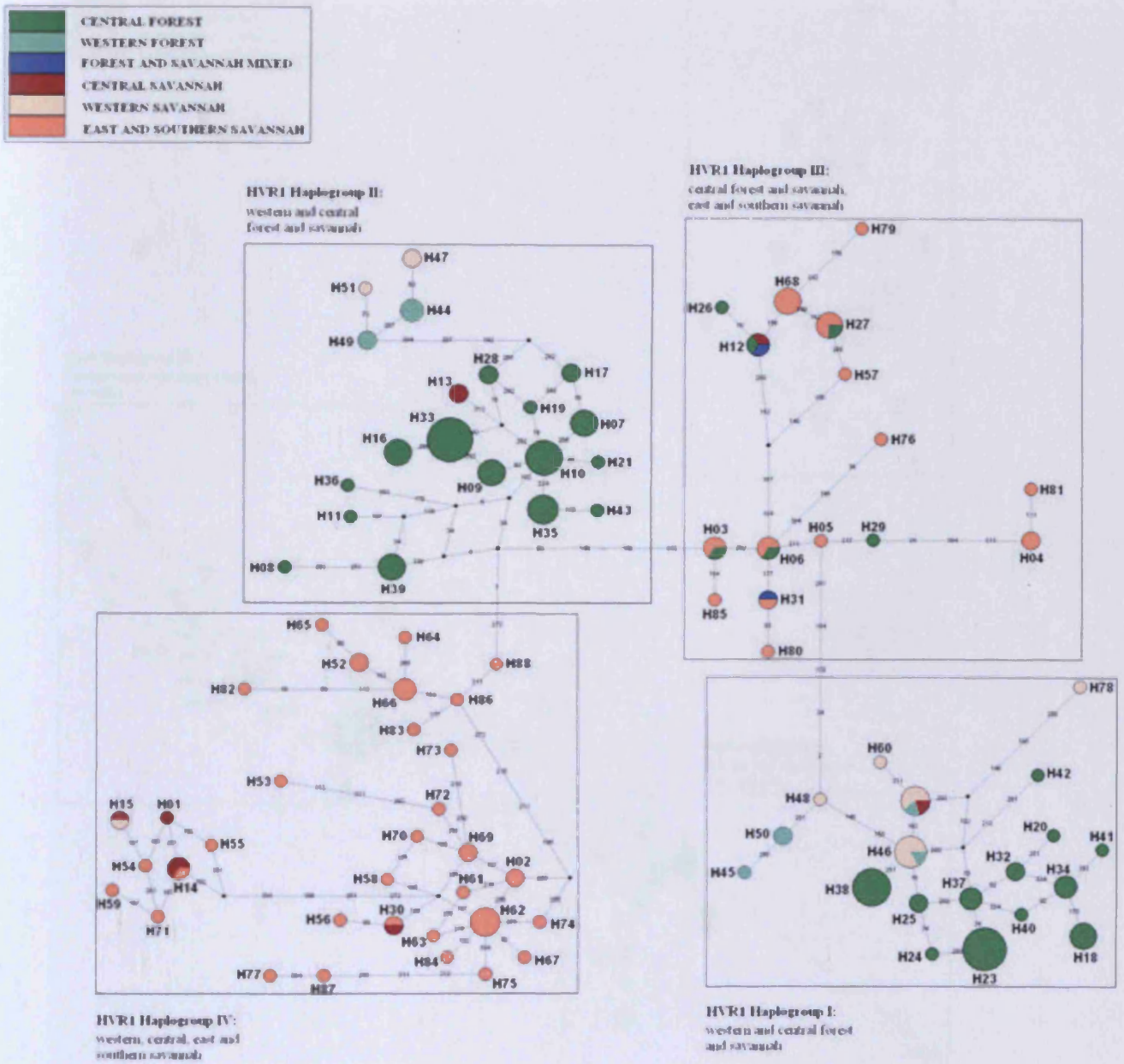

Figure 4.2. Median-joining networks for African elephants HVR1 mtDNA haplotypes. Circle size is proportional to haplotype frequency. The numbers on the connecting line determine the number of substitutions estimated by NETWORK V.4. 1. 1. 1. The entire list of haplotypes for both HVR1 and cytochrome *b* MJN can be found in **Table 4.2**.

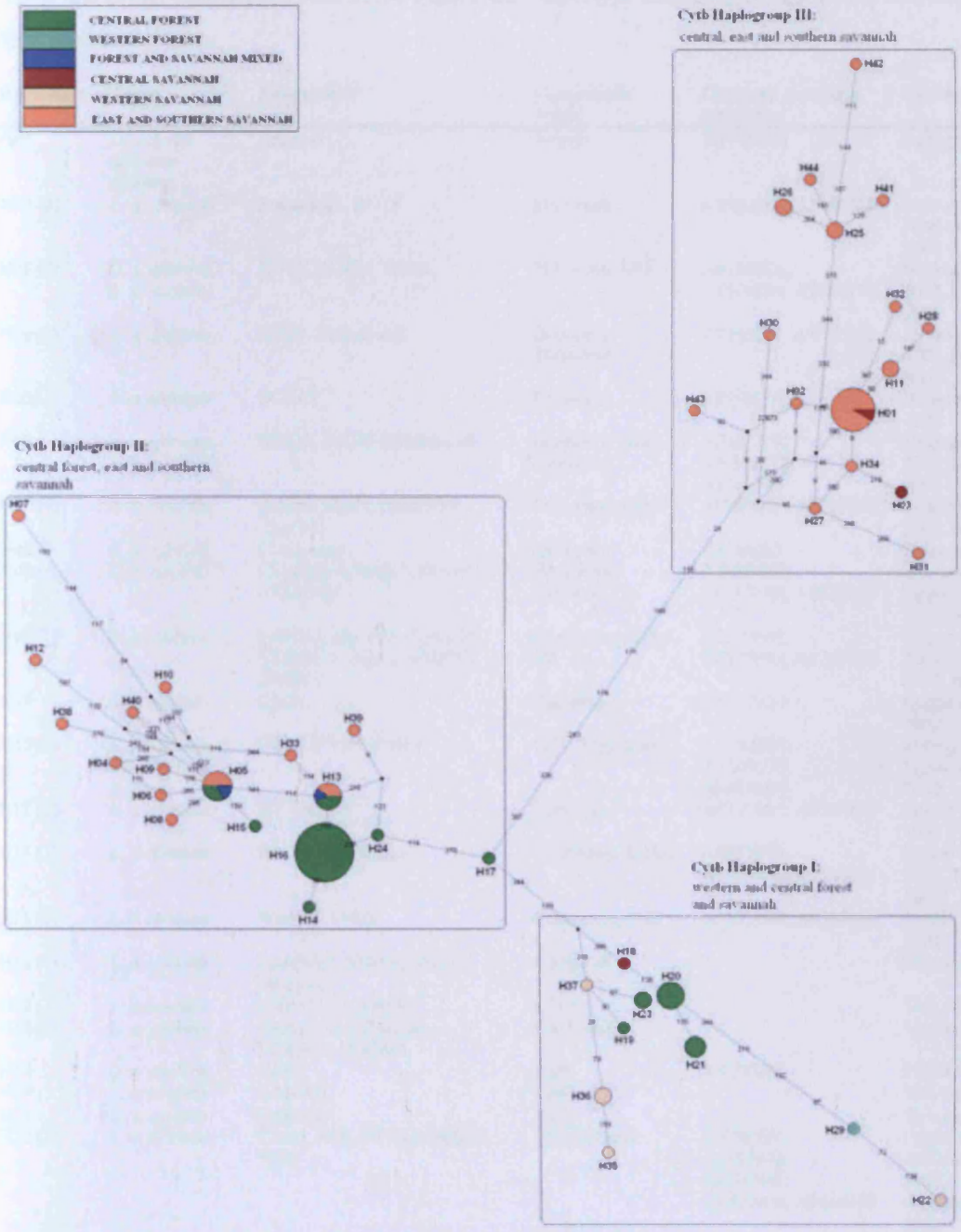

Figure 4.3. Median-joining networks for African elephant Cytochrome *b* mtDNA haplotypes. Circle size is proportional to the haplotype frequency. The numbers on the connecting line determine the number of substitutions estimated by NETWORK V.4. 1. 1. 1. The entire list of haplotypes for both HVR1 and cytochrome *b* MJN can be found in **Table 4.3**.

Table 4.2. HVR1 haplotypes used in the **Figure 4.2**. Haplotype frequency is indicated in brackets when there is more than one.

Haplotype	Taxon	Designation*	Geographic origin	Genbank accession numbers	Author
H01	*Loxodonta africana africana*	Angola1	Angola	AY741072	Debruyne 2005
H02 (2)	*L. a. africana*	Botswana1, BOT4	Botswana	AY741074, AF106230	Debruyne 2005, Nyakaana *et al.* 2002
H03 (3)	*L. a. africana, L. a. cyclotis*	BOT2, BOT21, DRC4	Botswana, DRC	AF106228, AF106234, AY359275	Nyakaana *et al.* 2002, Debruyne 2005
H04 (2)	*L. a. africana*	BOT9, Zimbabwe2	Botswana, Zimbabwe	AF106231, AY741329	Nyakaana *et al.* 2002, Debruyne 2005
H05	*L. a. africana*	BOT15	Botswana	AF106232	Nyakaana *et al.* 2002
H06 (3)	*L. a. africana, L. a. cyclotis*	BOT16, DRC1, Zimbabwe4	Botswana, DRC, Zimbabwe	AF106233, AY359277, AY742799	Nyakaana *et al.* 2002, Debruyne 2005
H07 (4)	*L. a. cyclotis*	Bmbo6, Dja39, CAR3214, CAR394	Cameroon, CAR	AF527653, AF527647	Eggert *et al.* 2002, this study
H08	*L. a. cyclotis*	Cameroon1	Cameroon	AY359267	Debruyne 2005
H09 (4)	*L. a. cyclotis*	Cameroon2, Bmbo1, Bmbo37, NYO0310	Cameroon, Gabon	AY359269, AF527646, AF527649	Debruyne 2005, Eggert *et al.* 2002, this study
H10 (7)	*L. a. cyclotis*	Bmbo16, Bmbo43, CAR274, CAR297, Congo2, NN0713, NN2911	Cameroon, CAR, CR	AF527648, AF527650, AY359268	Eggert *et al.* 2002, Debruyne 2005, this study
H11	*L. a. cyclotis*	Dja34	Cameroon	AF527651	Eggert *et al.* 2002
H12 (3)	*L.africana, L.a. fricana, L. a. cyclotis*	DRC13**, B1, DRC9	DRC, Cameroon	AY741081, AY359279, AF527654,	Debruyne 2005, Eggert *et al.* 2002
H13 (2)	*L. a. africana*	B7, Waza15	Cameroon	AF527655, AF527659	Eggert *et al.* 2002
H14 (3)	*L. a. africana*	B8, Waza10, Sudan1	Cameroon, Sudan	AF527656, AF527658, AY741073	Eggert *et al.* 2002, Debruyne 2005
H15 (2)	*L. a. africana*	Waza27, Mali2	Cameroon, Mali	AF527660, AF527666	Eggert *et al.* 2002
H16 (4)	*L. a. cyclotis*	CAR3622, NN059, NN279, NN3014	CAR, CR		This study
H17 (2)	*L. a. cyclotis*	CAR3315, CAR381	CAR		This study
H18 (4)	*L. a. cyclotis*	CAR5712, AFE82lan, MDC012, NOG053,	CAR, Gabon		This study
H19	*L. a. cyclotis*	CAR1	CAR	AY359272	Debruyne 2005
H20	*L. a. cyclotis*	CAR309	CAR		This study
H21	*L. a. cyclotis*	CAR3519	CAR		This study
H22 (5)	*L. a. africana*	Chad1, K68, RVV15, Mole13, WA6	Chad, Ghana	AY741080, AF527643, AF527641, AF527676, AF106243	Eggert *et al.* 2002, Debruyne 2005, Nyakaana *et al.* 2002
H23 (10)	*L. a. cyclotis*	NN3218, Lan027, LOP067, LOP51a14, NOG014, NOG025, NOG026, Mpa01, Mpa028, RAB0113	RC, Gabon		This study
H24	*L. a. cyclotis*	Congo1	RC	AY359266	Debruyne 2005
H25 (2)	*L. a. cyclotis*	CKT04a14, RAB275	RC, Gabon		This study
H26	*L. a. cyclotis*	DRC2	DRC	AY359270	Debruyne 2005
H27 (4)	*L. a. africana* and *L. a. cyclotis*	KV8, MF1, MF5, DRC3	Uganda, DRC	AF106206, AF106209, AF106210, AY359271	Nyakaana *et al.* 2002, Debruyne 2005
H28 (2)	*L. a. cyclotis*	DRC6, DRC8	DRC	AY359273, AY359274	Debruyne 2005

H29	*L. a. cyclotis*	DRC5	DRC	AY359276	Debruyne 2005
H30 (2)	*L. a. africana*	DRC11, AM1	DRC, Kenya	AY741078, AF106217	Nyakaana *et al.* 2002, Debruyne 2005
H31 (2)	*L. africana* and *L. a. africana*	DRC17**, QE13	DRC, Uganda	AY742802, AF106213	Nyakaana *et al.* 2002, Debruyne 2005
H32 (2)	*L. a. cyclotis*	IVI1011, RAB067	Gabon		This study
H33 (11)	*L. a. cyclotis*	Igl032, AFE85Igl, AFE86Igl, AFE88Igl, IVI1012, IVI043, LOA0310, LOP146, Mpa0319, RAB0215, WAK0410	Gabon		This study
H34 (3)	*L. a. cyclotis*	Lan015, Lan15911, RAB131	Gabon		This study
H35 (5)	*L. a. cyclotis*	Lan1566, IVI05a6, IVI05b8, RAB032, WAK0817	Gabon		This study
H36	*L. a. cyclotis*	Lan16014	Gabon		This study
H37 (3)	*L. a. cyclotis*	Gabon2, LOP0710, PBA023	Gabon	AY359265	Debruyne 2005, this study
H38 (8)	*L. a. cyclotis*	IVI06b2, Kes0721, Kes0819, LOA068, AFE79LOP, PBA0510, RAB044, RAB1118	Gabon		This study
H39 (4)	*L. a. cyclotis*	Kes0211, Kes0314, Kes0517, PBA0714	Gabon		This study
H40	*L. a. cyclotis*	Gabon1	Gabon	AY359278	Debruyne 2005
H41	*L. a. cyclotis*	NOG0810	Gabon		This study
H42	*L. a. cyclotis*	PBA0612	Gabon		This study
H43	*L. a. cyclotis*	IVI05a5	Gabon		This study
H44 (3)	*L. a. cyclotis*	Bia3, Bia69, Liberia1	Ghana, Liberia	AF527677, AF527680, AY741079	Eggert *et al* 2002, Debruyne 2005
H45	*L. a. cyclotis*	Bia48	Ghana	AF527678	Eggert *et al* 2002
H46 (6)	*L. a. cyclotis* and *L. a. africana*	Bia64, RVV22, Mole9, WA3, WA14,Mali7	Ghana, Mali	AF527679, AF527642, AF527675, AF106242, AF106245, AF527667	Eggert *et al* 2002, Nyakaana *et al* 2002
H47 (2)	**L. a. africana**	Mole3, Mali14	Ghana, Mali	AF527674, AF527668	Eggert *et al* 2002
H48	*L. a. africana*	Mole33	Ghana	AF527683	Eggert *et al* 2002
H49 (2)	*L. a. cyclotis*	Tai6, Tai17	Ivory Coast	AF527670, AF527671	Eggert *et al* 2002
H50 (2)	*L. a. cyclotis*	Tai19, Tai29	Ivory Coast	AF527672, AF527673	Eggert *et al* 2002
H51	***L. a. africana***	IvoryCoast1	Ivory Coast	AY741327	Debruyne 2005
H52 (2)	*L. a. africana*	SouthAfrica3, Zimbabwe1	South Africa, Zimbabwe	AY741320, AY741321	Debruyne 2005
H53	*L. a africana*	MM4	Kenya	AF106214	Nyakaana *et al* 2002
H54	*L. a. africana*	MM19	Kenya	AF106215	Nyakaana *et al* 2002
H55	*L. a. africana*	MM20	Kenya	AF106216	Nyakaana *et al* 2002
H56	*L. a. africana*	AM2	Kenya	AF106218	Nyakaana *et al* 2002
H57	*L. a. africana*	AM10	Kenya	AF106219	Nyakaana *et al* 2002
H58	*L. a. africana*	AM12	Kenya	AF106220	Nyakaana *et al* 2002
H59	*L. a. africana*	SA8	Kenya	AF106221	Nyakaana *et al* 2002
H60	*L. a. africana*	Mali28	Mali	AF527669	Eggert *et al* 2002
H61	*L. a. africana*	Mozambique1	Mozambic	AY741076	Debruyne 2005
H62 (5)	*L. a. africana*	Namibia1, Addo5, Uganda1, QE1, Zimbabwe10	Namibia, South Africa, Uganda, Zimbabwe	AY741325, AF527682, AF106211, AY741323,	Nyakaana *et al* 2002, Eggert*et al* 2002, Debruyne 2005

				AY742800	
H63	*L. a. africana*	KH2	Namibia	AF106239	Nyakaana *et al* 2002
H64	*L. a. africana*	Addo1	South Africa	AF527681	Eggert *et al* 2002
H65	*L. a. africana*	KG1	South Africa	AF106240	Nyakaana *et al* 2002
H66 (3)	*L. a. africana*	KG2, Tanzania2, Zimbabwe7	South Africa, Tanzania, Zimbabwe	AF106241, AY741070, AY741067	Nyakaana *et al* 2002, Debruyne 2005
H67	*L. a. africana*	Tanzania1	Tanzania	AY742801	Debruyne 2005
H68 (4)	*L. a. africana*	QE4, Zambia1, Af9, Af10	Uganda, Zambia, Kenya	AF106212, AY741328, AF527639, AF527640	Nyakaana *et al* 2002, Eggert*et al* 2002, Debruyne 2005
H69 (2)	*L. a. africana*	Uganda2, KV1	Uganda	AY741077, AF106203	Nyakaana *et al* 2002, Debruyne 2005
H70	*L. a. africana*	KV2	Uganda	AF106204	Nyakaana *et al* 2002
H71	*L. a. africana*	KV7	Uganda	AF106205	Nyakaana *et al* 2002
H72	*L. a. africana*	KV17	Uganda	AF106207	Nyakaana *et al* 2002
H73	*L. a. africana*	KV28	Uganda	AF106208	Nyakaana *et al* 2002
H74	*L. a. africana*	WC2	Namibia	AF106235	Nyakaana *et al* 2002
H75	*L. a. africana*	WC4	Namibia	AF106236	Nyakaana *et al* 2002
H76	*L. a. africana*	WC6	Namibia	AF106237	Nyakaana *et al* 2002
H77	*L. a. africana*	WC13	Namibia	AF106238	Nyakaana *et al* 2002
H78	*L. a. africana*	WA11	Ghana	AF106244	Nyakaana *et al* 2002
H79	*L. a. africana*	Af8	Kenya	AF527638	Eggert *et al* 2002
H80	*L. a. africana*	ZBE1	Zimbabwe	AF106222	Nyakaana *et al.* 2002
H81	*L. a. africana*	ZBE2	Zimbabwe	AF106223	Nyakaana *et al.* 2002
H82	*L. a. africana*	ZBE3	Zimbabwe	AF106224	Nyakaana *et al.* 2002
H83	*L. a. africana*	ZBE4	Zimbabwe	AF106225	Nyakaana *et al.* 2002
H84	*L. a. africana*	ZBE5	Zimbabwe	AF106226	Nyakaana *et al.* 2002
H85	*L. a. africana*	ZBE6	Zimbabwe	AF106227	Nyakaana *et al.* 2002
H86	*L. a. africana*	Zimbabwe3	Zimbabwe	AY741069	Debruyne 2005
H87	*L. a. africana*	Zimbabwe6	Zimbabwe	AY741071	Debruyne 2005
H88	*L. a. africana*	Zimbabwe5	Zimbabwe	AY741322	Debruyne 2005

* Original name from each author (Debruyne, 2005; Eggert*et al.* 2002; Nyakaana *et al.* 2002; and this study. ** Sample sharing both, forest and savannah haplotypes, according to the author (Debruyne, 2005).

Table 4.3. Cytochrome *b* haplotypes used in **Figure 4.3**. Haplotype frequency is indicated in brackets when there is more than one.

Haplotype	Taxon	Designation*	Geographic origin	Genbank accession numbers	Author
H01 (12)	*L. a. africana*	AM1, AM2, QE51, WC4, BO1, DRC11, MO1, NA1, TA1, UG1, UG3, ZI10	Kenya, Uganda, Namibia, Botswana, DRC, Mozambique, Tanzania, Zimbabwe	AY741074, AY741078, AY741076, AY741325, AY742801, AY741323, AY741324, AY742800	SN, Debruyne 2005
H02	*L. a. africana*	AM12	Kenya		SN
H03	*L. a. africana*	AN1	Angola	AY741072	Debruyne 2005
H04	*L. a. africana*	BOT13	Botswana		SN
H05 (6)	*L. a. cyclotis, L.a. africana, L. africana*	DRC1, DRC4, DRC17**, BOT17, ZI2, ZI4	DRC, Botswana, Zimbabwe	AY359275, AY359277, AY742802, AY741329, AY742799	Debruyne 2005, SN
H06	*L. a. africana*	BOT18	Botswana		SN
H07	*L. a. africana*	BOT1	Botswana		SN
H08	*L. a. africana*	BOT21	Botswana		SN
H09	*L. a. africana*	BOT25	Botswana		SN
H10	*L. a. africana*	BOT2	Botswana		SN
H11 (2)	*L.a. africana*	BOT4, ET1	Botswana		SN
H12	*L.a. africana*	BOT9	Botswana		SN
H13 (5)	*L. a. cyclotis, L.a.africana, L. africana*	DRC2, DRC9, DRC13**, KV8, MF5	DRC, Uganda	AY359270, AY359279, AY741081	Debruyne 2005, SN
H14	*L. a. cyclotis*	DRC3	DRC	AY359271	Debruyne 2005
H15	*L. a. cyclotis*	DRC5	DRC	AY359276	Debruyne 2005
H16 (22)	*Loxodonta africana cyclotis*	DRC6, DRC8, Cameroon2, CAR1, Congo2, CAR274, CAR297, CAR3315, CAR3417, CAR405, CAR3723, CAR4311, IVI1012, KES0819, LOP146, NN0713, NN232, NN267,NN279, NN2911, NN3116, NN3218	DRC, Cameroon, CAR, RC, Gabon	AY359268, AY359269, AY359272, AY359273, AY359274	Debruyne 2005, MJ
H17	*L. a. cyclotis*	Cameroon1	Cameroon	AY359267	Debruyne 2005
H18	*L.a. africana*	Chad1	Chad	AY741080	Debruyne 2005
H19	*L. a. cyclotis*	CKT04a14	RC		MJ
H20 (5)	*L. a. cyclotis*	Congo1, MPA01, MPA02, NOG014, NOG026	RC, Gabon	AY359266	Debruyne 2005, MJ
H21 (3)	*L. a. cyclotis*	Gabon2, Gabon1, NN255	Gabon, RC	AY359265, AY359278	Debruyne 2005, MJ
H22	*L.a. africana*	Ivory Coast1	Ivory Coast	AY741327	Debruyne 2005
H23 (2)	*L. a. cyclotis*	IVI06c4, LOPAFE79	Gabon		MJ
H24	*L. a. cyclotis*	KES0314	Gabon		MJ
H25 (2)	*L.a. africana*	Zi5, KG1	Zimbabwe, South Africa	AY741322	SN, Debruyne 2005
H26 (2)	*L.a. africana*	KG2, SouthAfrica3	South Africa	AY741320	Debruyne 2005, SN
H27	*L.a. africana*	KV19	Uganda		SN
H28	*L.a. africana*	KV2	Uganda		SN
H29	*L. a. cyclotis*	Liberia1	Liberia	AY741079	Debruyne 2005
H30	*L.a. africana*	MM19	Kenya		SN
H31	*L.a. africana*	MM20	Kenya		SN
H32	*L.a. africana*	Namibia2	Namibia	AY741326	Debruyne

					2005
H33	*L.a. africana*	QE48	uganda		SN
H34	*L.a. africana*	Sudan1	Sudan	AY741073	Debruyne 2005
H35	*L.a. africana*	WA13	Ghana		SN
H36 (2)	*L.a. africana*	WA14, WA15	Ghana		SN
H37	*L.a. africana*	WA6	Ghana		SN
H38	*L.a. africana*	WC6	Namibia		SN
H39	*L.a. africana*	Zambia1	Zambia	AY741328	Debruyne 2005
H40	*L.a. africana*	ZBE1	Zimbabwe		SN
H41	*L.a. africana*	ZBE3	Zimbabwe		SN
H42	*L.a. africana*	ZBE4	Zimbabwe		SN
H43	*L.a. africana*	ZBE5	Zimbabwe		SN
H44	*L.a. africana*	Zimbabwe1	Zimbabwe	AY741321	Debruyne 2005

* Original name from each author (Debruyne, 2005; this study SN=Silvester Nyakaana and MJ=Mireille Johnson)
** Sample sharing both, forest and savannah haplotypes, according to the author (Debruyne, 2005).

4.3.4 Demographic history

The HVR1 mismatch distribution of all African elephant haplotypes shows a bi-modal pattern, with the highest peak similar to that expected for an expanding population (**Figure 4.4**), and Fu's *Fs* was highly negative (-24.2605; P = 0.0006), strongly suggesting also a recent population expansion. When HVR1 sequences from forest and savannah elephants were examined separately (**Figure 4.5**), Fu's *Fs* was -14.2954 (P=0.0021) and -24.4427 (P<0.0001), respectively. Although significant values can indicate historical population expansion, the multimodal pattern for the forest elephant groups suggests that these populations encompass several subgroups as indicated in the networks. When we examined each haplogroup separately for signatures of demographic change (**Table 4.4**), a smooth and predominantly unimodal pattern was observed for HVR1 Haplogroup I, indicating a recent demographic expansion (**Figure 4.6**), while HVR1 Haplogroups II, III and IV were more complex, including the presence of some divergent haplotypes.

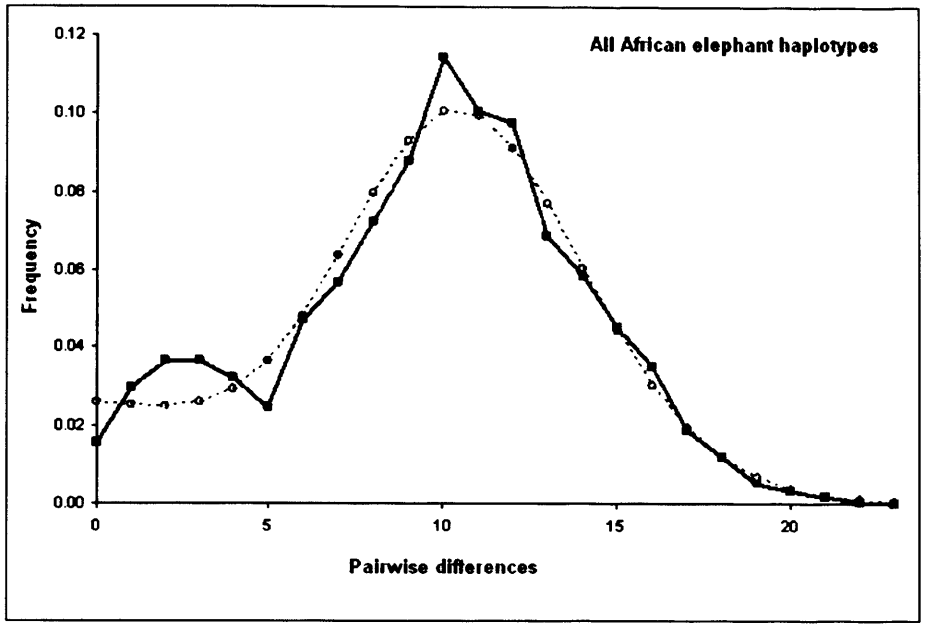

Figure 4.4. Mismatch distribution of the HVR1 haplotypes of: all African elephants, and forest and savannah African elephants, separately.

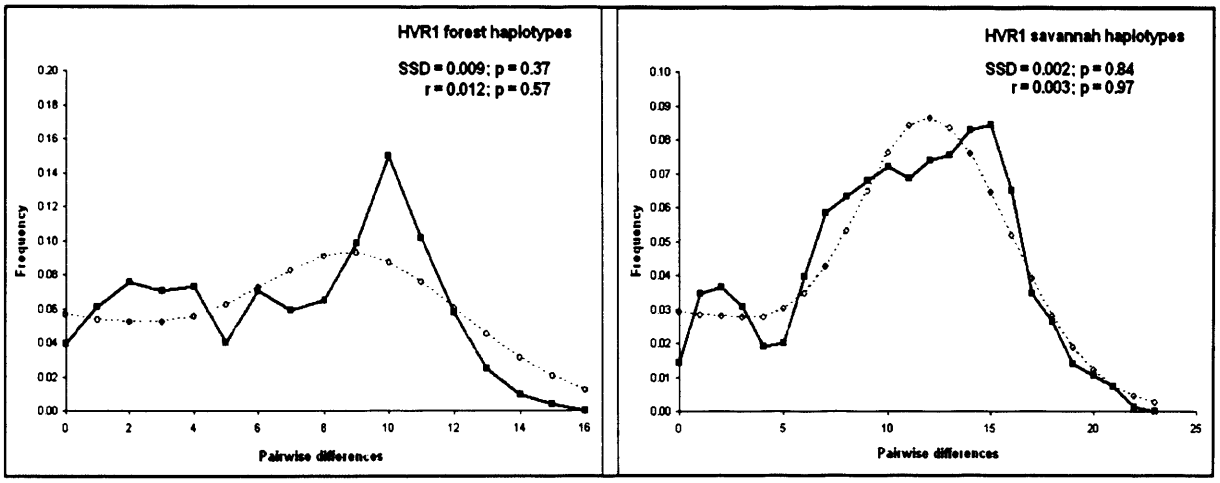

Figure 4.5. Mismatch distribution of the HVR1 forest and savannah African elephants haplotypes.

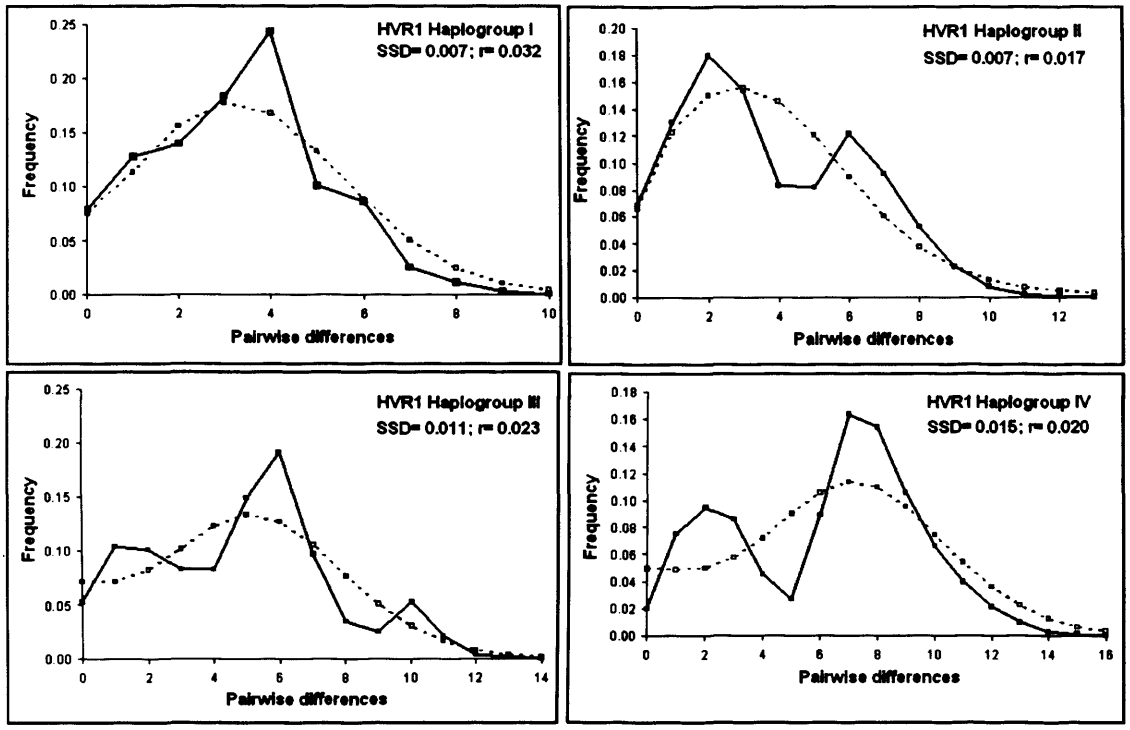

Figure 4.6. Mismatch distribution of the HVR1 haplogroups of African elephants.

Table 4.4. Indicators of demographic change in all African, forest and savannah elephants, and in different haplogroups with HVR1 mtDNA marker.

	All	Forest	Savannah	Haplogroup I	Haplogroup II	Haplogroup III	Haplogroup IV
Fu's Fs	-24.2605	-14.2954	-24.4427	-7.30	-6.34	-4.61	-22.44
p-value	0.0006	0.0021	<0.000	0.006	0.015	0.034	<0.0001

4.4 Discussion

In the light of the results obtained with the mitochondrial sequences used here, alternative explanations for the history of African elephants become evident and suggest that the conclusions drawn in previous studies may have been hampered by incomplete sample sets. The former genetic studies largely shared a pronounced lack of forest elephant data. The nuclear and mitochondrial DNA studies featured extremely limited sampling from central African forest elephants. The study by Roca *et al.* (2001) was limited on three populations from central African forest. Despite describing a narrow hybrid zone between the two elephant types (forest and savannah), only one population located in this zone (Garamba, (DRC)) was included and none from elsewhere in DRC or from west central Africa were examined. Eggert *et al.* (2002)'s study was limited on only two groups of forest elephant from central Africa both from the edge of the forest range in Cameroon, which may conceivably have influenced their conclusion of the genetic uniqueness of forest and western elephants. Elsewhere, Debruyne (2005) included elephants from across the DRC in his study but was again limited by small sample sizes. The results reported here have clearly shown that having a more extensive sample of forest elephants from the core of their range brought more information at the mitochondrial DNA sequences about the phylogeography, demographic history and the genetic structure of African elephants. Savannah populations, especially those in the south, would not have been affected by forest expansion since these areas remained unforested and thus habitat would not have been lost. Those savannah populations that may have been affected are those that may have occurred in areas that subsequently became forested. One explanation for the close genetic proximity between forest and savannah genotypes in DRC could be introgression of savannah mitochondrial haplotypes into forest elephants as the forests expanded and savannah habitat was lost. Such introgression would, in theory, be in the opposite direction to that proposed by Roca *et al* (Roca *et al.*, 2005; Roca *et al.*, 2001).

The results obtained for elephants in west and central Africa have strong implications for the division of elephants into forest and savannah species. These elephants are

taxonomically indeterminate (Frade, 1955) and have been described as having an intermediate morphology (Groves, 2000). Mitochondrially, all West African elephants are found in the same haplogroups as the (two) forest elephant lineages of central Africa. If ancient female-mediated introgression between the two forms followed by backcrossing into savannah populations is the reason why western savannah elephants possess largely 'forest' haplotypes then nuclear markers at these loci should resemble predominantly those of southern and eastern savannah elephants today. Alternatively these elephant populations could be an example of protracted gene flow between two forms of elephant, which is ongoing (or was until recently) and that west African savannah elephants are not distinguishable at the genetic or morphological level from their forest counterparts (thus undermining the two-taxon model). A third explanation could be a 'second movement' of elephants out of the forest (from either west or central Africa) and into the savannah. There are insufficient data to determine whether there was a single movement from forest to savannah habitat or whether these were multiple events, precipitating the morphological changes observed today. Whatever the origin of the two types, our data would support continued extensive hybridisation between the two proposed forms.

Our data do not support the separation of current African elephant populations into two different species. The evidence for this is most clear in west Africa where savannah elephants are indistinguishable at both the mitochondrial and morphological level from their forest African counterparts. The proposed two species model cannot be applied in this region and neither do west African elephants represent a third taxonomic entity. Central African elephant populations west of the Congo river also pose questions for the current classification. Forest elephants fall into two major mitochondrial DNA groups. Previous studies also found two major groups, savannah and savannah/forest, perhaps suggesting ancient introgression between forest females and savannah males in the past. However the inclusion of a larger central forest sample in this study suggests that this explanation is too simple and that African elephants were subject to a more complex demographic history. The classification of species is important for many reasons but with the massive extinction of species in the wild in the last 50 years accurate descriptions are

essential for management of wild resources. To develop management strategies incorporating the current two-taxon model could be misleading without further research and until further lines of evidence give us a clearer picture of the origins and current conservation needs of elephants populations throughout the continent. Future studies will need to analyse multiple nuclear DNA markers from across the range of forest and savannah elephants, especially in transition zones before any firm conclusions can be made.

4.5 References

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CHAPTER 5

Population genetic structure of forest elephants in west central Africa

5.0 Abstract

The genetic diversity and structure of the forest elephant (*Loxodonta africana cyclotis*) in west central Africa was examined using 12 polymorphic microsatellite loci identified from savannah African elephant. DNA was amplified from faecal samples collected from sites across Gabon, Nouabalé-Ndoki National Park in Congo, and Dzanga-Sangha National Park in the Central African Republic. High levels of genetic diversity were detected within samples with a mean number of alleles of 10.3 per locus; mean expected and observed heterozygosities of 0.81 and 0.79, respectively, with evidence for admixture in some regions. Genetic differentiation among sites was generally low with F_{ST} values ranging from 0.004 to 0.045, suggesting high levels of gene flow. No correlation was found between genetic and geographical distance (P = 0.943). Results also showed the existence of a contact zone in the area of Ivindo (Gabon) from where sampled individuals were symmetrically assigned to two genetically-based clusters inferred from Bayesian analyses. These genetic results should be considered when management strategies are established to ensure the conservation and sustainable management of elephants and their habitats in central Africa.

5.1 Introduction

Tropical rain forest is the predominant vegetation which defines the Congo Basin in Central Africa, but areas of savannah also occur such as the extensive and continuous grasslands of the highland Plateau Batéké highland in Gabon and Congo (Tutin *et al.*, 1997), and other areas of savannah mosaic with forest fragments (e.g. Lopé National Park, Gabon) adjacent to continuous forest, also exist (Tutin *et al.*, 1997). Additionally, mineral-rich clearings, often with permanent water bodies, surrounded by forest, (locally known as bais) are an important habitat feature found in tropical forest ecosystems (Turkalo & Fay, 1996). The majority of this tropical forest ecosystem is inhabited by forest elephants (*Loxodonta africana cyclotis*; Blanc *et al,* 2007). Unlike its savannah counterpart in eastern and southern Africa, little is known about the status, the distribution and population structure of the forest elephant (Barnes *et al.*, 1991; Blanc *et al.*, 2007). A substantial lack of reliable information on the situation of forest elephants exists in central African countries because of an absence of infrastructure necessary to monitor populations, the difficulties of accessing the forest and a substantial lack of institutional capacity and resources (Blanc *et al.*, 2007; Walsh & White, 1999). However, some studies have been carried out to monitor forest elephants (Blake *et al.*, 2001; Fay, 1991; Fay, 1999; Fay & Agnagna, 1991; Nzooh *et al.*, 2005) and to determine their distribution, density and biomass (Barnes *et al.*, 1991; Morgan, 2007), and the impact of human activities on their distribution (Barnes *et al.*, 1991; Buij *et al.*, 2007) . It has been widely reported that intense poaching for both elephant ivory and meat occurs in Central Africa and that the region is the main centre for the current ivory trade (Blanc *et al*, 2007). Activities such as logging, mining and oil extraction provide access to remote areas, increasing hunting, which is often targeted at elephants (Blanc *et al*, 2007). These threats almost certainly have had a negative impact on elephant populations, but this is difficult to determine because of a lack of information. There are, however, a few studies which have shown the effect of roads and hunting, or more broadly, the influence of human activities on forest elephants (Barnes *et al.*, 1991; Buij *et al.*, 2007; Laurance *et al.*, 2006). Surveys for estimating elephant abundance have provided the highest level of data quality only in Gabon and the Central African Republic (Blanc *et al*, 2007). Other

countries such as Congo and Democratic Republic of Congo currently have a lower level of data quality, with censuses based mainly on best guesses.

The wide variety of habitats where forest elephants occur would be expected to have an influence on their distribution, as human disturbance would similarly do. Few data exist on patterns of use by large mammals of natural forest fragments (Tutin *et al.*, 1997). Momont (2007) has shown that elephants in Lopé and Langoué bai, both in Gabon, exploit all different types of habitat according to seasonal changes, to benefit from the available food and mineral salts. Recent advances in global positioning system (GPS) technology have provided quality data to evaluate ranging, seasonal movements and the distribution of African elephants (Blake *et al.*, 2001; Douglas-Hamilton *et al.*, 2005). The same system was also used to study the influence of social relationships on spatial population structure and ranging strategies on the elephants inhabiting the Samburu and Buffalo Springs National Reserve in northern Kenya (Wittemyer *et al.*, 2007). It is essential to understand the ecology of any species studied genetically, since population dynamics, spatial distribution and genetic structure are closely tied to patterns of movement (Pough *et al.*, 1998). Forest elephant movements are related to the acquisition of necessary resources (Vanleeuwe & Gautier-Hion, 1998; White, 1994). Furthermore, their capacity to survive in variable habitats potentially allows wide dispersal thanks to their high mobility of up to 2000 km during their lifetime (Blake, 2007), although poaching and logging (with habitat loss) are a considerable menace (Blake & Hedges, 2004; Blanc *et al.*, 2007), and would impose a further cost on dispersal. African elephants have a matrilineal social structure characterised by a polygynous mating system where females are philopatric, strongly faithful to their natal group, and males have a high tendency to migrate and to exchange individuals between populations (Nyakaana & Arctander, 1999; Nyakaana *et al.*, 2002). Any dispersal from the natal area or group in a philopatric species will inevitably reduce the probability of mating with a close relative (Greenwood & Harvey, 1982). Recent studies have examined the evolution of dispersal as a mechanism of inbreeding avoidance in African elephants (Archie *et al.*, 2007; Moore, 2007) and also in birds (Szulkin & Sheldon, 2007). The former study has shown that elephants recognize close paternal kin and avoid mating with them. The consequence

of inbreeding, defined as the reproduction of individuals sharing ancestors (Wright 1922), is to increase the level of homozygosity in a population. Thus, some species develop strategies such as sex-biased dispersal or relative recognition to prevent inbreeding depression. Gene flow describes the movement of reproducing migrants from one population to another (Slatkin, 1985), although this migration can be restricted because of geographical features, such as rivers, highways, mountain ranges (Coulon *et al.*, 2006; Eriksson *et al.*, 2004; Keller *et al.*, 2004; Luiselli & Capizzi, 1997; Slatkin, 1987; Whitlock & McCauley, 1999) and human disturbance such as poaching and habitat fragmentation (Cegelski *et al.*, 2003; Luiselli & Capizzi, 1997).

Few studies have analysed the genetic diversity of African elephants using microsatellite markers. The studies of Nyakaana *et al.* (1999, 2002) based on four microsatellite loci have shown a high level of polymorphism in savannah elephant populations at regional and continental scales. They found a lack of concordance based on genetic structures when they compared nuclear and mitochondrial markers. Their microsatellite data depicted weak differentiation among populations compared to mtDNA, a finding they mainly explained to be the result of the matrilineal elephant social structure characterised by female natal philopatry favouring male biased gene flow (Nyakaana and Arctander, 1999). Meanwhile, Comstock *et al.* (2002) studied genetic variation among African elephant populations using 16 microsatellite loci across 20 populations and found lower genetic diversity in savannah elephants than in forest elephants. Only three sites were assessed from African equatorial rain forests. The aim of this Chapter was to investigate the genetic diversity and structure of *L. a. cyclotis* within and among populations using 12 polymorphic microsatellite DNA loci identified in the African elephant, and to compare these results with the mtDNA data in Chapter 3. The results were used to understand highlight mechanisms, which could be inferred from the observed population structure of forest elephants in Central Africa. I also expected to find, based on microsatellite analysis, (i) evidence for high levels of gene flow, (ii) a genetic signature of Pleistocene allopatric differentiation (see Chapter 3), coupled with (iii) detectable effects of human disturbance. However, the discussion of my findings will necessarily be limited because of the lack of data on social behaviour for *L. a. cyclotis*.

5.2 Materials and Methods

5.2.1 Study area

The study area included Gabon, northern Republic of Congo and south-west Central African Republic (CAR), where faecal samples were collected from 249 elephants from eight populations (see Figure 5.1 and Table 5.1 for details). Each location includes different forest types, from swamp forest to savannah-forest mosaic, including saline clearings (bais) surrounded by canopy forest

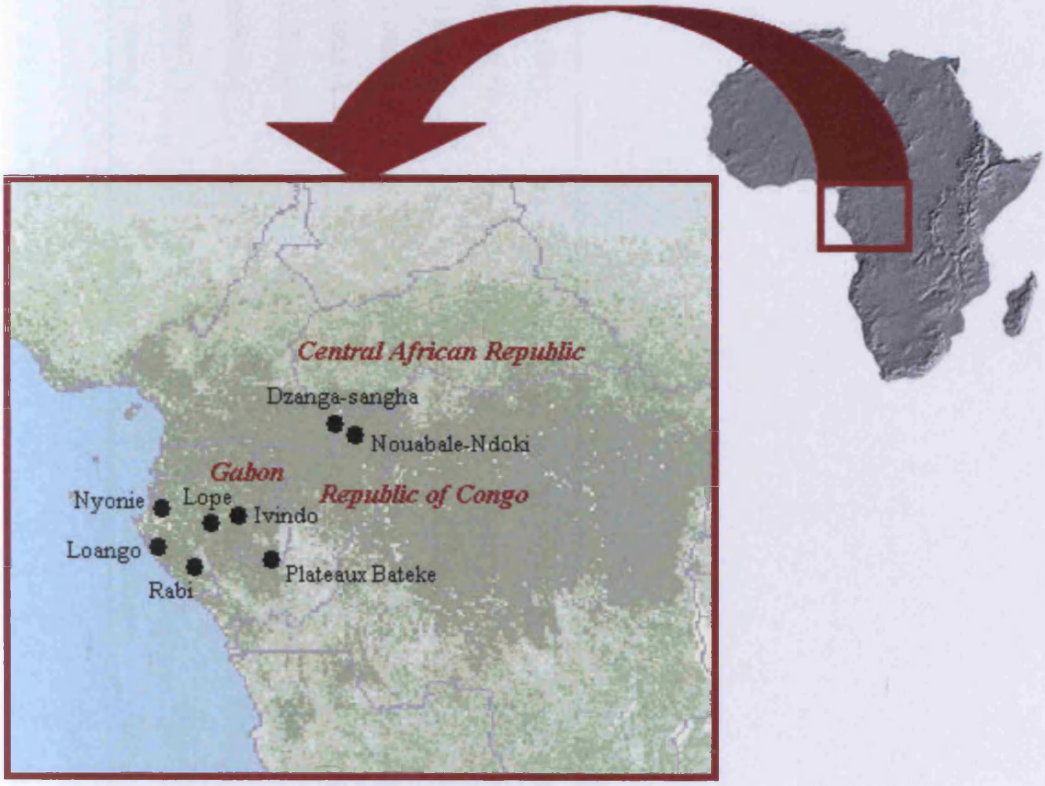

Figure 5.1. Map of the study area of forest elephant in the western part of the Congo Basin.

Table 5.1. Description of the 8 populations studied, their geographic origin, number of samples collected per site, and habitat features.

Geographic origin	Population	Code	*n*	Forest type
West coast Gabon	Rabi-Ndongo NP	RAB	21	Closed canopy forest
West coast Gabon	Loango NP	LOA	23	Mangroves and lagoons, inundated coastal
West coast Gabon	Nyonie	NYO	35	Mangroves and lagoons, inundated coastal
Central Gabon	Lope NP	LOP	77	Forest-savannah mosaic
Central Gabon	Ivindo NP	IVI	38	Saline clearing within forest
Southern CAR	Dzanga-sangha NP	CAR	35	Saline clearing within forest
North-eastern Congo	Nouabale-Ndoki NP	NN	40	Lowland rainforest
South-eastern Gabon	Plateaux Bateke NP	PBA	21	Degraded forest

NP = National Park

n = number of samples

5.2.2 Sampling and DNA amplification

Dung samples were collected and extracted as described in Chapter two. DNA was amplified using the Polymerase Chain Reaction (PCR) and genotyped with a multiplex panel of 12 polymorphic microsatellite loci: FH19, FH39, FH40, FH48, FH60, FH67 and FH71 (Comstock *et al.*, 2000); FH127 (Comstock *et al.*, 2002); LA6 (Eggert *et al.*, 2000); LAFMS03 (Nyakaana & Arctander, 1998); LAFMS07 (Nyakaana S *et al.*, 2005); and LAT08 (Archie *et al.*, 2003). Table 5.2 shows the constitution of each multiplex with the primer dye and size range (from the original study).

Table 5.2. Panel of microsatellite multiplexes used for genotyping in this study.

Multiplex	Locus	Dye	Size range	°C (annealing temp.) and # cycles
M1	FH39	NED (Yellow)	198-256	55 ° C : 38
	FH67	6-FAM (Blue)	90-116	
	FH127	6-FAM (Blue)	147-203	
M2	FH71	NED (Yellow)	61-137	58 ° C : 37
	LAFMS03	6-FAM (Blue)	137-157	
	LAMS07	VIC (Green)	132-168	
M3	FH60	6-FAM (Blue)	139-167	60 ° C : 37
	LA6	NED (Yellow)	153-175	
	LAT08	VIC (Green)	162-300	
M4	FH19	RED (Red)	187-213	60 ° C : 40
	FH40	6-FAM (Blue)	226-272	
	FH48	NED (Yellow)	152-180	

For each locus, the forward primer was dye-labeled and the PCR amplification was carried out in a 10 µl volume containing 5 µl of QIAGEN Multiplex PCR Master Mix (from the QIAGEN® Multiplex PCR Kit), 1 µl of the 10X primer mix (0.2µM of each primer, forward and reverse), 2µl of DNA, 1µl of 0.5X Q-Solution (provided in the kit)

and 1 µl of water. The amplification profile consisted of a denaturation step at 95°C for 15 min, followed by a 94°C denaturation for 30 sec; 1.5 min of primer annealing and 1.5 min of primer extension at 72°C. The number of cycles and the annealing temperature depended on the multiplex as shown in Table 5.2. A control extraction blank and PCR reaction control, to which no DNA was added, were included in each batch of amplification. Genotypes were determined using Peak Scanner (Applied Biosystems).

5.2.3 Population genetic analyses

Genetic diversity

Genotypes were assessed using MICRO-CHECKER 2.2.1 (Van Oosterhout *et al.*, 2004). The mean number of alleles per locus and population was calculated using GENETIX, and the observed (H_o) and expected (H_e) heterozygosities were also estimated per population and per locus using GENETIX and ARLEQUIN version 3.11 (Excoffier *et al.*, 2005), respectively. Deviation of observed genotype frequencies from those expected under Hardy-Weinberg equilibrium (HWE) was examined per population across loci and per population and locus by calculating Weir & Cockerham's inbreeding coefficient (F_{IS}) using 1000 permutations. Genotypic linkage disequilibrium (LD) and allele frequencies were also estimated using GENETIX. LD was measured using the correlation coefficient. A permutation approach was applied to determine the significance level ($P < 0.05$). Genetic differentiation and gene flow among populations were estimated using the F_{ST} analogue (theta) of Weir and Cockerham (1984) implemented by GENETIX, and a Mantel test was conducted using ARLEQUIN to test for the correlations between genetic and geographical distances (isolation by distance). GENETIX was used to visually explore patterns of genetic differentiation between individuals in all populations using Factorial Correspondence Analysis (FCA) based on allele frequencies (Belkhir *et al.*, 1998).

The significance of the population structure was analysed by analysis of molecular variance (AMOVA) with 10,000 permutations, among and within populations, executed by ARLEQUIN 3.11.

Population structure

Population structure was further inferred using the Bayesian clustering procedure implemented in STRUCTURE. Populations or individuals were assigned to one cluster if their proportion of membership (q) to that cluster was equal to or larger than an accepted threshold of 0.800 (Randi *et al.*, 2003). Individuals are assigned probabilistically to one (the population of origin) or more than one cluster (the parental populations) if their genotypes indicated that they were admixed. Most parameters were set to their default values as recommended in the STRUCTURE 2.0 user's manual (Pritchard & Wen, 2003). The admixture model and the option of correlated allele frequencies were chosen. The length of the burn-in period and the number of MCMC were set to 100,000 and 1,000,000 respectively. The range of possible numbers of partitions in the data (K) tested was 1 to 5. Ten runs were performed for each value of K, in order to verify that the estimates were consistent across runs. The mean posterior probability was calculated for each K over its runs, and the true K is the maximal value of the estimated logarithm of probability of the data ln Pr(X|K) (Pritchard *et al.*, 2000).

5.3 Results

5.3.1 Genetic diversity

A total of 249 individuals from eight populations were genotyped using 12 microsatellite loci (Appendix 5). Two hundred and four different alleles were observed in the whole sample and the mean number per locus was 17, ranging from eight (LAFMS07) to 48 (LAT08) (Figure 5.2). The level of polymorphism per population was also high, with a mean number of alleles of 10.3, ranging from 8.6 (PBA) to 12.3 (LOP and NN) (Table 5.3). The mean expected (H_e) and observed (H_o) heterozygosity per population were high across loci, ranging from 0.78 (NYO) to 0.84 (NN) and 0.73 (PBA) to 0.83 (RAB), respectively (Table 5.3). Allele frequency distribution by locus and population is shown in Appendix 6. The frequencies of the alleles generally showed multimodal distributions with more than two common alleles and a range of other alleles at low frequencies, except for the locus FH40, which had a single common allele with high mean frequency of 54 % over all populations.

Assessment of genotyping errors was implemented using MICRO-CHECKER (Van Oosterhout C *et al.*, 2004) as DNA degradation, low DNA concentrations and primer-site mutations may result in the incorrect assignment of microsatellite genotypes, biasing population genetic analyses. The results across populations for each locus gave no evidence for scoring error due to stuttering, no evidence for large allele dropout, but notified the potential presence of null alleles at some loci due to a general excess of homozygotes for most allele size classes. Populations were mainly found to conform to Hardy Weinberg equilibrium but some loci, such as FH127, LAT08, FH19, FH67, LA6, FH71 showed evidence of null alleles in LOP, CAR, NN and IVI. Only one locus showed evidence for null alleles in LOA, PBA and NYO (FH40, LAT08 and FH71, respectively). Since the calculated null allele frequencies (see Appendix 7) were generally negligible, little bias was expected in the analysis of population structure (Chapuis & Estoup, 2007;

Dakin & Avise, 2004). Hence, the downstream analyses were carried out with the twelve loci, with no adjustment of allele frequencies or removal of the affected loci.

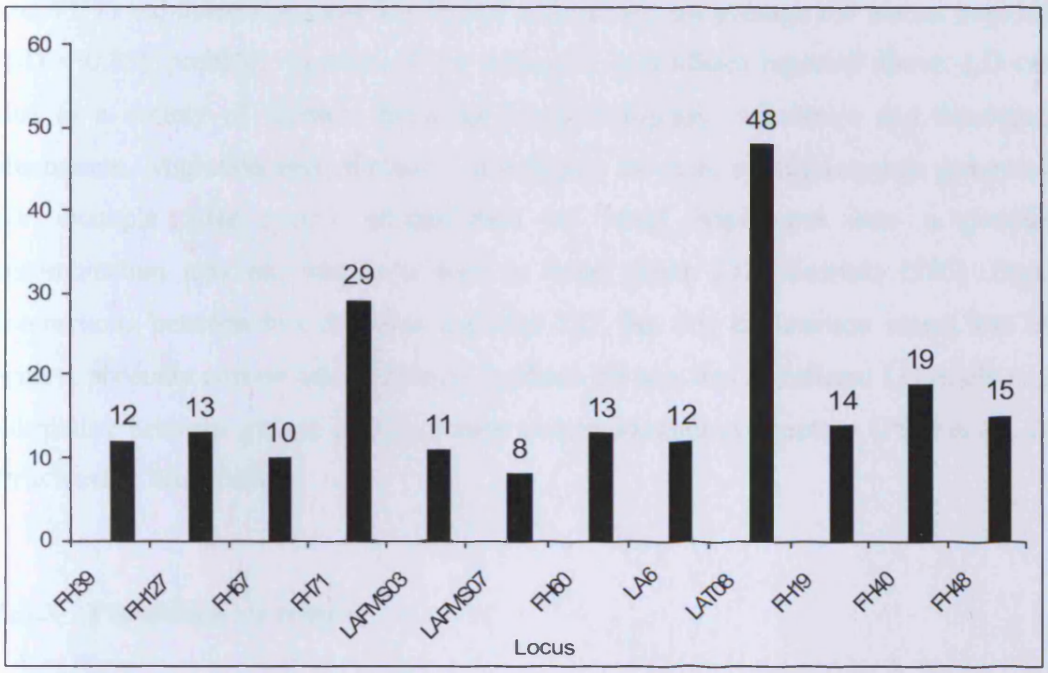

Figure 5.2. Total number of alleles per locus across all forest elephant populations.

5.3.2 Deviations from Hardy-Weinberg equilibrium and linkage disequilibrium (LD)

Across all loci, H_e varied from 0.78 (NYO) to 0.84 (NN), and H_o varied from 0.73 (PBA) to 0.83 (RAB) (Table 3). H_e values were slightly higher, generating low but significant F_{IS} values observed mainly for two loci (LAT08 and FH71). These loci exhibited significant deviation from HWE proportions for four and three populations, respectively, as differences were observed between H_e and H_o (Table 3). This departure could be due to null alleles detected, since these loci were implicated as demonstrating null alleles in most populations where nulls were suspected. A significant deviation from HWE was found in 4 populations (LOP, CAR, NN and PBA) across loci with a deficit of heterozygosity as shown in Table 5.3. NYO showed a low but not significant ($P = 0.445$)

excess of heterozygosity. Significant LD ($P < 0.05$) was found between some loci and in some populations (see appendix 8). LOP, RAB and PBA presented the highest number of significant comparisons (58, 59 and 55, respectively). Pairs of loci comprising LAT08 and FH71 exhibited the most significant LD values, on average 6.9 across populations (SD = 0.85), probably because of the suspected null alleles reported above. LD can be due to a variety of factors, including physical linkage, admixture and demographic fluctuation. Migration and admixture among two or more populations can generate LD, for example after recent introgression of novel haplotypes into a population, recombination may not had have time to break down LD (Hedrick, 1985). Epistatic interactions between loci can also maintain LD, but this explanation seems less likely with supposedly neutral microsatellites markers. Hence, this significant LD might suggest admixture between groups of populations and/or population structure (Pfaff *et al.*, 2001; Pritchard & Wen, 2003).

5.3.3 Population structure

Genetic differentiation among populations was low but significant for most of the pairwise comparisons, with the exception of CAR-NN (F_{ST} = 0.004), RAB-LOA (F_{ST} = 0.006), NN-PBA (F_{ST} = 0.009), NN-RAB (F_{ST} = 0.013) and LOA-PBA (F_{ST} = 0.017), indicating substantial movements between those populations and effective gene flow (Tables 5.4 and 5.5).

Table 5.3. Average number of alleles across populations (N_a), observed (H_o) and expected (H_e) heterozygosities and departures from Hardy-Weinberg proportions (F_{IS}) for populations and all loci, and mean number of alleles per population (MNA).

	Population	LOP	CAR	NN	IVI	RAB	LOA	PBA	NYO	
Locus	*n*	59	32	35	37	17	20	16	33	N_a
FH39	H_e	0.72	0.80	0.83	0.75	0.76	0.80	0.79	0.78	12
	H_o	0.73	0.70	0.94	0.97	0.71	0.90	0.73	0.78	
	F_{IS}	-0.022	0.137	-0.121	-0.279	0.077	-0.093	0.102	0.018	
		NS	NS	NS	*	NS	NS	NS	NS	
FH127	H_e	0.86	0.85	0.85	0.86	0.85	0.82	0.79	0.80	13
	H_o	0.74	0.81	0.97	0.84	0.94	0.75	0.69	0.94	
	F_{IS}	0.143	0.067	-0.126	0.042	-0.108	0.084	0.160	-0.149	
		NS	NS	NS	NS	NS	NS	NS	NS	
FH67	H_e	0.81	0.79	0.81	0.77	0.84	0.77	0.84	0.79	10
	H_o	0.78	0.66	0.78	0.70	0.94	0.90	0.87	0.90	
	F_{IS}	0.046	0.181	0.047	0.106	-0.123	-0.150	-0.014	-0.135	
		NS	NS	NS	NS	NS	NS	NS	NS	
FH71	H_e	0.82	0.78	0.86	0.86	0.94	0.92	0.78	0.92	29
	H_o	0.78	0.63	0.60	0.64	0.94	0.75	0.81	0.73	
	F_{IS}	0.068	0.205	0.319	0.274	0.004	0.187	-0.010	0.209	
		NS	NS	***	***	NS	NS	NS	**	
LAFMS03	H_e	0.80	0.82	0.83	0.74	0.70	0.78	0.80	0.66	11
	H_o	0.73	0.84	0.74	0.78	0.53	0.80	0.62	0.82	
	F_{IS}	0.103	-0.015	0.121	-0.042	0.271	0.002	0.227	-0.229	
		NS	NS	NS	NS	NS	NS	NS	NS	
LAFMS07	H_e	0.81	0.80	0.84	0.80	0.81	0.76	0.81	0.78	8
	H_o	0.90	0.81	0.83	0.83	0.82	0.74	0.81	0.85	
	F_{IS}	-0.097	0.015	0.033	-0.025	0.009	0.056	0.027	-0.078	
		NS	NS	NS	NS	NS	NS	NS	NS	
FH60	H_e	0.84	0.82	0.86	0.81	0.85	0.82	0.82	0.74	13
	H_o	0.83	0.74	0.83	0.83	0.82	0.89	0.73	0.88	
	F_{IS}	0.015	0.109	0.048	-0.012	0.037	-0.059	0.135	-0.164	
		NS	NS	NS	NS	NS	NS	NS	NS	
LA6	H_e	0.74	0.79	0.80	0.68	0.82	0.74	0.76	0.77	12
	H_o	0.73	0.58	0.74	0.66	0.88	0.67	0.75	0.85	
	F_{IS}	0.019	0.284	0.090	0.050	-0.041	0.134	0.048	-0.088	

		NS	*	NS	NS	NS	NS	NS	NS	
LAT08	H_e	0.95	0.93	0.96	0.94	0.94	0.94	0.93	0.93	48
	H_o	0.77	0.79	0.84	0.86	0.94	0.85	0.75	0.88	
	F_{IS}	0.203	0.165	0.143	0.106	0.004	0.099	0.224	0.053	
		***	*	*	NS	NS	NS	*	NS	
FH19	H_e	0.86	0.88	0.89	0.89	0.82	0.86	0.84	0.86	14
	H_o	0.77	0.90	0.76	0.83	0.76	0.95	0.71	0.73	
	F_{IS}	0.116	-0.009	0.163	0.078	0.098	-0.082	0.182	0.155	
		NS	NS	NS	NS	NS	NS	NS	NS	
FH40	H_e	0.81	0.58	0.66	0.66	0.64	0.67	0.66	0.62	19
	H_o	0.72	0.59	0.73	0.65	0.82	0.47	0.61	0.73	
	F_{IS}	0.119	0.002	-0.085	0.028	-0.255	0.296	0.103	-0.164	
		NS	NS	NS	NS	NS	NS	NS	NS	
FH48	H_e	0.83	0.84	0.86	0.85	0.85	0.87	0.85	0.79	15
	H_o	0.94	0.80	0.84	0.83	0.82	0.90	0.69	0.81	
	F_{IS}	-0.129	0.063	0.038	0.033	0.061	-0.038	0.197	-0.004	
		NS	NS	NS	NS	NS	NS	NS	NS	
Total	H_e	0.82	0.81	0.84	0.80	0.81	0.80	0.80	0.78	
	(SD)	(0.06)	(0.08)	(0.07)	(0.08)	(0.08)	(0.07)	(0.06)	(0.09)	
	H_o	0.78	0.74	0.80	0.79	0.83	0.80	0.73	0.82	
	(SD)	(0.07)	(0.10)	(0.10)	(0.10)	(0.12)	(0.13)	(0.08)	(0.07)	
	F_{IS}	0.052	0.102	0.060	0.035	0.004	0.034	0.117	-0.038	
		***	***	*	NS	NS	NS	*	NS	
	MNA	12.3	11.0	12.3	11.2	8.8	8.9	8.6	9.3	

n = sample size, NS = non significant, * = $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table 5.4. Pairwise genetic differentiation (F_{ST}; Weir & Cockerham, 1984) between forest elephant populations (below diagonal) and straight line geographical distances in km (above diagonal).

	LOP	CAR	NN	IVI	RAB	LOA	PBA	NYO
LOP	-	580	620	76	288	328	352	248
CAR	0.030	-	52	512	856	902	588	796
NN	0.025	**0.004**	-	552	896	940	604	844
IVI	0.020	0.022	0.018	-	348	388	304	328
RAB	0.039	0.031	**0.013**	0.027	-	50	436	124
LOA	0.031	0.033	0.020	0.019	**0.006**	-	488	258
PBA	0.026	0.018	**0.009**	0.017	0.020	**0.017**	-	572
NYO	0.045	0.045	0.032	0.030	0.019	0.020	0.033	-

Bold numbers are non significant values of F_{ST} ($P > 0.05$)

Table 5.5. Pairwise estimates of Nm gene flow between forest elephant populations.

	LOP	CAR	NN	IVI	RAB	LOA	PBA
CAR	8.07	-					
NN	9.89	62.15	-				
IVI	12.12	11.09	13.58	-			
RAB	6.14	7.86	19.15	8.91	-		
LOA	7.79	7.36	12.10	13.18	38.23	-	
PBA	9.38	13.66	26.23	14.13	12.37	14.76	-
NYO	5.31	5.24	7.66	8.13	12.68	12.03	7.29

NN and RAB F_{ST} was low despite a straight line geographic distance of 896 km between them. The estimation of gene flow (Tables 5.4 and 5.5) showed that the populations, which have exchanged more individuals mostly have a shorter geographical distance between them (Table 5.4). This is not the case for LOP and IVI, which were approximately 76 km distant, with a significant ($P < 0.001$) , but low $F_{ST} = 0.020$. NN and LOA are geographically separated by 940 km and had the same genetic differentiation ($F_{ST} = 0.020$) as between LOP and IVI, as shown in Table 5.4. A Mantel test showed no correlation (r = -0.29, $P = 0.943$) between genetic and geographical distance (isolation by distance) in forest elephant populations (Figure 5.3). Little structure was observed from the FCA plot of individual microsatellite genotypes (Figure 5.4) although there is some evidence of two groups and an intermediate zone where the groups are in contact. One group included LOP, CAR, NN and PBA, and the second is mainly the coastal populations, with LOA, RAB and NYO. IVI was distributed between both groups, and overlaps the contact zone. It is important to point out that these groups are not supported by 95% confidence elipses and are indicative only. The AMOVA test for the whole sample showed that 97 % of the genetic variation was within populations, with a significant but low differentiation among populations ($P < 0.05$, $F_{ST} = 0.028$).

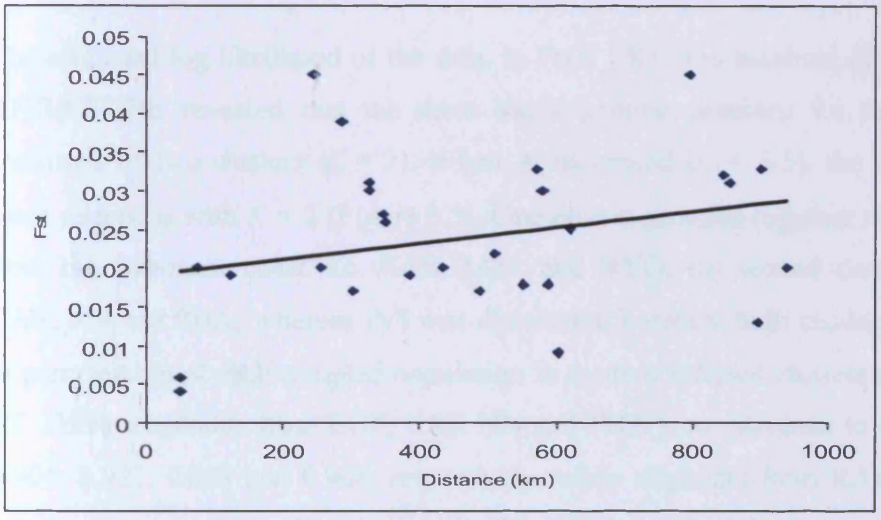

Figure 5.3. Correlation between pairwise genetic distances (F_{ST}) and geographical distances (km) in 8 populations of forest elephants ($P = 0.943$).

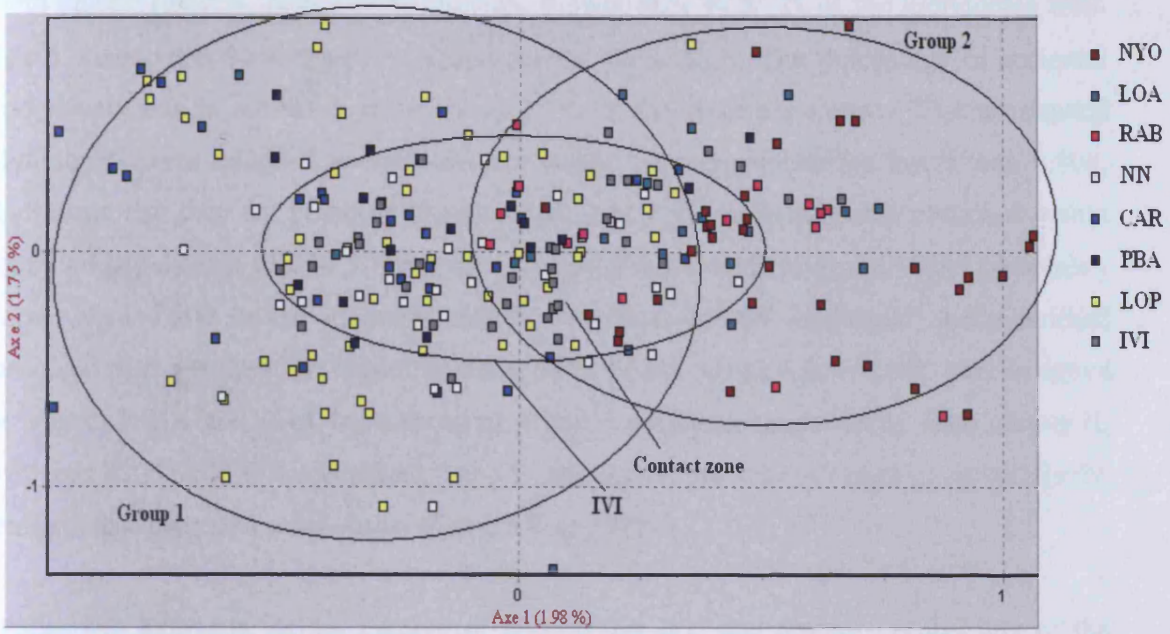

Figure 5.4. Factorial Correspondence Analysis showing relationship among multilocus genotypes of individual forest elephants from 8 populations in west central Africa.

Bayesian clustering

The estimated log likelihood of the data, ln Pr(X | K), was maximal at $K = 2$ (Table 5.6). STRUCTURE revealed that the most likely genetic structure for the whole data set consisted of two dusters ($K = 2$). When K increased (K = 3-5), the results showed the same pattern as with $K = 2$ (Figure 5.5). One cluster grouped together all three populations from the Gabonese coast, i.e. RAB, LOA and NYO, the second cluster grouped LOP, CAR, NN and PBA, whereas IVI was distributed between both clusters. The proportions of membership of each sampled population in the two inferred clusters are shown in Table 5.7. Forest elephants from LOP, CAR NN and PBA were assigned to cluster I with q_I = 0.904, 0.922, 0.898 and 0.908, respectively, while elephants from RAB, LOA and NYO were assigned to cluster II with q_{II} = 0.935, 0.907, 0.928. IVI comprised individuals from both clusters I and II assigned with a probability larger than 0.800 (q_I = 0.836 and q_{II} = 0.901, respectively). Thirty-eight and 27 % of the individuals from IVI were assigned in

both cluster I and II, respectively. Results showed that 90.91 % of the individuals from NYO were correctly assigned to their original population. The percentage of assigned individuals was in the range of 59 % to 75 % in the other populations. The unassigned individuals were assigned to both clusters (I and II) with probability lower than 0.800, indicating that they are admixed (Randi *et al.* 2003). Potential migrants observed within sampled populations (Table 5.7) are the number of individuals assigned with a probability larger than 0.800 in the opposite cluster, for instance LOP contained one individual assigned with q = 0.935 in cluster II while 68 % of the sampled population were assigned in cluster I. NN and PBA have received 4 and 2 migrants, respectively, from cluster II, whereas RAB and LOA comprised 2 and 3 migrants at the time of sampling, respectively. No potential migrants were observed at CAR and NYO.

Table 5.6. Inference for the number of populations (K). The posterior probability of the number of populations was maximum with $K = 2$.

K	ln Pr(X|K)
1	-12713.2
2	**-12599.8**
3	-12692.1
4	-12873.5
5	-12706.0

Table 5.7. Bayesian clustering analysis in forest elephants performed using STRUCTURE (Pritchard *et al.* 2000). The table shows the proportion of membership (q) of each predefined sampled population in each of 2 inferred clusters. Each sampled population was assigned to a single cluster if q_i (i = I-II) = 0.800. The number (in parentheses) and percentage of total individuals assigned are indicated. Proportions of membership of potential migrants are indicated with their original cluster (I or II) (see text for details). n = population size.

Population (n)	Cluster		Potential migrants	Unassigned individuals	% of total* assigned individuals
	I	II			
LOP (59)	0.904 (40)	0.096 (0)	0.935 $(1)_{II}$	18	69.49
CAR (32)	0.922 (20)	0.078 (0)	0	12	62.50
NN (35)	0.898 (23)	0.102 (0)	0.873 $(4)_{II}$	8	77.14
IVI (37)	0.836 (14)	0.901 (10)	-	13	64.86
RAB (17)	0.065 (0)	0.935 (8)	0.828 $(2)_{I}$	7	58.82
LOA (20)	0.093 (0)	0.907 (12)	0.892 $(3)_{I}$	5	75.00
PBA (16)	0.908 (8)	0.092 (0)	0.879 $(2)_{II}$	6	62.50
NYO (33)	0.072 (0)	0.928 (30)	0	3	90.91

* including potential migrants

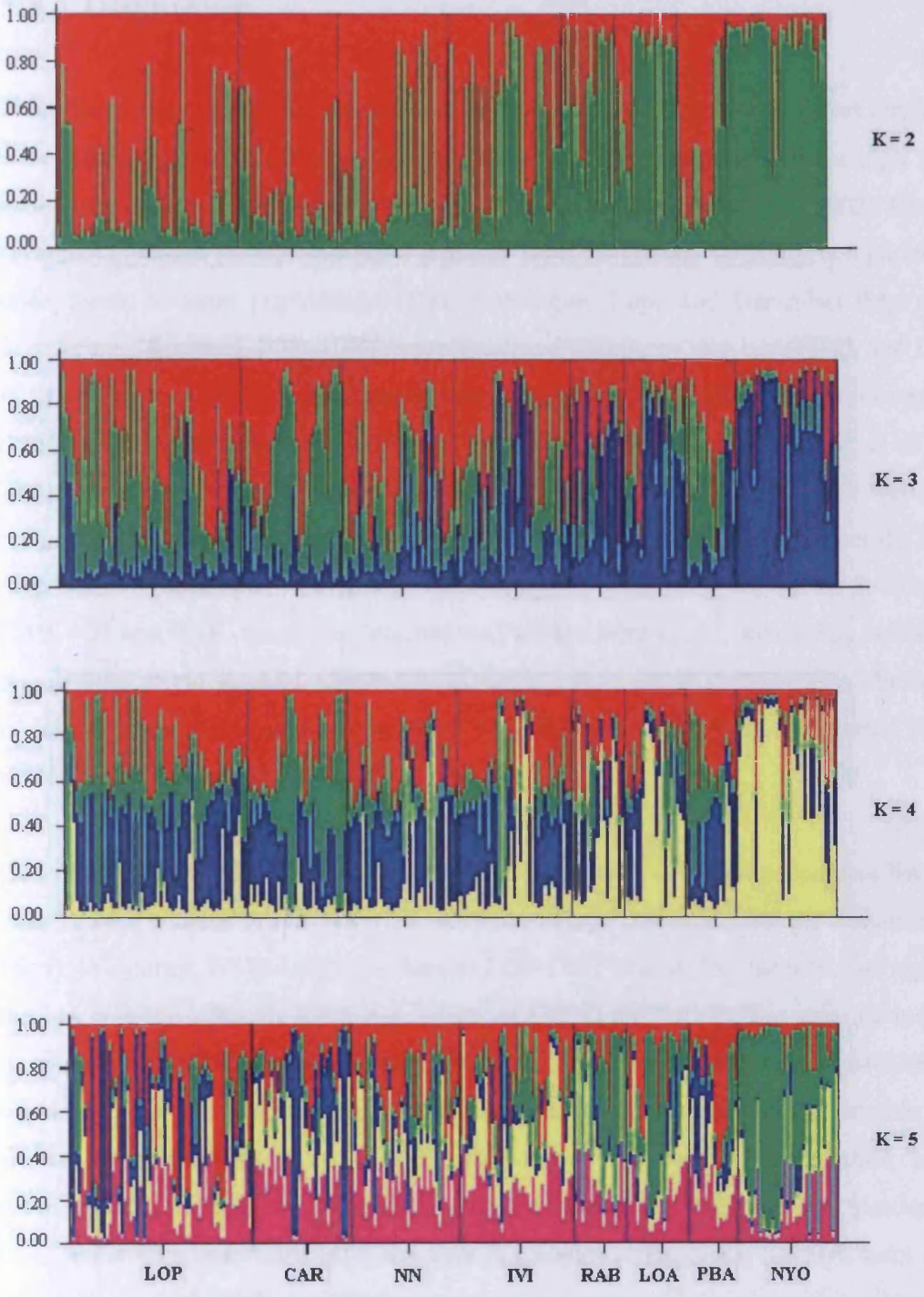

Figure 5.5. Clustering results (K = 2) for all sites, according to STRUCTURE analysis. Each individual is represented as a vertical line partitioned into coloured segments. Sampled sites are separated by black vertical line and labelled below the figure.

5.4 Discussion

This study presents the first extensive analysis of genetic structure in forest elephants in west central Africa based on microsatellite markers. The results showed a high level of genetic diversity within forest elephant populations. Comstock *et al* (2002) found that savannah elephant populations have a lower level of genetic diversity compared to the three forest elephant populations (Dzanga-Sangha, Lopé and Garamba) they studied. Significant departures from HWE were observed mainly at two bci (FH71 and LAT08) in three and four populations, respectively. Departure from HWE and the observed LD could be the result of recent admixture, migration or hybridization (Randi *et al.* 2003). These deviations may have been also due to null alleles detected by MICRO-CHECKER, but as their frequencies were negligible, they are extremely unlikely to affect the analysis of population structure. A deficit of heterozygosity was observed across loci in LOP, CAR, NN and PBA. Since the detected null alleles were minor, inbreeding within those populations might explain significant positive values of F_{IS}. However, inbreeding is perhaps less plausible, as the analysis of AMOVA showed 97 % of the genetic variation segregating within populations.

Gene flow in forest elephants seems to be sporadic, with dispersal sometimes being high over short distances (CAR-NN) but also occurring over much longer distances (NN-PBA). In contrast, NYO-LOP (248 km) or LOP-IVI (76 km), for instance, had significant but low restricted genetic exchange. Momont (2007) did not observe any one individual in both LOP and IVI during his study, although these sites are not far from each other. He concluded that the two elephant populations may be demographically separate. These unpredictable movements are corroborated by the lack of correlation between geographical and genetic distance shown by the Mantel test in Figure 5.3. Similar results were found with mitochondrial DNA data in Chapter 3. However, the high level of gene flow between NN, CAR and PBA suggests the existence of 2 main corridors, along which elephants regularly move (between NN and CAR, and between NN and PBA), confirming the observation of cross-border movements between Congo, Central African Republic and Gabon (Blanc *et al.*, 2007). Elephants moving between NN and CAR have

been reported to form a single trans-boundary population (Blanc *et al.*, 2007). Clearly, another corridor occurs between LOA and RAB.

The results showed that NYO was slightly differentiated from other populations, with the exception of its immediate neighbours LOA and RAB. This differentiation was shown with Bayesian analyses and F_{ST} values among populations, although these values are considered to be low when they range from 0 to 0.05 (Balloux & Lugon-Moulin, 2002). Various landscape traits such as estuaries, the Atlantic Ocean, a national road, a railway, and a zone of lakes and swamps surrounding NYO could be partly responsible for this partial isolation, as the population is located inside and in the vicinity of Wonga-Wongué National Park. It has been reported that coastal populations do not move or disperse very much, since they are able to find all necessary resources in the vicinity (Blake, pers. comm.). Furthermore, it is well known that in many species, populations are often subdivided into smaller units because of ecological or behavioural factors (Hedrick, 1985), which could be the case in this study. However, because elephants are highly mobile, gene flow must occur frequently enough to produce widespread genetic homogeneity (Slatkin, 1985), reducing differentiation among populations. Despite the low differentiation observed, elephants from NYO have apparently received significant migration from other populations. The low and significant ($P < 0.001$) deficit of heterozygotes observed in LOP ($F_{IS} = 0.05$) could be attributable to a recent effect of human disturbance. Although, LOP is a well protected National Park with limited human activities inside, it also has a history of logging, and is surrounded by a large river (Ogooué), a railway, a national road, and villages (Momont, 2007).

How much does human disturbance affect the structure of forest elephants in central Africa? A regional forest elephant status survey was carried out about two decades ago, and reported that forest elephants have undergone slaughter by poaching, as have elephants in the rest of Africa (Blake, 2007). No reliable data are available in terms of the number of elephant killed across the Congo Basin with the exception of MIKE survey teams (2003-2004) which reported recently that poaching for ivory in remote national parks has had a devastating impact on at least some populations (Blake, 2007). Thus,

LOP, CAR, NN and PBA elephants might have suffered intensive poaching for ivory recently since they inhabit areas where there is little or no effective policy against elephant poachers (Blake, 2007).

Table 5.3 presents different estimates of heterozygosity per population, but when all populations were pooled together, a deficiency of heterozygotes, assuming Hardy-Weinberg proportions, was observed (H_e = 0.81, H_o = 0.79), illustrating a potential Wahlund effect in the forest elephant of west central Africa. This observation is consistent with the two groups of populations with an intermediate population shared between groups, shown by FCA (Figure 5.4), and also well supported by posterior Bayesian analysis, with an inferred value of $K = 2$ clusters. This result agrees with the two haplogroups found in Chapter 3, based on the mitochondrial genome. One group was made up of populations from the coastal area (NYO, LOA, RAB), and a second group consisted of populations farther from the coast (CAR, NN, LOP, PBA). Individuals from IVI were more or less equally distributed amongst both clusters. IVI is thus likely to be a contact zone. Fragmentation of forests in Central Africa during the Pleistocene possibly led to a bottleneck (but with rapid recovery), resulting in the observed low deficiency of heterozygotes in different populations that were fragmented in the past (Wahlund effect).

Populations of forest elephants in west central Africa have likely experienced different degrees of admixture. All sampled populations had a proportion of individuals assigned to both clusters. The results of STRUCTURE (Table 5.7 and Figure 5.5) show evidence of intermixing between populations or clusters. IVI in particular presents almost equal proportions of individuals assigned to both clusters. This site, located in the eastern part of Gabon, has been already described as a population "made up of a mixture of highly divergent haplogroups" or a "heavily admixed" population in lowland gorillas (Anthony *et al.* 2007). In Chapter 3, the same site of Ivindo was the most variable in terms of mitochondrial DNA haplotypes when compared to CAR, which has the same number of samples. Again this may be the signature of Pleistocene changes when the forest underwent contraction and fragmentation during the drier and colder periods and

expanded in the interglacial (Hewitt, 2004). Secondary contact has possibly occurred at Ivindo for both lowland gorilla and forest elephant.

Pleistocene retraction and expansion is likely to have shaped the genetic structure of elephants, as is the case for lowland gorillas (Anthony *et al.*, 2007; Clifford *et al.*, 2004) in central Africa and for numerous bovid species in east and southern Africa (Arctander *et al.*, 1999; Flagstad *et al.*, 2001; Nersting & Arctander, 2001; Van Hooft *et al.*, 2002). However, human activity has been shown to be a major determinant of forest elephant distribution (Barnes *et al.*, 1991; Buij *et al.*, 2007), and the negative impact of this disturbance should not be neglected since several studies have reported a loss of genetic diversity following intensive poaching and habitat loss (Nyakaana S *et al.*, 2001; Nyakaana & Arctander, 1999; Whitehouse & Harley, 2001).

Forest cover in central Africa is about 2 million km^2 and all of this is believed to be suitable elephant habitat (Blake S, 2007). Furthermore, it has been reported that DRC and Gabon together account for nearly half of the elephant estimates in Central Africa (Blanc *et al*, 2007). Hoare & du Toit (1999) showed that there is no correlation between the observed elephant density and human population density until a threshold of human density is reached about 15.6 inhabitants / km^2.,Human density is still relatively low in central Africa, ranging from 1 to 6 inhabitants / km^2 (IUCN, 2005). Therefore, in theory, forest elephants should be unaffected. However, the reality seems very different since the genetic diversity assessed in this study does not reflect the current situation, but rather past history. The high level of genetic diversity currently observed may be explained by the past presence of a large population of elephants throughout the central African rain forest and the long generation time of this species, allowing the retention of diversity within populations for long periods. Similar explanations have been proposed for orang-utans in Sabah (Malaysia), which show a high level of genetic diversity in fragmented populations (Goossens *et al.*, 2005). Nevertheless, the more immediate threats to elephants in the west central African rainforest are illegal killing for ivory and habitat loss with the increase of logging, road expansion associated with human population growth (Cropper & Griffiths, 1994; IUCN, 2005; Laurance *et al.*, 2006).

In conclusion, this study revealed a high level of genetic diversity and confirms the existence of connectivity between forest elephant populations, which are split into two clusters. It also showed that the site of Ivindo is a likely admixture zone for elephants. This has also been shown for gorillas in the west central African rainforest (Anthony *et al.*, 2007). Genetic studies should be regarded as important information sources, able to produce outputs that can help to achieve the main objectives of strategies established by conservation organisms such as IUCN to "*ensure the conservation and sustainable management of elephants and their habitats in central Africa*" (IUCN, 2005).

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CHAPTER 6

General Discussion

There has been continued debate over the taxonomy of the forest elephant, which eventually led to its reclassification to species level based on both morphological (Grubb *et al.*, 2000) and genetic characters (Barriel *et al.*, 1999; Comstock *et al.*, 2002; Roca *et al.*, 2001). However, this information has been based on remarkably few samples and the IUCN (2002) has urgently requested more extensive genetic studies to resolve the taxonomy of African elephants across their range. Poaching and habitat destruction pose serious threats to the survival of forest elephants in central Africa. Illegal hunting for ivory has massively reduced the number of African elephants over the past three decades. From five to 10 million individuals estimated in 1930 (Stuart & Stuart, 1997), only 600,000 African elephants remained by 1992 (Spinage, 1994; Stiles, 2004). Consequently, African elephant populations have become fragmented with animals concentrated in central, east and northern parts of southern Africa.

The current study used non-invasive techniques to investigate the genetic structure within and among African elephant populations, and to assess migration patterns of forest elephants in central Africa. Findings from this study could have substantial implications for the conservation of forest elephant populations by guiding future management planning and decision-making processes.

The central African forest elephant exhibits a low level of nucleotide diversity (1.3 %) compared to that observed in its savannah counterpart (2 %, Nyakaana *et al.*, 2002) and other mammals such as western lowland gorillas (6.2 %, Clifford *et al.*, 2004), African buffalo (5.0 %, Simonsen *et al.*, 1998) and Grant's gazelle (6.2 %, (Arctander *et al.*, 1996). Low levels of nucleotide diversity generally indicate long-term small effective population sizes, which could be the result of extended demographic bottlenecks

(Nyakaana *et al.*, 2002). However, the high level of mitochondrial haplotype diversity and bimodal mismatch distribution in the forest elephant sample analysed here reflects a more complex population history and / or the presence of substantial population structure. That two haplogroups were found within forest elephants (Chapters 3 and 4) with no apparent geographical structure, was unexpected and is consistent with a scenario involving expansion from refugia and subsequent introgression (Hewitt, 1996). Indeed, the phylogeographic patterns found in forest elephants are comparable with those observed in savannah populations of the African buffalo (Van Hooft *et al.*, 2002). In contrast, other large African mammalian taxa have been shown to exhibit strong genetic subdivision despite their potential for high rates of genetic exchange. This is the case of numerous bovids such as, for example, the hartebeest and bushbuck (Arctander *et al.*, 1999; Flagstad *et al.*, 2001; Moodley & Bruford, 2007). Further, lineage diversification does not always coincide with major climatic change factors as found by Brown *et al.*, (2007) in African giraffe, suggesting a potentially important role for reproductive isolation among previously isolated populations. There appear to be no simple generalisations currently possible for African mammals.

Chapters 3 and 5 present the first spatially extensive genetic study of mitochondrial and nuclear markers in the forest elephant populations of central Africa. MtDNA analysis revealed low genetic divergence between most groups. Haplotypes were distributed with little restriction to geographical localities, indicating high levels of gene flow. Similarly, nuclear microsatellite loci revealed that genetic differentiation among sites was generally low, suggesting high levels of gene flow as well as regional admixture. No correlation was found between genetic and geographical distances. Further the existence of a contact zone between formerly allopatric populations from different putative refugia (Chapter 5) was also indicated for the area of Ivindo (eastern Gabon). MtDNA and microsatellite data were concordant, both indicating a high level of gene flow in forest elephants. It is important to note through, that although both mtDNA and microsatellites identified two groups (or genetic clusters), assignment of individuals to these clusters was not consistent across genomes, possibly a result of differential admixture in nuclear and mitochondrial DNA, due to variation between the sexes in introgression or dispersal. Indeed, 28.3% of

the individuals in Haplogroup A (in Chapter 3, which corresponds to HVRI Haplogroup II in Chapter 4) grouped together in cluster I, while 7.5% individuals grouped in cluster II and 20.75% were unassigned neither in cluster I nor in cluster II. Similarly, 18.6% of the individuals in Haplogroup B (= HVRI Haplogroup I) co-assigned to cluster I, 6.9% were from cluster II and 13.9% individuals were unassigned. A lack of strict genomic concordance between genomes has been previously observed in savannah elephants (Nyakaana & Arctander, 1999; Nyakaana *et al.*, 2002). This discordance was explained by the social organization observed in savannah elephants (Nyakaana & Arctander, 1999), which have a strong matrilineal social structure characterised by female natal philopatry favouring male-biased gene flow. This social structure is usually reflected in a low level of genetic differentiation at nuclear loci. A high level of gene flow and haplotype exchange was found between the closest but also the most distant forest elephant populations with both nuclear and mtDNA markers. This may be the result of their high mobility and ability to live in various ecological habitats, thus no geographic barriers have obviously shaped the observed structure of forest elephant populations. One comparable study of African buffalo (*Syncerus caffer*) showed a similar lack of differentiation among populations despite the reported strong philopatric social structure of this animal (Simonsen *et al.*, 1998; Van Hooft *et al.*, 2002).

Bayesian analysis suggested that forest elephants form two distinct genetic clusters, whose origins possibly relate to climatic changes during the Pleistocene. They therefore seem to have initially diverged allopatrically in refugia, followed by population expansion, bringing the isolated populations into secondary contact within an admixture zone around Ivindo. This same location was also found to be an admixture zone for lowland gorillas (Anthony *et al.*, 2007) and the observed admixture may explain the high level of genetic variation (Arruga *et al.*, 2007) and reduced bottleneck effect with the low deficiency of heterozygotes in past fragmented populations (Wahlund effect). During the Pleistocene, a substantial reduction in population size (bottleneck) was likely, which is predicted to have decreased genetic diversity in forest elephants (Chapter 4). Demographic events, such as bottlenecks, migration or admixture, may also explain the linkage disequilibrium (Hedrick, 1985) observed in each population. Forest elephants

have undergone complex evolutionary histories (see Chapters 3, 4 and 5): a similar finding to studies of western lowland gorillas (Anthony *et al.* 2007; Clifford *et al.* 2004) found into the same equatorial rain forest. The latter authors proposed refugia located in the Monts de Cristal, Massifs du Chaillu and Monts Doudou in Gabon. Muloko-Ntoutoume *et al* (2000) suggested that okoumé (*Aucoumea klaineana*), an endemic pioneer forest tree species in Gabon, had similar refugial origins as gorillas. A fluvial refuge for gorillas (Anthony *et al.*, 2007) was also suggested in the restricted southern of Central African Republic (CAR) and adjacent Congo. In Clifford *et al.* (2004)'s study, the authors identified one haplogroup (D) comprising western gorillas from CAR, Congo, Equatorial Guinea and Gabon, and one museum sample from southern Cameroon. This haplogroup, genetically divided in three subgroups D1, D2 and D3, coincided with the locations of several major forest refugia in Equatorial Guinea, CAR, Gabon and adjacent Congo, respectively. Unfortunately, no samples from Monts de Cristal in northwestern Gabon and Equatorial-Guinea were available in the current study in order to corroborate the existence of a possible Monts de Cristal refuge.

6.1 Two-species model versus a complex evolutionary history of African elephant

The studies by Roca et al (2005, 2007) suggest a two species model for African elephants. These studies proposed that a limited nuclear gene flow from savannah elephants into forest elephant population is consistent species-level distinction. Though the existence of a hybrid zone with identified intermediate morphotypes (Groves and Grubb, 2000), Roca et al (2005, 2007) suggested that an extrinsic mechanism would prevent forest or hybrid males from reproducing successfully with savannah elephant populations, strongly reducing the contribution of forest elephants in the savannah nuclear genome. Recurrent backcrossing would mostly have occurred between hybrid females and large savannah males, a repeated unidirectional hybridization leading to a savannah morphotype in savannah elephant habitats. However, a major weakness of Roca's studies and consequently, potentially, this hypothesis is limited sampling, in both central forest areas and more even importantly in West Africa.

The current study revealed a complex evolutionary history for African elephants during the Pleistocene. Forest elephant populations in central Africa form two distinct lineages, the origin of which possibly relates to populations separated during past climatic changes. These populations seem likely seem to have diverged in allopatry in Pleistocene refugia, followed by population expansion. These isolated populations have come back into secondary contact within an admixture zone. However our data do not support the separation of current African elephant populations into two different species. The evidence for this is most clear in West Africa where savannah elephants are indistinguishable at the mitochondrial level from their west African forest counterparts and where, most importantly, all individuals in our study are found in the same haplogroups as the forest elephant mitochondrial lineages of central Africa. The most parsimonious explanation for this observation implies a forest ancestor for both modern west African forest and savannah elephants, implying rapid morphological change for elephants in forest and savannah habitats regardless of their mitochondrial (or nuclear) DNA affiliation. Further, even in the unlikely event that the converse was true (that west African elephants gave rise to modern central African forest elephants) this would still imply rapid morphological adaptation within a mitochondrial haplogroup.

The existence of two divergent nuclear genomes has been cited by Roca et al (2005, 2007) as evidence of species-level divergence between forest and savannah elephants. However, given the rapid rate of morphological evolution implied from our studies of central and west African elephants and the fact nuclear DNA divergence seemingly fails to impede introgression where it is present, the tempo and mode of nuclear DNA evolution seems irrelevant to the question of the establishment of savannah and forest morphologies. Thus, while two nuclear DNA lineages clearly exist, there is no *a priori* reason to suppose that these lineages correlated with savannah or forest elephant morphologies in the past and are likely to have been engendered by a long period of allopatric isolation in the Pleistocene which could have involved forest, savannah, or a mixture of both.

African forest and savannah elephant populations could therefore be an example of long-lasting gene flow between two ecological forms, which is ongoing (or was until recently). If ancient female-mediated introgression between the two forms followed by backcrossing into savannah populations is the reason why western savannah elephants possess largely 'forest' haplotypes then nuclear markers at these loci should resemble predominantly those of southern and eastern savannah elephants today. This is not the case (Eggert et al 2002) and west African elephants most closely resemble central African elephants at nuclear microsatellite markers.

Another explanation could be a 'second movement' of elephants out of the forest (from either west or central Africa) and into the savannah. It is difficult to determine whether there was a single movement from forest to savannah habitat or whether these were multiple events, precipitating the morphological changes observed today. Further data are necessary to confirm the origin of West African elephant. Whatever the origin of the two types, our data would support continued extensive hybridisation between the two proposed forms. Thus the classification of species into savannah and forest may not reflect their recent evolutionary history. However, West African elephants seem to group with, and potentially have originated from, forest elephant lineages in central Africa (and do not share mtDNA with widespread savannah lineages) and seem to have subsequently diverged into West African forest and west African savannah elephants. This seems likely to have happened in sympatry through ecological divergence (Rice & Hostert, 1993) since there is no evidence for mtDNA monophyly in west African forest and savannah haplotypes associated with either of the two rainforest haplogroups. Therefore this phylogeographic history of African elephants does not obviously support the suggested two-taxa model: although two clearly differentiated nuclear genomes exist (Roca et al 2005), these do not correlate with four demographic groups identified here and have evolved over different timescales (e.g. Zink & Barrowclough, 2008).

Additionally to previous morphological, mitochondrial and nuclear DNA sequence studies, research on adaptive genes could provide relevant information in order to detect local adaptations that elephants might develop according to their habitat. Further

sampling in areas of hybrid zone and West Africa is also needed to understand the historical evolutionary and population movement of African elephants.

To develop management strategies, incorporating current simple two-taxon model could therefore be misleading without further research and until further lines of evidence give us a clearer picture of the origins and current conservation needs of elephant populations throughout the continent. Hence, the taxonomic uncertainty of African elephants persists.

6.2 Conservation implications for central African forest elephants

Two major mitochondrial lineages have been defined in central African forest elephants with no geographical structure, while microsatellite loci described two genetic clusters with one comprising coastal populations from Gabon (Nyonié, Loango and Rabi) and the other inland populations (Lopé, Plateaux Batéké, Ivindo, Nouabalé-Ndoki and Dzanga-Sanga; see Chapter 5). Three main corridors were detected (Chapter 5) implying elephant movements between sites at short (Rabi-Loango, Dzanga-Sanga-Noubalé-Ndoki) and longer (Nouabalé-Ndoki-Plateaux Batéké) distances. Haplotype exchange between adjacent populations (Chapter 3), enable inference of movements between Rabi and Conkouati (southwest Congo). It is important to maintain this gene flow to ensure long-term genetic diversity, given that intensive poaching and the loss of forest habitat currently threaten elephants. A number of factors have been reported (IUCN, 2005) which directly or indirectly negatively impact on elephant populations in central Africa. Threats with direct impact are for example illegal killing, habitat loss and fragmentation. Many studies have indicated that elephants tend to avoid human settlement areas (Blake & Hedges, 2004). Unfortunately logging roads give poachers access to remote forest where elephants occur, thus facilitating the ivory trade. Other, indirect effects include institutional weakness and political instability (Lee & Graham, 2006) with insufficient and / or ineffective legal frameworks and weak application of the law, encouraging poachers often equipped with powerful firearms. Conservationists met in 2005 in Limbé

(Cameroon) in order to establish a sub-regional strategy for the conservation and management of central African elephants (IUCN, 2005). They identified four main objectives to:

1. reduce the illegal killing of elephants and trade elephant products
2. ensure connectivity between elephant populations
3. improve knowledge of elephant populations and their habitats
4. gain more support from the public for elephant conservation.

A series of activities (including genetic studies) were suggested in order to achieve these objectives. This study has already identified potential corridors, which could contribute to achieving Objective 2 above. Objective 1 could be assisted by using highly polymorphic DNA markers (e.g. Wasser *et al.*, (2004) combined with new statistical methods (Bayesian assignment tests) (Manel *et al.*, 2002). In this way, the source of slaughtered individuals could be detected in order to identify areas of illegal activity and enable steps to be put in place to reduce illegal killing of elephants in central Africa. The high level of genetic diversity found here in the current populations of forest elephants, coupled with the maintenance of uninterrupted forest blocks in central Africa, if managed correctly, does provide suitable refuge for diverse and large elephant populations. Hence, the implementation of the above strategy is crucial for the long-term survival of forest elephants.

Future studies will need to analyse multiple nuclear DNA markers from across the range of forest and savannah elephants, especially in transition zones before any final taxonomic or phylogenetic conclusions can be made. Phylogeographic analyses have found two main mitochondrial DNA haplogroups in central Africa, indicating the existence of populations originating from different Pleistocene refugia. It is important to extend the sampling in south Cameroon, Equatorial Guinea, northwest Gabon in Monts de Cristal since these areas are also known to encompass candidate refugia (Anthony *et al.*, 2007; Clifford *et al.*, 2004; Maley, 1996). More samples from West Africa, north and central Cameroon, north CAR and the Democratic Republic of Congo (e.g. Garamba, Salonga), where in some cases savannah and forest elephants may co-occur (Blanc *et al.*,

2007), would help to elucidate movements of elephants during the contraction and expansion phases of the last glacial maxima and in some cases will enable a detailed study of ongoing introgression. Furthermore, in order to improve conservation efforts for forest elephants, it is essential to better understand their social behaviour.

The outcomes of this thesis provide novel and potentially useful information to challenge the validity of the two-taxon model of the African elephant. The use of mitochondrial and microsatellite DNA showed broadly congruent results that forest elephants are grouped in two genetic units with little geographical structure. However, they have indicated the existence of refugial divergence, an important degree of population admixture with a contact zone located in Ivindo (Gabon), and a number of corridors allowing movement of elephants. Therefore, further management recommendations should be informed by detailed genetic studies.

6.3 References

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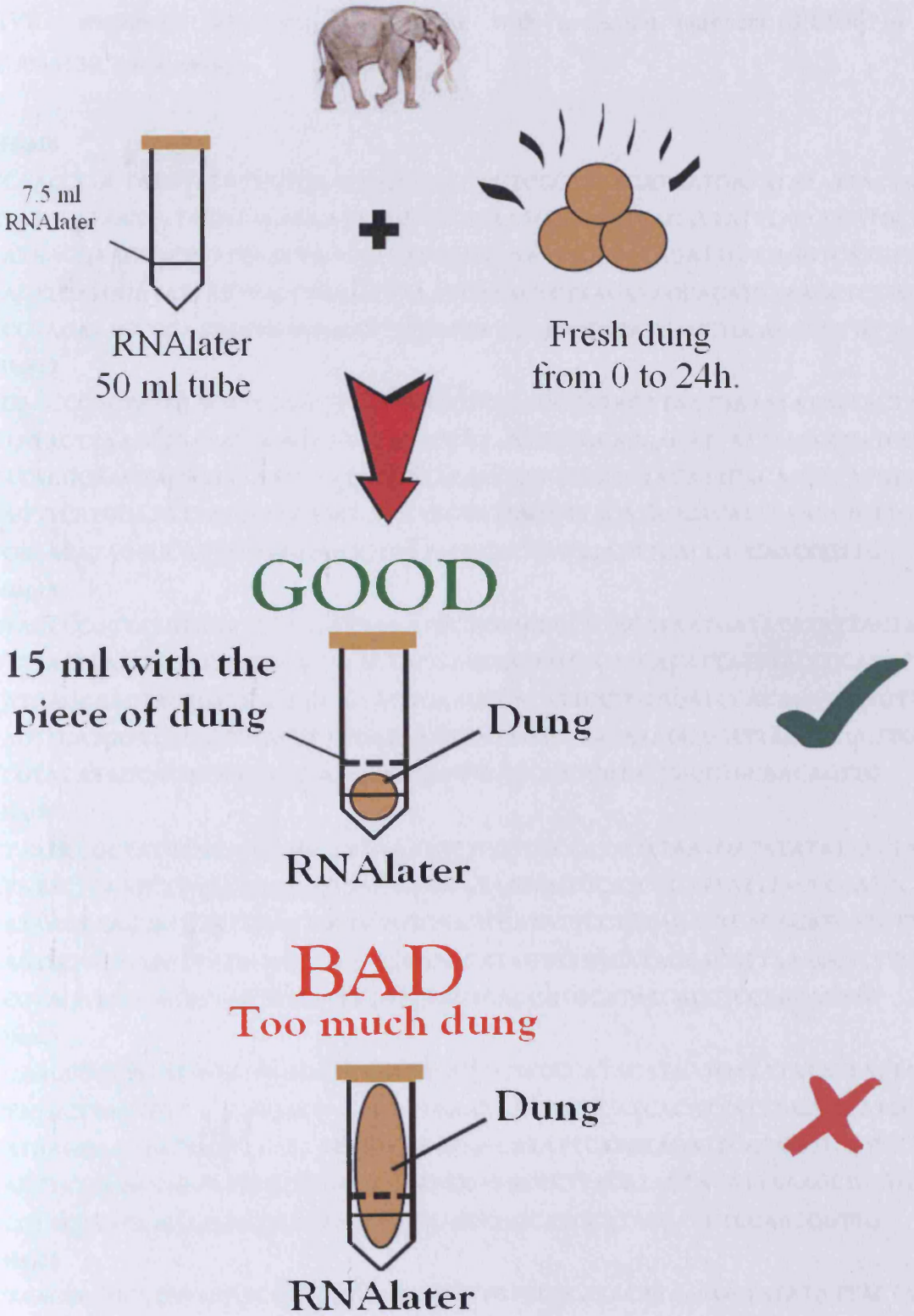
APPENDIX

Appendix 1. Handout showing how to collect elephant dung sample.



Appendix 2.

HVR1 sequences submitted on Genbank with accession numbers: EU096114 – EU096130, respectively.

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Appendix 3.

Cytochrome *b* sequences submitted on Genbank with accession number EU115995 – EU116019, respectively.

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CATTTCATTCTTCCATTTACTATAACTGCACTAGCAGGAGTACACCTAACCTTTCTTCACGAAA
CAGGCTCAAACAACCCACTAGGCCTCACTTCAGACTCAGACAAAATCCCCTTTCACCCGTACT
ATACCATCAAAGACTTC
>SNHap_41
ATTGGACGAAACATCTACTATGGGTCCTACCTATACTCGGAAACTTGAAATACCGGCATTATA
TTACTACTAATCACCATAGCCACCGCCTTCATAGGATATGTCCTTCCGTGAGGACAAATATCA
TTTTGAGGGGCAACCGTAATCACTAACCTTTTCTCAGCAATCCCTTATATCGGCACAAACCTA
GTAGAATGAATCTGAGGAGGCTTTTCAGTAGATAAAGCAACCTTAAATCGATTTTTCGCCCTC
CATTTCATTCTTCCATTTACTATAATTGCACTAGCAGGAGTACACCTAACCTTTCTTCACGAAA
CAGGCTCAAACAATCCACTGGGCCTCACTTCAGACTCAGACAAAATCCCCTTTCACCCATACT
ATACCATTAAGGACTTC
>SNHap_42
ATTGGACGAAACATCTACTATGGGTCCTACCTATACTCGGAAACTTGAAATACCGGCATTATA
TTACTACTAATCACCATAGCCACCGCCTTCATAGGATATGTCCTTCCGTTAGGACAAATATCAT
TCTGAGGGGCAACCGTTATCACTAACCTTTTCTCAGCAATCCCTTATATCGGCACAAACTTA GT
AGAATGAATCTGAGGAGGCTTTTCAGTAGATAAAGCAACCTTAAATCGATTTTTCGCCCTCCA
TTTCATTCTTCCATTTACTATAATTGCACTAGCAGGAGTACACCTAACCTTTCTTCACGAAACA
GGCTCAAACAATCCACTGGGCCTCACTTCAGACTCAGACAAAATCCCCTTTCACCCATACTAT
ACCATTAAGGACTTC
>SNHap_43
ATTGGACGAAACATCTACTATGGGTCCTACCTATACTCAGAAACCTGAAATACCGGCATTATA
TTACTACTAATCACCATAGCCACCGCCTTCATAGGATATGTCCTTCCGTGAGGACAAATATCA
TTCTGAGGGGCAACCGTAATCACTAACCTTTTCTCAGCAATCCCTTATATCGGCACAAACCTA
GTAGAATGAATCTGAGGAGGCTTTTCAGTAGATAAAGCAACCTTAAATCGATTTTTCGCCCTC
CATTTCATTCTTCCATTTACTATAATTGCACTAGCAGGAGTACACCTAACCTTTCTTCACGAAA
CAGGCTCAAACAACCCACTGGGCCTCATTTCAGACTCAGACAAAATCCCCTTTCACCCGTACT
ATACCATTAAAGACTTC

>MJHap_19

ATTGGACGGAACATCTACTATGGATCCTATCTATACTCGGAAACCTGAAATACCGGCATTATA
TTACTACTAATCACCATAGCCACCGCCTTCATAGGATATGTCCTTCCGTGAGGACAAATATCA
TTCTGAGGGGCAACCGTAATCACTAACCTCTTCTCAGCAATTCCCTACATCGGCACAAGCCTA
GTAGAATGAATCTGAGGAGGCTTTTCGGTAGATAAAGCAACCTTAAATCGATTCTTCGCCCTC
CATTTCATTCTTCCATTTACTATAATTGCACTAGCAGGAGTACACCTAACCTTTCTTCACGAAA
CAGGCTCAAACAACCCACTAGGCCTCACTTCAGACTCAGACAAAATCCCCTTTCACCCGTACT
ATACCATCAAAGACTTC

>MJHap_23

ATTGGACGGAACATCTACTATGGATCCTACCTATACTCGGAAACCTGAAATACCGGCATTATA
TTATTACTAATCACCATAGCCACCGCCTTCATAGGATATGTCCTTCCGTGAGGACAAATATCAT
TCTGAGGGGCAACCGTAATCACTAACCTCTTCTCAGCAATTCCCTACATCGGCACAAGCCTAG
TAGAATGAATCTGAGGAGGCTTTTCGGTAGATAAAGCAACCTTAAATCGATTCTTCGCCCTCC
ATTTCATTCTTCCATTTACTATAATTGCACTAGCAGGAGTACACCTAACCTTTCTTCACGAAAC
AGGCTCAAACAACCCACTAGGCCTCACTTCAGACTCAGACAAAATCCCCTTTCACCCGTACTA
TACCATCAAAGACTTC

>MJHap_24

ATTGGACGGAACATCTACTATGGATCCTACCTATACTCGGAAACCTGAAATACCGGCATTATA
TTACTACTAATCACCATAGCCACCGCCTTCATAGGATATGTCCTTCCGTGGGGACAAATATCA
TTCTGAGGGGCAACCGTAATCACTAACCTCTTCTCAGCAATTCCCTACATCGGCACAAACCTA
GTAGAATGAATCTGAGGAGGCTTTTCAGTAGATAAAGCAACCTTAAATCGATTCTTCGCCCTC
CATTTCATTCTTCCATTTACTATAACTGCACTAGCAGGAGTACACCTAACCTTTCTTCACGAAA
CAGGCTCAAACAACCCACTAGGCCTCATTTCAGACTCAGACAAAATCCCCTTTCACCCGTACT
ATACCATCAAAGACTTC

Appendix 4. Details of list of samples used in Chapter 3. Sample ID is mentioned with its locality, coordinates, type of sample and preservation, date of collection and the collector. * indicated samples collected while the animal was collared.

Sample ID	Locality	Country	GPS	Type	Preservation	Date	Collector
Lop067	Lopé NP	Gabon	789788 / 9978573	Faecal	RNAlater	27/03/2001	L. Momont
Lop0710	Lopé NP	Gabon	791843 / 9978868	Faecal	RNAlater	04/04/2001	L. Momont
Lop0914	Lopé NP	Gabon	790757 / 9979849	Faecal	RNAlater	20/11/2001	L. Momont
Lop1016	Lopé NP	Gabon	790757 / 9979849	Faecal	RNAlater	21/11/2001	L. Momont
Lop146	Lopé NP	Gabon	790180 / 9978751	Faecal	RNAlater	21/11/2001	L. Momont
Lop154	Lopé NP	Gabon	791027 / 9979045	Faecal	RNAlater	21/11/2001	L. Momont
Lop167	Lopé NP	Gabon	790180 / 9978751	Faecal	RNAlater	22/11/2001	L. Momont
Lop175	Lopé NP	Gabon	789432 / 9978176	Faecal	RNAlater	23/11/2001	L. Momont
Lop1810	Lopé NP	Gabon	790180 / 9978751	Faecal	RNAlater	23/11/2001	L. Momont
Lop51a14	Lopé NP	Gabon	789499 / 9977702	Faecal	RNAlater		K. Abernethy
AFE79Lop*	Lopé NP	Gabon		Blood		29/07/2003	L.Momont
Loa0310	Loango NP	Gabon		Faecal	Silica gel	10/10/2002	S. Lahm
Loa068	Loango NP	Gabon		Faecal	Silica gel	15/10/2002	S. Lahm
Igl032	Loango NP	Gabon	S2°20.150/E9°36.510	Faecal	Silica gel	02/12/2002	N. Anthony
AFE85Igl*	Loango NP	Gabon	S2°21.505/E9°36.893	Blood			S. Blake
AFE86Igl*	Loango NP	Gabon	S1°53.162/E9°17.244	Blood			S. Blake
AFE87Igl*	Loango NP	Gabon		Blood			S. Blake
AFE88Igl*	Loango NP	Gabon		Blood			S. Blake
AFE89Igl*	Loango NP	Gabon		Blood			S. Blake
Rab0113	Rabi NP	Gabon		Faecal	RNAlater	17/06/2002	S. Lahm
Rab0215	Rabi NP	Gabon		Faecal	RNAlater	17/06/2002	S. Lahm
Rab032	Rabi NP	Gabon	S1°56.229/E9°51.336	Faecal	RNAlater	18/06/2002	S. Lahm
Rab044	Rabi NP	Gabon	S1°56.872/E9°51.542	Faecal	RNAlater	17/06/2002	S. Lahm
Rab067	Rabi NP	Gabon	S1°54.859/E9°52.392	Faecal	RNAlater	18/06/2002	S. Lahm
Rab1118	Rabi NP	Gabon	S1°52.083/E9°51.217	Faecal	RNAlater	17/06/2002	S. Lahm
Rab131	Rabi NP	Gabon	S1°54.859/E9°52.392	Faecal	RNAlater	30/05/2002	S. Lahm
Rab275	Rabi NP	Gabon	S1°56.229/E9°51.336	Faecal	RNAlater	15/06/2002	S. Lahm

Sample ID	Locality	Country	GPS	Type	Preservation	Date	Collector
Wak0410	Waka NP	Gabon	S1°46.864/E9°53.113	Faecal	RNAlater		G. Abitsi
Wak0512	Waka NP	Gabon	S1°51.168/E9°51.306	Faecal	RNAlater		G. Abitsi
Wak0613	Waka NP	Gabon		Faecal	RNAlater		G. Abitsi
Wak0715	Waka NP	Gabon		Faecal	RNAlater		G. Abitsi
WAk0817	Waka NP	Gabon		Faecal	RNAlater		G. Abitsi
AFE82Lan	Ivindo NP	Gabon		Blood			S. Blake
Lan015	Ivindo NP	Gabon		Faecal	Silica gel		L. Momont
Lan027	Ivindo NP	Gabon		Faecal	Ethanol		L. Momont
Lan1566	Ivindo NP	Gabon		Faecal	RNAlater		E. Spotswood
Lan15911	Ivindo NP	Gabon		Faecal	RNAlater		E. Spotswood
Lan16014	Ivindo NP	Gabon	S0°11.304/E12°32.627	Faecal	RNAlater		E. Spotswood
Lan209	Ivindo NP	Gabon	S0°11.263/E12°33.501	Faecal	Silica gel		L. Momont
Ivi043	Ivindo NP	Gabon	S0°11.374/E12°33.585	Faecal	Silica gal	26/07/2002	S. Lahm
Ivi05a6	Ivindo NP	Gabon		Faecal	RNAlater	27/07/2002	S. Lahm
Ivi05b8	Ivindo NP	Gabon	N0°15.290/E12°27.901	Faecal	RNAlater	27/07/2002	S. Lahm
Ivi06b2	Ivindo NP	Gabon	N0°14.899/E12°26.470	Faecal	RNAlater	29/07/2002	S. Lahm
Ivi06c4	Ivindo NP	Gabon	N0°14.899/E12°26.470	Faecal	RNAlater	29/07/2002	S. Lahm
Ivi088	Ivindo NP	Gabon	N0°15.027/E12°24.565	Faecal	RNAlater	31/07/2002	S. Lahm
Ivi0910	Ivindo NP	Gabon	N0°15.027/E12°24.565	Faecal	RNAlater	01/08/2002	S. Lahm
Ivi1011	Ivindo NP	Gabon	N0°14.717/E12°22.257	Faecal	RNAlater	02/08/2002	S. Lahm
Ivi1012	Ivindo NP	Gabon	N0°14.717/E12°22.257	Faecal	RNAlater	02/08/2002	S. Lahm
Kes0211	Plateaux Batéké NP	Gabon	N0°14.310/E12°22.045	Faecal	RNAlater	03/08/2003	H. Ontsana
Kes0314	Plateaux Batéké NP	Gabon	N0°14.310/E12°22.045	Faecal	RNAlater	03/08/2003	H. Ontsana
Kes0415	Plateaux Batéké NP	Gabon		Faecal	RNAlater	07/09/2003	H. Ontsana
Kes0721	Plateaux Batéké NP	Gabon		Faecal	RNAlater	07/09/2003	H. Ontsana
Kes0819	Plateaux Batéké NP	Gabon		Faecal	RNAlater	07/09/2003	H. Ontsana
PBa023	Plateaux Batéké NP	Gabon		Faecal	Silica gel	20/02/2005	N. Bout
PBa0612	Plateaux Batéké NP	Gabon		Faecal	Silica gel	21/02/2005	N. Bout
PBa0714	Plateaux Batéké NP	Gabon	S1°58.523/E14°00.217	Faecal	Silica gel	21/02/2005	N. Bout
Mpa01	Plateaux Batéké NP	Gabon	S1°57.778/E13°58.741	Tissue	Silica gel	25/11/2001	P. Henschel

Sample ID	Locality	Country	GPS	Type	Preservation	Date	Collector
Mpa028	Plateaux Batéké NP	Gabon	S1°56.940/E13°57.596	Tissue	Silica gel	25/11/2001	P. Henschel
Mpa0319	Plateaux Batéké NP	Gabon	S2°18.356/E14°03.260	Tissue	Silica gel	01/12/2001	P. Henschel
MDC012	Mts de Crystal NP	Gabon	S2°18.356/E14°03.260	Faecal	RNAlater	04/12/2004	R. A. Nseme
MDC024	Mts de Crystal NP	Gabon	S2°13.110/E13°50.516	Faecal	RNAlater	04/12/2004	R. A. Nseme
Nog014	North of Ogooué River	Gabon	N0°78.515/E10°23.842	Faecal	RNAlater		S. Clifford
Nog025	North of Ogooué River	Gabon	N0°79.040/E10°23.644	Faecal	RNAlater		S. Clifford
Nog026	North of Ogooué River	Gabon		Faecal	Silica gel		S. Clifford
Nog038	North of Ogooué River	Gabon		Faecal	RNAlater		S. Clifford
Nog053	North of Ogooué River	Gabon		Faecal	RNAlater		S. Clifford
Nog066	North of Ogooué River	Gabon		Faecal	RNAlater		S. Clifford
Nog078	North of Ogooué River	Gabon		Faecal	RNAlater		S. Clifford
Nog0810	North of Ogooué River	Gabon		Faecal	RNAlater		S. Clifford
Nyo0310	North Wonga-Wongué Res.	Gabon		Faecal	Silica gel		B. Goossens
Ckt04a14	Conkouati-Douli NP	Congo		Faecal	RNAlater		M. Johnson
Nn059	Nouabalé-Ndoki NP	Congo		Faecal	Silica gel		A. Turkalo
Nn0713	Nouabalé-Ndoki NP	Congo		Faecal	Silica gel		A. Turkalo
Nn232	Nouabalé-Ndoki NP	Congo	N2°15.546/E16°24.680	Faecal	Silica gel	16/10/2003	A. Turkalo
Nn267	Nouabalé-Ndoki NP	Congo	N2°15.546/E16°24.680	Faecal	Silica gel	02/12/2003	A. Turkalo
Nn279	Nouabalé-Ndoki NP	Congo	N2°20.327/E16°52.517	Faecal	Silica gel	03/12/2003	A. Turkalo
Nn2911	Nouabalé-Ndoki NP	Congo	N2°17.981/E16°52.601	Faecal	Silica gel	23/02/2004	A. Turkalo
Nn3014	Nouabalé-Ndoki NP	Congo	N2°19.089/E16°52.094	Faecal	Silica gel	27/02/2004	A. Turkalo
Nn3116	Nouabalé-Ndoki NP	Congo		Faecal	Silica gel	01/03/2004	A. Turkalo
Nn3218	Nouabalé-Ndoki NP	Congo	N2°18.820/E16°51.621	Faecal	Silica gel	20/03/2004	A. Turkalo
CAR274	Dzanga-Sangha NP	CAR	N2°56.533/E16°21.116	Faecal	Silica gel	11/02/2004	A. Turkalo
CAR297	Dzanga-Sangha NP	CAR	N2°56.607/E16°21.227	Faecal	Silica gel	11/03/2004	A. Turkalo
CAR309	Dzanga-Sangha NP	CAR	N2°56.676/E16°21.203	Faecal	Silica gel	11/03/2004	A. Turkalo
CAR3111	Dzanga-Sangha NP	CAR	N2°56.718/E16°21.220	Faecal	Silica gel	11/03/2004	A. Turkalo
CAR3214	Dzanga-Sangha NP	CAR	N2°56.257/E16°21.600	Faecal	Silica gel	12/03/2004	A. Turkalo
CAR3315	Dzanga-Sangha NP	CAR	N2°56.542/E16°21.301	Faecal	Silica gel	12/03/2004	A. Turkalo
CAR3417	Dzanga-Sangha NP	CAR	N2°56.501/E16°21.210	Faecal	Silica gel	12/03/2004	A. Turkalo

Sample ID	Locality	Country	GPS	Type	Preservation	Date	Collector
CAR3519	Dzanga-Sangha NP	CAR	N2°56.606/E16°21.229	Faecal	Silica gel	15/03/2004	A. Turkalo
CAR3622	Dzanga-Sangha NP	CAR	N2°56.617/E16°21.215	Faecal	Silica gel	15/03/2004	A. Turkalo
CAR381	Dzanga-Sangha NP	CAR	N2°56.808/E16°21.175	Faecal	Silica gel	15/03/2004	A. Turkalo
CAR394	Dzanga-Sangha NP	CAR	N2°56.533/E16°21.195	Faecal	Silica gel	17/03/2004	A. Turkalo
CAR405	Dzanga-Sangha NP	CAR	N2°56.540/E16°21.196	Faecal	Silica gel	17/03/2004	A. Turkalo
CAR4210	Dzanga-Sangha NP	CAR	N2°56.523/E16°21.199	Faecal	Silica gel	18/03/2004	A. Turkalo
CAR441	Dzanga-Sangha NP	CAR	N2°56.749/E16°21.260	Faecal	Silica gel	18/03/2004	A. Turkalo
CAR5712	Dzanga-Sangha NP	CAR	N2°56.688/E16°21.206	Faecal	Silica gel	04/04/2004	A. Turkalo
CAR5813	Dzanga-Sangha NP	CAR	N2°56.665/E16°21.200	Faecal	Silica gel	04/04/2004	A. Turkalo

Appendix 5.

249 forest elephant individuals from 8 populations were genotyped using 12 polymorphic microsatellite loci (below at the left-hand column). Pop 1 to 8 are populations (LOP, CAR, NN, IVI, RAB, LOA, PBA and NYO, respectively). Lines represent individuals with their genotypes at each locus (1 to 12).

1 FH39
2 FH127
3 FH67
4 FH71
5 LAFMS03
6 LAFMS07
7 FH60
8 LA6
9 LAT08
10 FH19
11 FH40
12 FH48

Pop 1	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
LOP024,	240240	155155	096100	075081	141149	152164	161161	155167	000000	191191	230242	172174
LOP036,	240242	161163	100104	069085	147149	132152	139141	163167	202278	191195	228240	156164
LOP041,	246252	153155	096100	081085	141145	160164	157165	157167	294294	193197	248250	166170
LOP0710,	240246	153155	096100	075081	145147	160164	141167	155165	274278	195201	228260	166170
LOP0812,	242246	153159	096100	085095	139145	148160	139157	155157	266266	195207	228260	156166
LOP1016,	240250	159163	096100	069081	137143	132156	141157	165169	212218	191209	240268	166178
LOP202,	240250	159161	092100	081085	147149	132164	149155	155155	210252	205205	228250	156178
LOP2313,	240250	153163	096100	085085	147147	132164	139155	163167	278294	187191	240268	156170
LOP2415,	240246	169169	092094	069089	141143	160160	139155	155167	256274	191205	228268	156164
LOP284,	238240	159161	092100	081085	145149	132160	149155	155155	252256	201205	228250	170178
LOP347,	240250	155163	102102	081085	141147	148164	139141	155163	278286	191197	240240	156166
LOP366,	240240	155163	096104	075085	147147	160164	139155	163167	234294	191201	228268	156172
LOP509,	238240	159161	092100	081085	145149	132160	149155	155155	252256	201205	228250	170178
LOP51a15,	240242	159163	096100	085095	139147	152160	139139	155163	220294	191191	228250	156164
LOP5315,	238240	155161	096100	081093	139143	132132	157157	155165	234290	201205	228240	166170
LOP5418,	234242	147153	094098	081093	139147	152164	161163	155165	214294	195203	228228	166170
LOP631,	240240	147147	094098	067081	137143	132148	141157	155155	250274	189201	240268	156174
LOP753,	240250	155163	098102	081085	141147	148164	139141	155163	278286	191197	240240	156166
LOP765,	240240	155159	092102	069075	137145	148160	139141	159159	212212	193193	228242	156166
LOP777,	236242	147147	096098	073075	139145	160164	139141	157165	238238	195201	252252	166170
LOP8312,	240240	147159	092098	081081	143147	132168	141155	155167	206242	000000	228228	156164
LOP8613,	240242	155161	092096	081085	139145	132164	141155	155167	194226	213213	228240	164170
LOP8715,	240242	161163	098104	069085	145147	132152	139141	163167	202278	191195	228240	156164
LOP9718,	240240	153153	092098	095095	143147	132160	141155	157167	262290	195209	228252	156166
LOP9819,	240240	155163	096104	075085	147147	160164	139155	163167	234294	191201	228268	156172
LOP9922,	242246	159165	094096	081085	139139	160164	159163	155157	230230	199201	230250	156166
LOP1476,	240240	147155	096096	075085	145145	152156	153155	155169	186190	201205	240240	156178
LOP1499,	000000	000000	100100	069081	137143	000000	157163	155155	212230	207207	240252	166166
LOP15112,	240240	159161	092094	079079	143147	148164	139155	165167	194222	195205	226248	164174
LOP15214,	242246	147161	092102	081085	141147	156164	141157	155155	206214	199205	228228	156160
LOP15823,	240240	165165	096096	081091	141147	164164	141157	155169	190190	203205	000000	170174
LOP17016,	240240	147161	094102	085085	143143	160164	155155	157157	190190	191191	228228	164170
LOP1391,	244256	161161	096096	075075	147147	148148	157157	155167	000000	000000	000000	000000
LOP1403,	236240	155155	096098	069075	143147	156164	155157	155155	212278	193207	252268	172178
LOP1411,	240250	155161	094096	067081	147147	132164	153157	167171	262290	189191	240258	164178
LOP1423,	242250	157161	092094	067069	143147	132164	153163	167171	256262	189189	228240	156178
LOP17117,	240240	147157	096096	085093	147147	132164	141155	155167	000000	191191	000000	000000
LOP17322,	240240	149155	096102	081081	143143	148152	139163	155155	000000	000000	240240	164170

LOP1741, 240240 147169 094094 069081 143145 132148 139139 165167 256256 191195 228230 156166
LOP17523, 234240 147147 094094 069081 143145 132132 139139 167167 256256 191191 230230 156166
LOP1763, 000000 155155 096102 081081 143143 148152 139139 153155 000000 000000 000000 000000
LOP177A5, 242246 155157 096096 081089 143143 148164 139157 155155 194208 197205 228240 156164
LOP178a10, 240246 159163 096100 085095 143143 132152 153155 155169 226270 191191 228228 166166
LOP179b15, 236242 147161 096098 073075 139139 160164 139141 163165 218234 191201 252268 172178
LOP18019, 240242 155159 102102 085093 139141 148160 139141 155167 266270 193213 240240 152164
LOP1818, 242244 155161 092094 085087 141157 152156 141157 167167 000000 000000 228240 000000
LOP18210, 234244 161161 092102 081081 139147 148164 141153 165167 234234 195197 228228 156166
LOP18312, 240242 159159 094096 081089 145147 132148 161163 155155 194242 191201 226260 156172
LOP1889, 238252 153153 096104 067067 139147 132164 139151 167169 194226 201209 230260 164178
LOP121, 240242 147159 094100 067067 139145 132164 149159 155167 238242 197213 268268 166178
LOP153, 242246 159169 096096 067069 139145 132148 139149 155167 238242 191213 242268 166170
LOP176, 240242 157157 092096 075089 141143 152160 155157 155167 274278 193205 262268 164174
LOP197, 234248 161161 092096 071073 139147 156164 139157 155157 290290 191205 230266 166166
LOP219, 240242 155203 096102 081085 141147 152164 139161 155161 178234 201201 228268 166174
NOG013, 234240 147161 092100 081095 143147 152152 139139 155171 242290 211213 228228 156166
NOG026, 000000 147157 096100 000000 143147 152164 139159 155155 000000 000000 000000 000000
NOG037, 242250 155161 096100 081081 145145 152160 155159 155155 166270 201203 228260 000000
NOG05, 240248 149161 098098 085093 143143 132164 141141 155165 222222 195203 228234 000000
NOG09, 240240 155159 094094 085085 143145 152156 139141 155167 208262 187191 228250 156180

Pop 2

CAR187, 234236 151161 096098 069069 147155 156160 153157 155167 188218 191199 228228 160170
CAR199, 238242 151157 098102 085089 143143 152168 155159 155157 216226 207207 242260 000000
CAR2114, 238240 147157 098098 075075 141143 152168 155155 155157 212266 197209 228228 156172
CAR2216, 242248 153157 096102 081089 145147 148152 157159 155155 174216 187207 228260 164166
CAR273, 240240 147159 094110 077085 141145 156164 141155 155167 210238 195205 228228 164172
CAR286, 234238 161161 096098 067081 145145 164168 141141 167169 218238 191197 228248 170172
CAR298, 234234 155161 098098 069085 141145 148152 163163 157157 230230 205205 228260 158170
CAR3010, 242256 147159 094098 085085 145147 152160 141159 165171 252274 199205 228268 162166
CAR3112, 236244 153161 098100 075081 141155 152152 141159 155171 226230 191205 000000 156164
CAR3213, 242248 147153 100100 069069 147147 000000 155155 165165 210210 191203 000000 158166
CAR3316, 242248 157165 096104 085109 143155 132160 159163 155155 202206 191201 228260 156156
CAR3418, 234242 149161 092098 069073 141145 152152 155163 157157 220266 191201 228228 156172
CAR3520, 234248 155161 098098 069069 139145 132148 155157 157167 230294 197201 228228 166172
CAR3621, 240240 159159 098104 081085 145147 152152 141155 157159 278282 197203 228228 156156
CAR3724, 240248 153159 098098 069085 143147 152160 155155 157167 170190 201203 228240 156166
CAR382, 000000 000000 092096 069085 147155 148160 000000 000000 190190 195205 228228 000000
CAR393, 242250 155157 092096 000000 141141 132164 139139 169169 220266 197197 228228 164172
CAR406, 240240 159159 092092 073081 139141 132164 141159 155167 190216 203205 228258 160164
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CAR454, 234242 161163 096096 073091 141143 132160 163163 155159 196196 191213 228258 156164
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Pop 3

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NN4914, 238252 153165 098098 081081 147147 148168 155161 157167 246246 203203 228248 156166
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NN684, 234244 147153 092094 081087 141147 132148 155157 167169 222276 193201 232248 170170
NN7110, 240242 153159 100100 085085 139139 152156 139153 167169 000000 193211 226268 164164

NN7416, 242248 155157 094098 075075 139143 160160 165165 155155 212212 203203 000000 164170
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NN1616, 238242 147151 098102 061085 139151 132152 141161 165169 194294 191209 228248 156168
NN1718, 240240 147159 092096 067085 139147 148168 163165 155155 208218 195201 228228 168168
NN2121, 234256 147157 092102 079087 139147 152152 153155 169169 198206 191207 228242 170178
NN035, 240242 153161 098098 071085 139147 132148 155159 167169 178226 000000 228248 170172
NN047, 242252 155159 000000 067085 143155 148152 139155 155157 190190 197197 228258 164172
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NN3811, 000000 151157 098102 069069 141145 160160 155159 155155 000000 209209 228250 000000
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NN4016, 240244 147157 096098 067085 147151 148168 141141 165175 234238 197199 228260 166170
NN4219, 242250 153159 096110 081081 145145 132160 151155 155169 220282 195203 228228 168170

Pop 4

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LAN044, 240250 151157 096096 081081 143145 156168 157157 155169 206224 195209 228242 156164
LAN056, 240246 161161 092100 069081 139147 132132 141155 157169 222226 203211 256258 172174
LAN068, 240256 159161 094100 069075 145147 148164 141141 155167 218286 197213 262268 170172
LAN0812, 238240 147165 092100 067093 145147 148160 155159 157157 000000 201201 228230 154166
LAN0914, 000000 153157 092100 073093 141143 000000 141155 165165 000000 197205 228228 000000
LAN1118, 242246 155163 092094 000000 137143 160164 159161 155155 220220 191197 228250 164174
LAN1220, 238240 151161 096096 085085 145147 160164 139163 157157 220246 187197 228242 152164
LAN1322, 240242 147151 100102 089089 143145 148164 155155 155157 186220 205209 228228 170178
LAN2010, 240242 155159 094100 081085 147147 132148 159163 155167 196226 187209 266268 156170
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LAN232, 240242 153161 096096 081095 145145 132160 141159 000000 194194 201209 228240 166170
LAN2812, 236240 147157 094098 069081 139145 164164 141155 155169 194274 209211 228258 156156
LAN1541, 242248 159159 100100 085093 143147 160164 141141 155167 190270 195195 228230 156156
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LAN15912, 240242 153159 092100 089089 143145 132168 155159 155155 194294 187191 228262 166166
LAN16013, 240240 157161 092100 071081 143147 160160 155157 155155 234234 191197 228258 160166
LAN1619, 240242 151159 094100 085093 143145 160164 155163 155165 244248 195199 228228 156172
LAN16211, 240242 151155 100100 071095 143145 148164 155159 155157 216234 191197 228248 174176
LAN1664, 234240 147153 094100 069085 143143 148148 000000 155157 208208 193193 260260 164174
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IVI1713, 240250 151157 096100 081081 143145 156168 157157 155169 206224 195209 228240 156164
IVI1815, 242250 157165 096096 085085 143145 156160 139157 157169 186206 195197 228268 164174
IVI1917, 240248 151161 094100 085085 137145 160164 157159 157165 186234 187193 228228 164164
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IVI2121, 240242 147159 094094 085085 143145 148160 141163 155155 178246 197203 228230 156156
IVI2223, 000000 161161 096096 075075 143143 132148 155159 000000 220234 000000 228228 156164
IVI2314, 246256 151161 100102 069085 145145 132148 141163 155165 226242 193207 228228 166170
IVI249, 242244 147151 096100 069081 143145 164164 155159 155155 186210 193199 260268 156164

Pop 5

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RAB067, 240240 157161 092100 067113 145145 144156 157159 165169 190220 187203 228248 164166
RAB0914, 242256 157159 096100 085091 141141 132160 159159 159169 202222 191195 228248 154166
RAB155, 242244 153157 094102 081097 141141 132148 155157 157157 186226 191205 228228 156178
RAB167, 240240 157161 092100 067113 145145 144156 157159 165169 190220 187203 228248 164166
RAB1812, 242246 157161 096100 081089 141145 164164 155157 155155 212222 197203 228260 164164
RAB1914, 240250 151161 096098 085099 141145 156164 141153 157167 220278 195205 228240 164174
RAB2322, 240246 155159 096100 063073 141143 148164 153155 155169 190212 191201 228250 168170
RAB263, 236240 155165 096100 075137 139141 148148 159161 165167 260274 205205 228248 166166
RAB275, 242244 153157 094096 089089 139143 132156 155163 155159 202270 195195 228250 164170
RAB288, 242242 155161 098098 081089 139141 148148 139161 155167 212230 191201 228260 170178
RAB299, 242242 153153 094102 097123 139141 148156 161167 155157 194198 191191 228228 156178
RAB3012, 234242 147157 092096 135137 143143 132164 139155 155159 220230 195197 228260 170172
RAB3215, 242244 153157 094096 089091 139143 132156 155163 155159 202270 195195 228250 164170
RAB3317, 242242 151155 092094 071089 141145 148164 159159 163167 206206 191213 228228 154170
RAB3522, 242250 151153 098102 083095 141141 144156 155157 159165 186224 191213 252268 172172
RAB3623, 234242 147157 092096 135137 143143 132164 139155 155159 220230 195197 228260 170172

Pop 6

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LOA0110, 234246 147159 096100 075095 141145 144160 139141 155169 220224 203205 228228 156164
LOA0212, 240242 153161 094096 075085 143147 132156 141159 155155 202242 197201 228228 166170
LOA031, 238242 157163 096100 071071 143145 132160 155161 155155 242246 207209 260272 170178
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LOA079, 240242 155157 092096 071075 143145 000000 000000 000000 202212 195205 000000 170178
LOA0811, 240242 155161 092104 075105 143145 164164 159159 155159 202212 195203 228260 166170
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LOA1514, 240250 157157 092094 081123 139143 132148 139153 159159 194242 187193 228250 156164
LOA1615, 236242 155157 096100 075091 145145 160164 139141 157167 218230 201205 228228 164166
LOA339, 242242 157157 098100 123123 139143 148164 139159 155157 270274 201203 228228 164170
LOA3514, 248250 153159 092094 069093 143147 132132 141153 155169 190208 191205 242260 154172
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PBA117, 242242 159161 098102 085097 147155 156160 141141 163169 192206 197205 228228 164166
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PBA2422, 246246 159159 092094 071081 147147 160160 141159 167167 222222 000000 000000 156156
KES019, 240242 153159 090100 081081 139139 148164 139155 155167 190238 197209 258260 156178
KES0415, 240242 159161 090100 081081 143145 148156 157159 155155 210250 209211 228228 174178
KES0721, 242252 153159 098100 085089 145145 132164 139139 155167 194214 191201 228228 156164

KES0819, 240250 159159 096098 071097 143145 156160 155155 159165 242246 191197 258268 156164
MPA01, 000000 153155 094096 085091 141141 132160 141153 155165 194198 195195 000000 164164
MPA02, 250250 155155 094096 091097 143145 152152 000000 155165 194198 195195 000000 164166
MPA0319, 234242 157159 096096 081085 139141 132164 157159 155165 178216 203209 228230 156164

Pop 8

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WW47 , 234242 147155 096100 085089 145145 160164 139155 155169 216270 193197 228228 166172
WW59 , 240242 155157 096102 085087 141145 148164 139153 157161 202294 205205 228228 172176
WW611, 240242 151153 096100 087091 143145 132148 139141 155161 290294 197205 228258 160164
WW816, 240246 155157 098100 075075 143145 132148 139141 155155 182194 195197 228248 166170
WW917, 238242 151153 096100 091091 143145 132148 139141 155155 290294 197205 228258 160164
WW1019, 242246 147157 096100 075097 141145 164164 139157 171173 220252 195205 228250 166166
NZA19, 242246 151157 098100 067087 143143 160164 141159 155167 274274 195199 228260 170170
NZA211, 242256 157167 000000 067081 141143 132160 139159 159167 260290 195195 228260 170172
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NZA416, 240242 157161 098100 067067 141143 148160 139159 159167 274282 195197 228260 166170
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NZA721, 240242 157161 098100 067067 141143 148160 139159 159167 274282 195195 228260 166170
NZA823, 234242 155157 094098 067075 141143 148164 141159 155157 274286 199201 250264 170174
NZA91, 242250 155161 098100 067085 143145 148168 159163 155159 294294 193195 228228 156170
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IFK39, 242242 153155 094098 073089 143145 132156 139159 155167 212290 197197 228250 170172
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RIO323, 242242 155157 096096 129129 141143 152152 157159 159169 222286 191201 228250 156172
RIO41, 240240 155157 096102 085091 145145 148164 159159 155165 216286 193193 228250 164170
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RIO65, 234240 157157 096096 089089 141145 160164 159159 155167 274290 195195 228258 164170
RIO77, 000000 000000 092098 081095 143145 148160 141157 155167 216234 191205 228258 166170
RIO89, 234234 157159 092098 081095 143145 148160 141157 155167 216234 191205 228258 166170
RIO911, 234240 155157 096100 089089 141145 160164 159159 155167 274290 195197 228258 164170
RIO1013, 240250 153155 094098 071085 143145 148160 139159 155155 190224 195207 228228 166176
RIO1217, 240240 155157 096102 085091 145145 164164 159159 155165 216286 193193 228228 164170
RIO1320, 234250 149161 092098 091093 141145 148160 139159 155171 224290 191205 228228 166170
RIO1421, 234234 153161 092100 061091 141145 148160 157159 171171 282290 205209 228240 166166
RIO1523, 234242 155157 096096 071089 143145 148160 139159 155167 290290 195195 228256 000000
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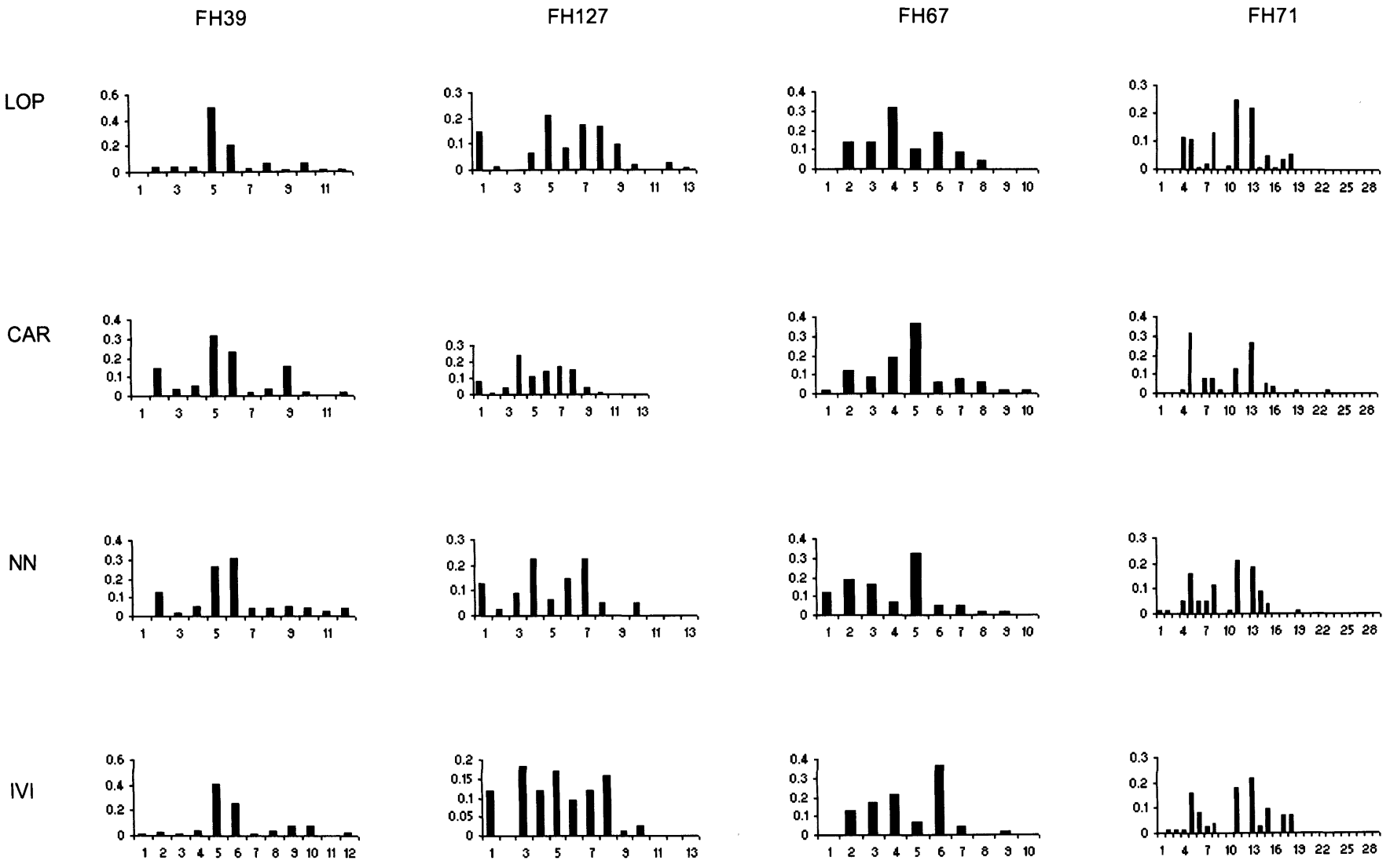
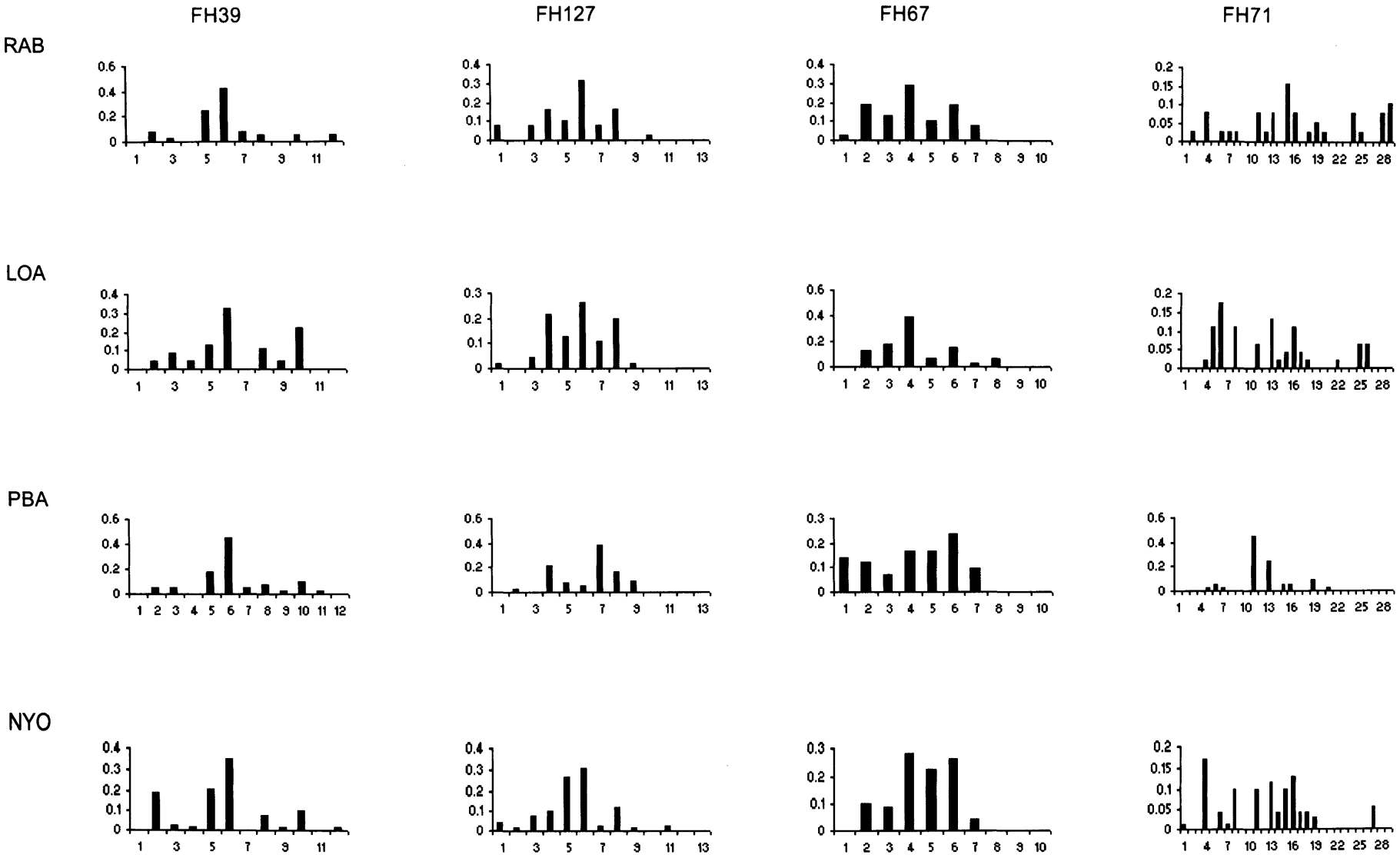
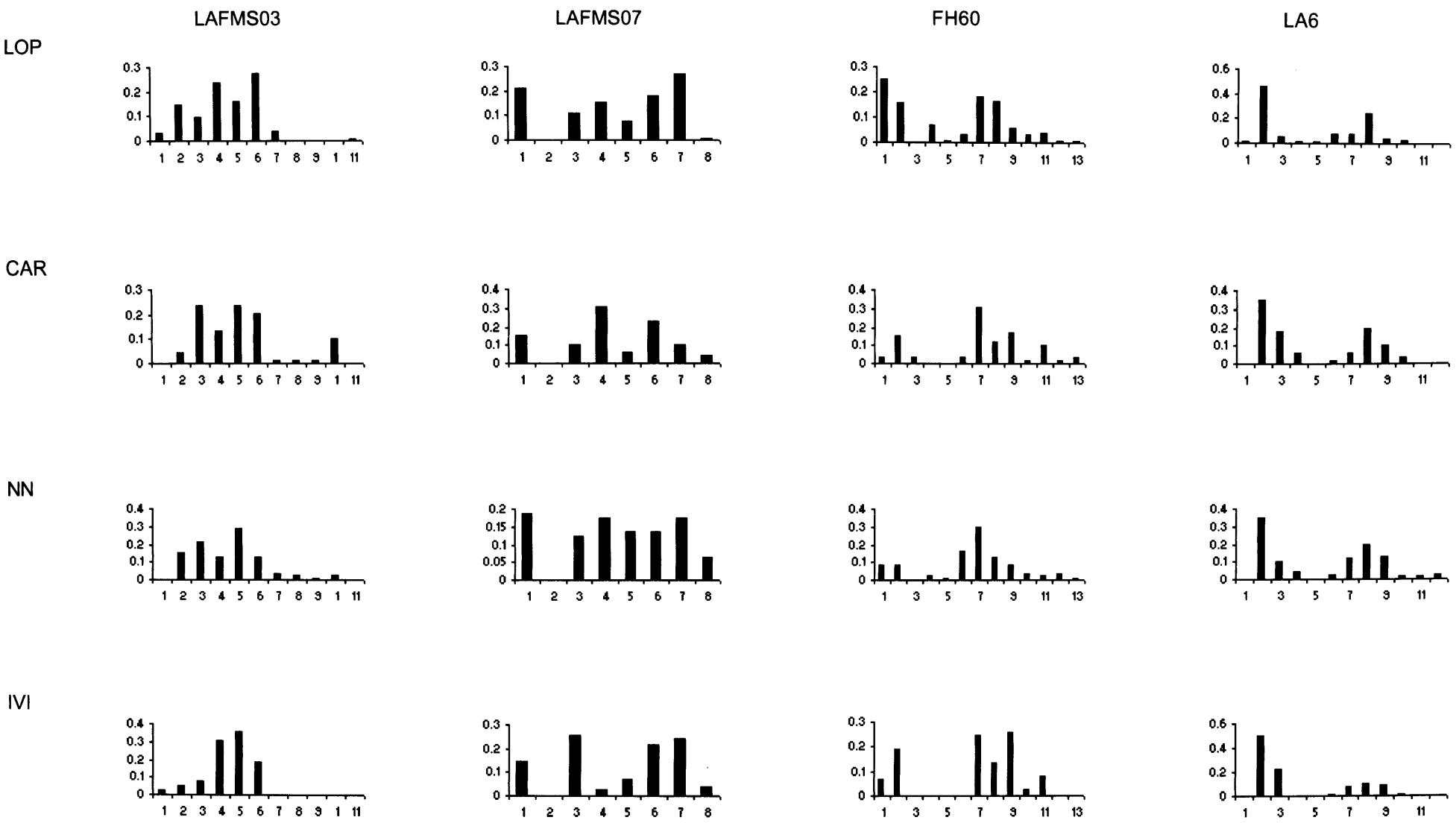
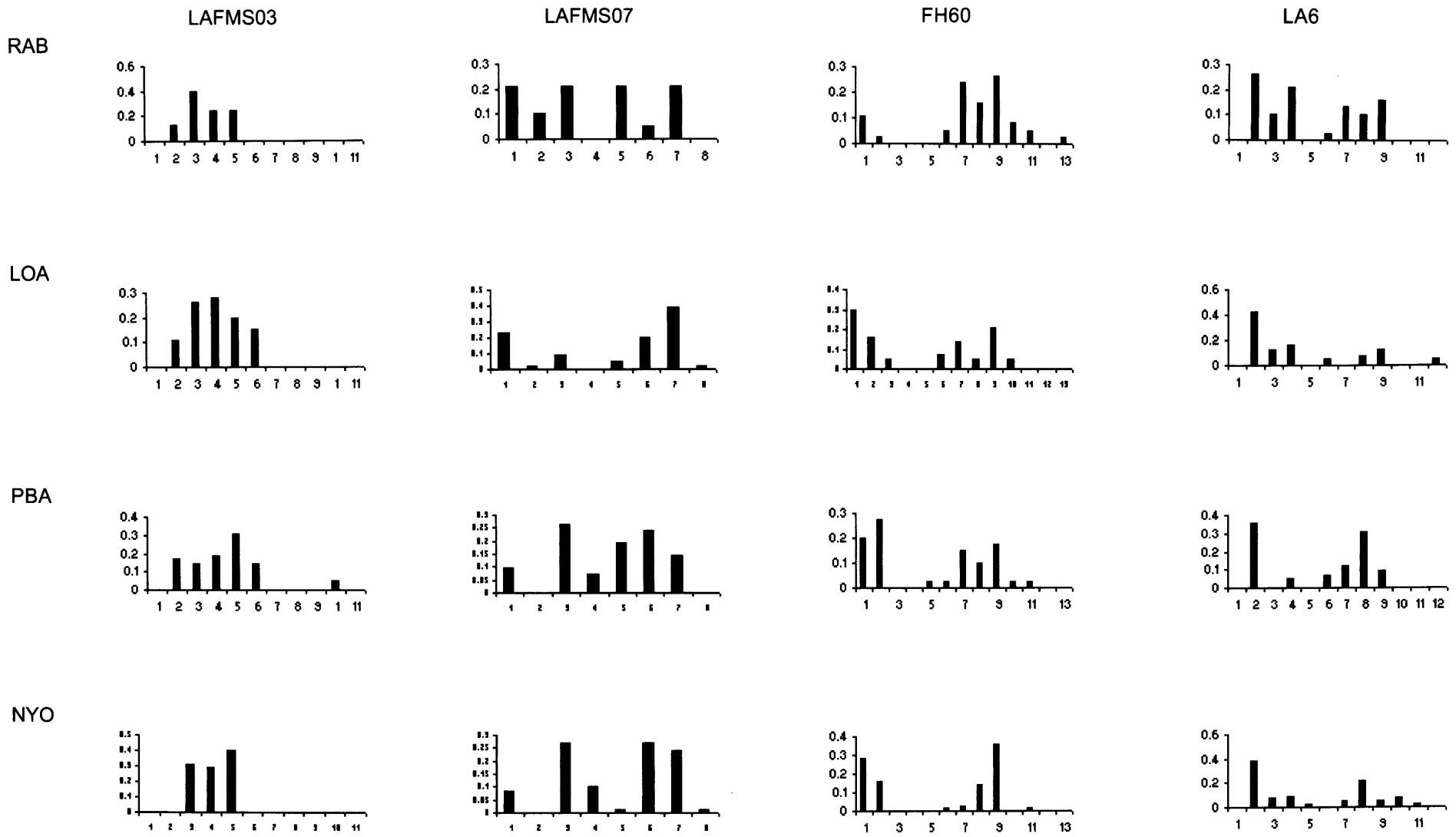
Appendix 6. Allele frequency distribution by locus and population.




FH39
FH127
FH67
FH71
RAB
LOA
PBA
NYO


LAFMS03
LAFMS07
FH60
LA6
LOP
CAR
NN
IVI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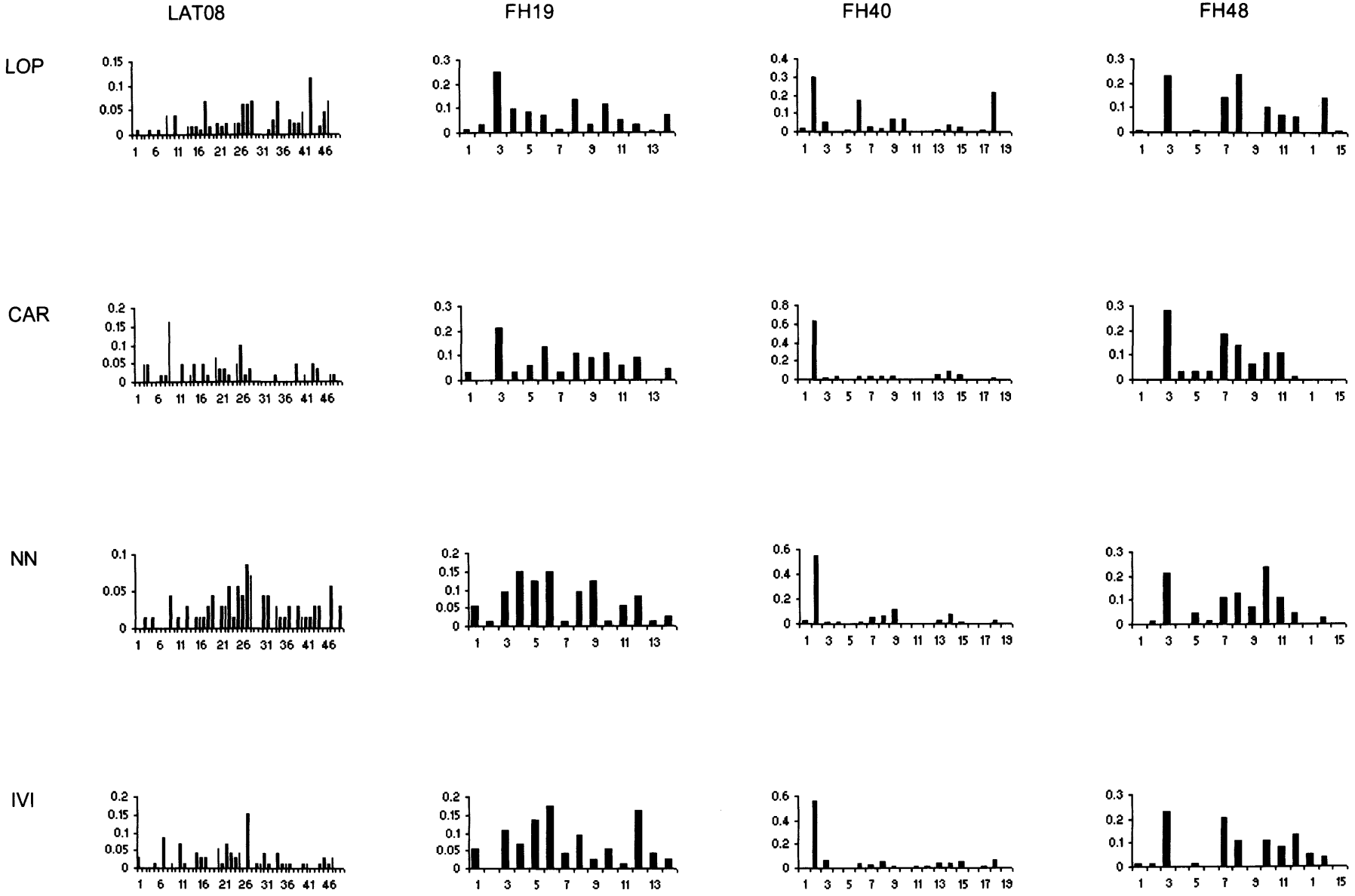
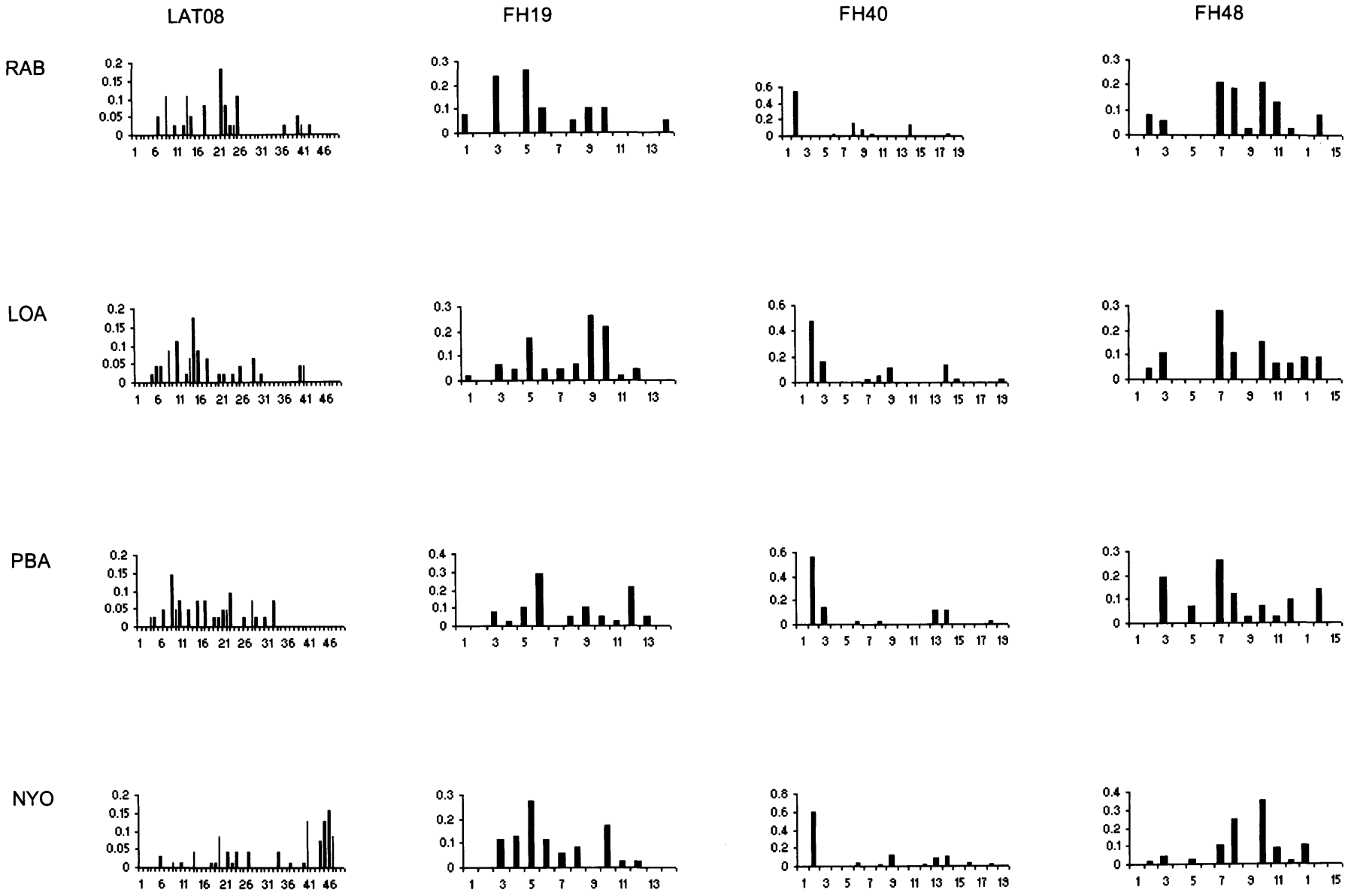
LAFMS07
FH60
LA6
RAB
LOA
PBA
NYO


LAT08
FH19
FH40
FH48
LOP
CAR
NN
IVI


LAT08
FH19
FH40
FH48
RAB
LOA
PBA
NYO

Appendix 7.

Summary statistics null allele estimates

No evidence for scoring error due to stuttering.
No evidence for large allele dropout.
Null alleles may be present at this locus, as is suggested by the general excess of homozygotes for most allele size classes.

LOP

Locus	Null Present	Oosterhout	Chakraborty	Brookfield 1	Brookfield 2
FH39	no	-0.0031	-0.0154	-0.013	0.1234
FH127	yes	0.0638	0.0721	0.062	0.1015
FH67	no	0.0165	0.0193	0.017	0.017
FH71	no	0.0246	0.0304	0.0266	0.0746
LAFMS03	no	0.0495	0.0495	0.042	0.042
LAFMS07	no	-0.0569	-0.05	-0.0472	0.0317
FH60	no	0.0028	0.0031	0.0028	0.0028
LA6	no	0.0062	0.0052	0.0044	0.0044
LAT08	yes	0.0968	0.1074	0.0947	0.2669
FH19	yes	0.0541	0.0564	0.0496	0.2187
FH40	no	0.0545	0.058	0.0491	0.2045
FH48	no	-0.0724	-0.0647	-0.0626	0.1499

Several loci show evidence for a null allele.
This population is possibly in Hardy Weinberg equilibrium with loci FH127, LAT08, FH19, showing signs of a null allele.

CAR

Locus	Null Present	Oosterhout	Chakraborty	Brookfield 1	Brookfield 2
FH39	no	0.0701	0.0642	0.0535	0.178
FH127	no	0.026	0.0258	0.0231	0.096
FH67	yes	0.0807	0.09	0.0727	0.0727
FH71	no	0.1026	0.1045	0.083	0.2037
LAFMS03	no	-0.0194	-0.0153	-0.014	0
LAFMS07	no	0.0053	-0.0006	-0.0006	0.0842
FH60	no	0.0433	0.0484	0.0415	0.1147
LA6	yes	0.1343	0.1552	0.1189	0.1822
LAT08	yes	0.0774	0.08	0.0714	0.2172
FH19	no	-0.0169	-0.0125	-0.0119	0.0635
FH40	no	0.001	-0.0077	-0.0057	0.2154
FH48	no	0.0258	0.0237	0.0211	0.1452

Several loci show evidence for a null allele.
This population is possibly in Hardy Weinberg equilibrium with loci FH67, LA6, LAT08, showing signs of a null allele.

NN

Locus	Null Present	Oosterhout	Chakraborty	Brookfield 1	Brookfield 2
FH39	no	-0.0737	-0.0637	-0.0616	0.0773
FH127	no	-0.0743	-0.0654	-0.0643	0
FH67	no	0.0137	0.0157	0.0138	0.1781
FH71	yes	0.1535	0.1804	0.1417	0.1417
LAFMS03	no	0.0566	0.0565	0.0486	0.0486

LAFMS07	no	0.0056	0.0095	0.0086	0.0086
FH60	no	0.0089	0.0172	0.0156	0.0156
LA6	no	0.048	0.0393	0.0337	0.0337
LAT08	yes	0.0634	0.0677	0.0621	0.229
FH19	yes	0.073	0.0798	0.0695	0.1714
FH40	no	-0.0633	-0.0476	-0.0398	0.1249
FH48	no	0.01	0.0114	0.0105	0.1637

Several loci show evidence for a null allele.
This population is possibly in Hardy Weinberg equilibrium with loci FH71, LAT08, FH19, showing signs of a null allele.

IVI

Locus	Null Present	Oosterhout	Chakraborty	Brookfield 1	Brookfield 2
FH39	no	-0.1865	-0.1276	-0.1255	0.0897
FH127	no	0.0139	0.0144	0.0131	0.0131
FH67	no	0.0419	0.0485	0.0403	0.0403
FH71	yes	0.1289	0.1499	0.1209	0.1723
LAFMS03	no	-0.0311	-0.0273	-0.0239	0
LAFMS07	no	-0.0241	-0.0193	-0.0176	0.0659
FH60	no	-0.0196	-0.0129	-0.0117	0.0681
LA6	no	0.0169	0.018	0.0143	0.1511
LAT08	yes	0.0471	0.0478	0.0443	0.1357
FH19	no	0.0302	0.0329	0.03	0.0891
FH40	no	-0.0119	0.007	0.0055	0.0055
FH48	no	0.0123	0.0096	0.0088	0.0773

Several loci show evidence for a null allele.
This population is possibly in Hardy Weinberg equilibrium with loci FH71, LAT08, showing signs of a null allele.

RAB

Locus	Null Present	Oosterhout	Chakraborty	Brookfield 1	Brookfield 2
FH39	no	0.0175	0.0239	0.0199	0.0199
FH127	no	-0.0791	-0.0646	-0.0625	0
FH67	no	-0.0885	-0.0709	-0.0686	0
FH71	no	-0.0114	-0.013	-0.0126	0
LAFMS03	no	0.1146	0.138	0.0998	0.0998
LAFMS07	no	-0.0104	-0.0106	-0.0096	0
FH60	no	0.0061	0.0031	0.0028	0.0028
LA6	no	-0.0387	-0.0345	-0.0323	0
LAT08	no	-0.0156	-0.013	-0.0126	0
FH19	no	0.035	0.0349	0.0304	0.0304
FH40	no	-0.2249	-0.124	-0.1106	0
FH48	no	0.0136	0.0155	0.014	0.014

No loci show evidence for a null allele.
This population is probably in Hardy Weinberg equilibrium.

LOA

Locus	Null Present	Oosterhout	Chakraborty	Brookfield 1	Brookfield 2
FH39	no	-0.0593	-0.0557	-0.0526	0
FH127	no	0.0359	0.0299	0.0257	0.0257
FH67	no	-0.0898	-0.0803	-0.0757	0
FH71	no	0.0827	0.0881	0.0765	0.0765
LAFMS03	no	-0.014	-0.0119	-0.0105	0
LAFMS07	no	0.018	0.0148	0.0126	0.1321
FH60	no	-0.0453	-0.0411	-0.0387	0.0839
LA6	no	0.0241	0.0557	0.0451	0.2325

LAT08	no	0.0399	0.0382	0.0352	0.0352
FH19	no	-0.0566	-0.0512	-0.0498	0
FH40	yes	0.149	0.1566	0.1066	0.2156
FH48	no	-0.0285	-0.0308	-0.0291	0

One locus shows evidence for a null allele.
This pop ulation is possibly in Hardy Weinberg equilibrium with locus FH40, showing signs of a null allele.

PBA

Locus	Null Present	Oosterhout	Chakraborty	Brookfield 1	Brookfield 2
FH39	no	-0.0036	0.0351	0.0299	0.1606
FH127	no	0.0585	0.0688	0.0568	0.0568
FH67	no	-0.0224	-0.0228	-0.0213	0
FH71	no	-0.0191	-0.0209	-0.0187	0
LAFMS03	no	0.0965	0.1086	0.0857	0.0857
LAFMS07	no	-0.0057	-0.0024	-0.0022	0
FH60	no	0.053	0.0531	0.0453	0.1686
LA6	no	0.0039	0.0078	0.0067	0.0067
LAT08	yes	0.0976	0.107	0.0931	0.0931
FH19	no	0.0676	0.0789	0.0667	0.2681
FH40	no	0.0612	0.0326	0.025	0.343
FH48	no	0.0804	0.0904	0.0749	0.0749

One locus shows evidence for a null allele.
This population is possibly in Hardy Weinberg equilibrium with locus LAT08, showing signs of a null allele.

NYO

Locus	Null Present	Oosterhout	Chakraborty	Brookfield 1	Brookfield 2
FH39	no	-0.0074	0.0009	0.0008	0.0866
FH127	no	-0.0905	-0.0759	-0.0733	0.0417
FH67	no	-0.0768	-0.07	-0.0664	0.0476
FH71	yes	0.0973	0.1074	0.092	0.092
LAFMS03	no	-0.1294	-0.1086	-0.0966	0
LAFMS07	no	-0.0559	-0.0447	-0.0408	0
FH60	no	-0.0902	-0.082	-0.0763	0
LA6	no	-0.062	-0.0491	-0.0449	0
LAT08	no	0.0186	0.0192	0.018	0.018
FH19	no	0.0718	0.075	0.0639	0.0639
FH40	no	-0.1264	-0.082	-0.0681	0
FH48	no	-0.0245	-0.0168	-0.015	0.0756

One locus shows evidence for a null allele.
This population is possibly in Hardy Weinberg equilibrium with locus FH71, showing signs of a null allele.

Appendix 8.

Tests of linkage disequilibrium (LD). LD was measured using the correlation coefficient. The significance at $P < 0.05$ by pair of loci and for each population is represented with *. NS = non significant.

Locus 1	Locus 2	LOP	CAR	NN	IVI	RAB	LOA	PBA	NYO
FH39	FH127	*	*	*	*	*	*	*	*
FH39	FH67	*	NS	NS	NS	*	NS	*	*
FH39	FH71	*	NS	*	NS	*	*	*	*
FH39	LAFMS03	*	NS	NS	NS	*	NS	*	NS
FH39	LAFMS07	*	*	*	*	*	*	*	NS
FH39	FH60	*	*	NS	*	*	*	*	NS
FH39	LA6	NS	*	NS	NS	*	*	NS	NS
FH39	LAT08	*	*	*	*	*	*	*	*
FH39	FH19	*	*	NS	*	*	NS	*	*
FH39	FH40	*	NS	NS	*	*	*	*	NS
FH39	FH48	NS	NS	*	NS	*	*	*	NS
FH127	FH67	*	*	NS	NS	*	NS	*	NS
FH127	LAFMS03	*	NS	*	NS	*	*	NS	NS
FH127	LAFMS07	*	NS	NS	NS	*	*	*	NS
FH127	FH60	*	*	NS	*	*	NS	*	NS
FH127	LA6	*	NS	*	*	*	*	*	*
FH127	LAT08	*	*	*	*	*	*	*	*
FH127	FH19	*	*	*	NS	*	*	*	*
FH127	FH40	*	NS	NS	*	NS	*	*	NS
FH127	FH48	*	NS	NS	NS	*	NS	*	NS
FH67	FH71	*	*	*	*	*	NS	*	*
FH67	LAFMS03	*	*	NS	*	*	NS	*	NS
FH67	LAFMS07	*	*	*	*	NS	NS	*	NS
FH67	FH60	*	*	*	NS	NS	*	NS	NS
FH67	LA6	*	*	NS	*	*	*	*	NS
FH67	LAT08	*	*	*	*	*	NS	*	*
FH67	FH19	*	*	*	*	*	NS	*	*
FH67	FH40	*	NS	NS	NS	NS	NS	NS	NS
FH67	FH48	*	*	NS	*	*	NS	*	NS
FH71	LAFMS03	*	NS	*	*	*	*	*	*
FH71	LAFMS07	*	*	*	*	*	*	*	*
FH71	FH60	*	*	*	*	*	NS	*	NS
FH71	LA6	*	NS	*	*	*	*	*	*
FH71	LAT08	*	*	*	*	*	*	*	*
FH71	FH19	*	*	*	*	*	NS	*	*
FH71	FH40	*	NS	*	NS	*	*	*	*
FH71	FH48	*	NS	*	*	*	*	*	*
LAFMS03	LAFMS07	NS	*	*	NS	*	NS	*	NS
LAFMS03	FH60	*	*	*	NS	*	NS	*	NS
LAFMS03	LA6	*	NS	*	*	*	*	*	*
LAFMS03	LAT08	*	NS	*	NS	*	*	*	NS
LAFMS03	FH19	*	NS	*	NS	*	NS	*	*
LAFMS03	FH40	NS	NS	*	*	*	NS	*	NS

LAFMS03	FH48	*	*	NS	NS	*	NS	*	NS
LAFMS07	FH60	NS	NS	*	*	*	NS	*	*
LAFMS07	LA6	NS	*	NS	NS	*	NS	NS	*
LAFMS07	LAT08	*	*	*	*	*	*	*	*
LAFMS07	FH19	*	NS	*	NS	*	NS	*	*
LAFMS07	FH40	*	NS	NS	NS	NS	NS	NS	NS
LAFMS07	FH48	*	NS	NS	NS	*	NS	*	NS
FH60	LA6	*	*	*	NS	*	*	*	*
FH60	LAT08	*	*	*	*	*	*	*	*
FH60	FH19	*	*	*	NS	*	*	*	*
FH60	FH40	*	NS	NS	NS	*	NS	*	NS
FH60	FH48	*	NS	*	NS	*	NS	*	NS
LA6	LAT08	*	*	*	NS	*	*	*	*
LA6	FH19	*	*	*	NS	*	*	*	*
LA6	FH40	*	*	NS	NS	NS	*	NS	NS
LA6	FH48	NS	*	*	NS	*	*	*	*
LAT08	FH19	*	*	*	*	*	NS	*	*
LAT08	FH40	*	NS	*	*	*	*	*	*
LAT08	FH48	*	*	*	NS	*	*	NS	*
FH19	FH40	*	NS	*	*	*	*	NS	*
FH19	FH48	*	*	NS	*	*	NS	*	*
FH40	FH48	*	NS	NS	NS	*	*	NS	*

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Complex phylogeographic history of central African forest elephants and its implications for taxonomy

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Abstract

Background: Previous phylogenetic analyses of African elephants have included limited numbers of forest elephant samples. A large-scale assessment of mitochondrial DNA diversity in forest elephant populations here reveals a more complex evolutionary history in African elephants as a whole than two-taxon models assume.

Results: We analysed hypervariable region I of the mitochondrial control region for 71 new central African forest elephants and the mitochondrial cytochrome b gene from 28 new samples and compare these sequences to other African elephant data. We find that central African forest elephant populations fall into at least two lineages and that west African elephants (both forest and savannah) share their mitochondrial history almost exclusively with central African forest elephants. We also find that central African forest populations show lower genetic diversity than those in savannahs, and infer a recent population expansion.

Conclusion: Our data do not support the separation of African elephants into two evolutionary lineages. The demographic history of African elephants seems more complex, with a combination of multiple refugial mitochondrial lineages and recurrent hybridization among them rendering a simple forest/savannah elephant split inapplicable to modern African elephant populations.

Background

The taxonomic status of the African elephant (*Loxodonta africana*) has been debated since the turn of the 20th century [1] and up to 22 subspecies have been described [2]. However, modern taxonomy refers to two types, with their names reflecting the habitat in which they are found, namely the larger savannah (*Loxodonta africana africana*) (Blumenbach 1797) and the smaller forest (*Loxodonta africana cyclotis*) (Matschie 1900) elephants. It has become increasingly established in the literature that forest and savannah elephants are distinct species (*L. Africana and L. cyclotis*) [3-7], with recent publications considering their

datasets in the light of this concept. The most persuasive genetic basis for a two-taxon model originates from a series of studies exploring patterns of differentiation at nuclear loci, culminating in a study using male inherited *Y-chr*, andbi-parentally inherited *X-chr* sequences [6] that concluded "*there was a deep and almost complete separation between African forest and African savannah elephants.*" In this study, divergent nuclear DNA sequences segregated with either forest or savannah elephant morphological types. There were, however, a number of exceptions, including a forest elephant from Garamba in the Democratic Republic of Congo (DRC, where forest and savannah populations are sympatric) that had nuclear sequences typical of savannah elephants and two savannah elephants from Cameroon (at the limit of the forest-savannah transition zone) that had nuclear sequences typical of forest elephants [6]. The study estimated the divergence between the savannah and forest elephants to be 3 million years. The two-taxon argument has also been used to explain data from two nuclear microsatellite DNA [5,7] and one morphological study [8,9]. However, subsequently Debruyne [10] performed a morphometric analysis of museum elephant skulls, and found evidence for a continuum between two morphotypes, suggesting that, despite historical separation that promoted subdivision, these two forms freely interbreed wherever their ranges intersect.

Molecular studies using mitochondrial (mt) DNA [10,11] including data from the study by Roca *et al* [6] have pointed to a more complex scenario for African elephants. Debruyne [10] examined several thousand base pairs of mtDNA from elephants across Africa and although he also reported two highly divergent molecular clades, these did not conform to the morphological delineations of *cyclotis* and *africana*. He interpreted these results as a consequence of incomplete isolation between forest and savannah African elephant populations, followed by recurrent and ongoing introgression between the two forms. Roca *et al.* [6] obtained very similar mitochondrial results but explained the non-concordance between mitochondrial and nuclear markers as a result of cytonuclear genomic disassociation such that the mitochondrial tree did not reflect the species tree. The mtDNA results observed were explained as having arisen during episodes of backcrossing between successive generations of savannah males with forest females, leading to half of extant savannah elephants surveyed possessing 'forest' typical mitochondrial haplotypes but almost exclusively 'savannah' nuclear X and Y-chromosomal DNA. Eggert *et al.* [11] (in addition to Nyakaana et al.'s mitochondrial sequences [12]) included samples from west Africa and found a more complex picture using mtDNA and nuclear microsatellites, suggesting that western savannah and forest elephants formed a potential third *Loxodonta* taxonomic unit. Finally, Roca *et al.*[13]recently revisited the question with a statistical re-analysis of eight morphological and genetic datasets (nuclear and mitochondrial) including their own and those of Eggert *et al.*[11] and Debruyne [10] and reconfirmed their initial interpretation of a two taxon model with cyto-nuclear genomic dissociation.

The above-mentioned studies largely share a pronounced lack of forest elephant data. The nuclear DNA studies [4,11] featured limited sampling from central African forest elephants. Despite describing a narrow hybrid zone between the two elephant types, only one population located in this zone (Garamba, (DRC)) was included and none from elsewhere in DRC or from west central Africa were examined. Elsewhere, Debruyne [10] included elephants from across DRC in his study but was again limited by sample size. The study by Eggert *et al.* [11] was limited by the inclusion of only two populations of Central African forest elephants, both from the edge of the forest range in Cameroon which may conceivably have influenced their conclusion of the genetic uniqueness of forest and western elephants. To date, no study has addressed the partitioning of genetic diversity in the equatorial forests of Africa. Further, the potential effect of Pleistocene forest refugia was partially addressed by Eggert *et al.*[11] and also previously reported as having a major influence on large mammal distribution and range dynamics [14-18] has yet to be addressed in African elephants. Here we report results from the most extensive sample of forest elephants to date, from the core of their range, and compare these results with previously published mitchondrial DNA sequences for savannah elephants from east and southern Africa and populations from west Africa and DRC.

We examined the phylogeographic history, population structure and past demography of African elephants using patterns of molecular diversity for the mtDNA control region and cytochrome b gene. Since mtDNA is maternally inherited, this marker provides a female-biased view of population history and structure. We included the most variable mtDNA segment, the hypervariable region 1 (HVR1) of the control region since it has a high rate of nucleotide change, allowing recently diverged lineages to be distinguished [19-21]. This segment is equivalent to data previously published by Eggert *et al.* [11] and Debruyne [10], allowing us to examine forest elephant sequences within the context of a sample set with the largest geographic coverage. We could not use Roca's mtDNA sequences as he studied a different fragment (ND5 instead of control region).

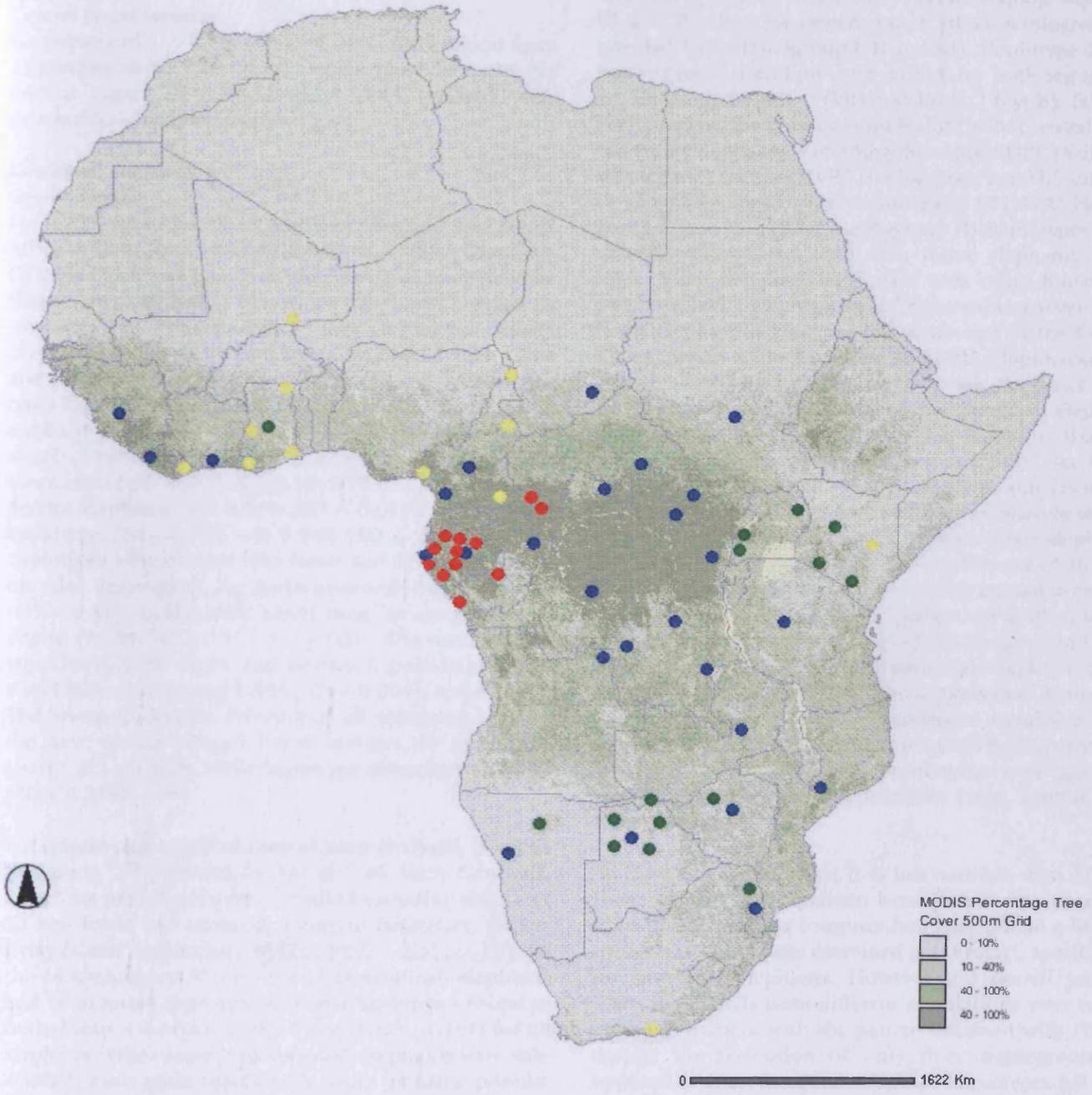

Figure 1
Map of Africa showing approximate sampling sites from previous mtDNA studies combined with those from this study. The green, yellow and blue dots are sampling sites from Nyakaana et al. [12], Eggert *et al.* [11] and Debruyne [10], respectively. The red dots are the sites from this study.

Results

Central forest samples

We sequenced 316 bp of HVR1 of the control region from 71 samples and 396 bp of the cytochrome b from 28. No nuclear copies of mitochondrial DNA (*Numts*) were detected for either sequence.

Combined sequences

Genetic diversity

For HVR1, we analysed 189 sequences from 66 sites across Africa in both forest and savannah elephants (Figure 1). Of these 102 were from forest elephants (71 samples from the present study and 31 from Genbank) and 87 savannah elephants (all from Genbank). The combined dataset comprised eighty-eight haplotypes (33 and 51 from forest and savannah elephants, respectively) and four haplotypes found in both types. Of the 21 central African forest elephant haplotypes identified in this study, 17 were novel (Genbank accessions EU096114 - EU096130). Mean nucleotide diversity (π) for HVR1 sequences for all African elephants was 0.030 (SD = 0.015), while mean haplotype diversity (h) was 0.985 (SD = 0.003). When haplotypes were divided into forest and savannah, based on prior designation, the forest population π was 0.022 (SD = 0.11), significantly lower than for savannah elephants (0.034, SD = 0.017; $p < 0.001$). The mean haplotype diversity for forest and savannah populations was 0.960 (SD = 0.007) and 0.986 (SD = 0.004), respectively. The lowest nucleotide diversity of all groupings was for the new central African forest samples in this study (0.013, SD = 0.007), while haplotype diversity was 0.947 (SD = 0.009).

For cytochrome *b*, 100 sequences were analysed, 28 from this study, 27 provided by SN and 45 from Genbank. Forty-four haplotypes were identified including three and 22 new forest and savannah elephant sequences, respectively (Genbank accessions EU115995 - EU116019). Of the 44 haplotypes, 32 were found in savannah elephants and 10 in forest elephants, with two haplotypes found in both. Mean π for cytochrome *b* was 0.023 (0.012) for all elephants. When forest and savannah elephants were subdivided, π was again significantly lower for forest populations (0.009, SD = 0.005) than for savannah populations (0.026, SD = 0.013; $p < 0.001$). These results contrast with the study of Roca *et al.* (2005) who reported 15 haplotypes for 281 elephants at the mitochondrial ND5 locus and described low genetic diversity as being typical for savannah elephants.

Population structure

The median joining networks for the HVR1 and cytochrome *b* sequences (Figures 2 and 3, respectively), exhibit patterns consistent with a complex demographic history. The HVR1 pattern is more complex (comprising four haplogroups – here labelled HVR1 Haplogroup I, II, III and IV) than for cytochrome *b* (three haplogroups - labelled Cytb Haplogroup I, II and III). Haplotype designations for this and previous studies for both sequences are found in Table 1 (HVR) and Table 2 (cyt b). For the HVR1 region, the most obvious feature is that central African forest elephants (excluding those from DRC) fall into two separate groups (HVR1 Haplogroups I and II) with little geographic structuring, consisting of 19 (HVR1 Haplogroup I) and 20 (HVR1 Haplogroup II) haplotypes with variable frequencies. Only two forest elephants from DRC, share the same haplotype with other forest elephants in HVR1 Haplogroup II. The remaining seven DRC forest elephant haplotypes (all south-east of the Congo River), group with sequences in HVR1 Haplogroup III (which additionally comprises savannah elephants from eastern and southern Africa and one savannah elephant from Cameroon). The other striking feature is that for West African elephants (from Eggert et al 2002, see Table 1 for haplotype designations), both forest and savannah types possess haplotypes found almost exclusively within the same haplogroup as central African forest elephants (HVR1 Haplogroups I and II). Twenty-five out of 26 haplotypes from west Africa are more closely related to central Forest elephants from Gabon, Congo and CAR. A single western savannah sequence (H15) can be found in HVR1 Haplogroup IV grouping with savannah elephants from eastern, southern and central Africa. Analysis of Molecular Variance (AMOVA) of HVR1 sequences revealed a non-significant (p = 0.065) genetic structure (18.62% variation among populations) when populations were grouped according to geographic distribution (west, central, east and southern Africa).

As expected, Cytochrome b is less variable than HVR1. However, direct comparison between patterns obtained from both regions is compromised here due to a lack of equivalent individuals examined at both loci, specifically for savannah elephants. However the overall pattern when individuals from different populations were examined is consistent with the pattern obtained with HVR1, despite the resolution of only three haplogroups as opposed to four. Savannah elephant haplotypes fall into two distinct haplogroups (Cyt b Haplogroup II and III) as do forest elephant haplotypes (Cyt b Haplogroups I and II). Cytochrome b Haplogroup II, which is divided into two haplogroups for HVR1, is characterised by a network structure in which forest and savannah elephant samples are not overlaid (see Figure 3). Again all western elephants, both forest and savannah, cluster with central African forest elephants (Cyt b Haplogroup I).

Demographic history

When HVR1 sequences from forest and savannah elephants were examined separately, Fu's *Fs* was -14.2954

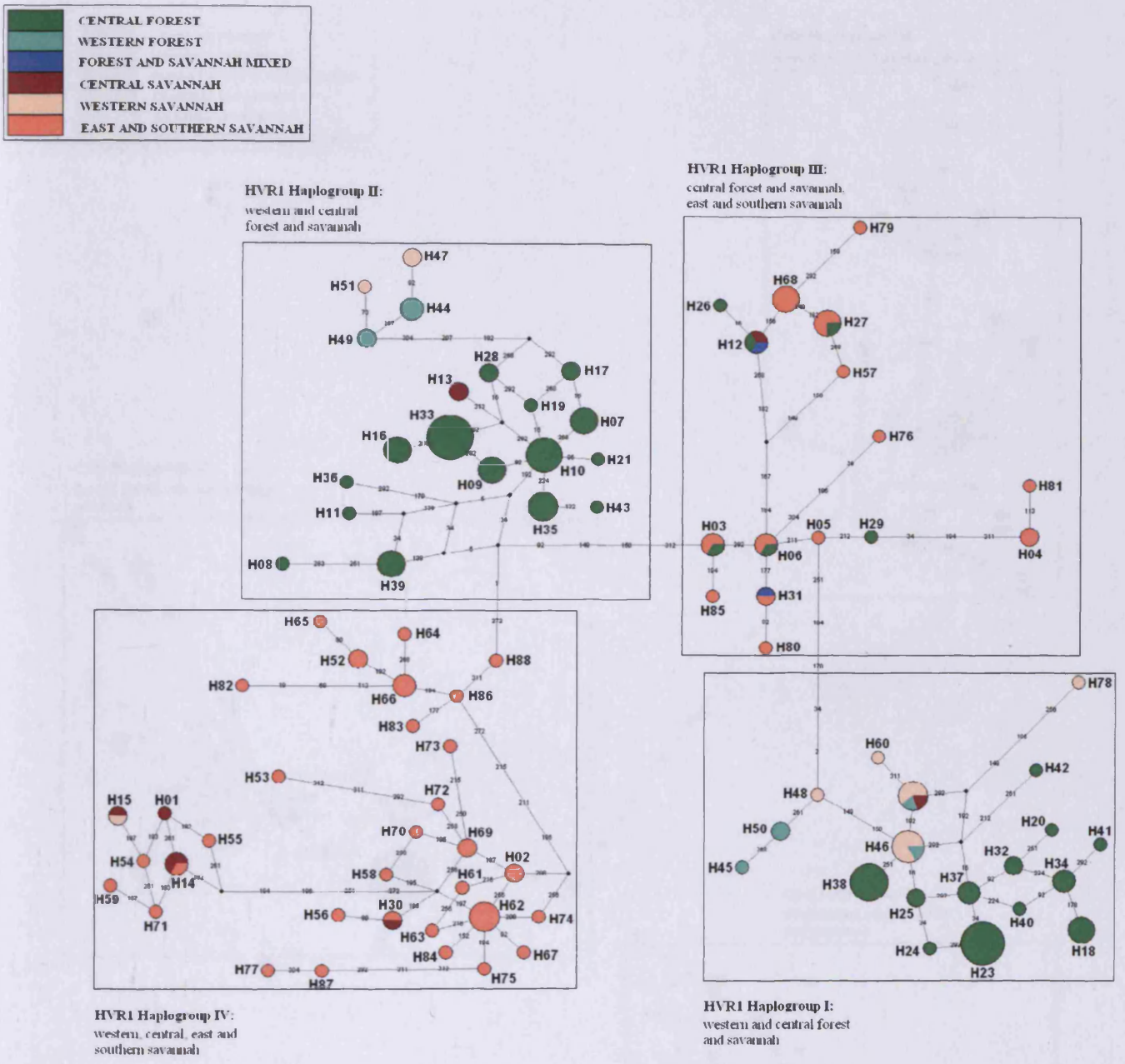

Figure 2

Median-joining networks for African elephants HVR1 mtDNA haplotypes. Circle size is proportional to the haplotype frequency. The numbers on the connecting line determine the number of substitutions estimated by NETWORK V.4. 1. 1. 1. The entire list of haplotypes for HVR1 MJN can be found in **Table 1**.

($P = 0.0021$) and -24.4427 ($P < 0.0001$), respectively. Although significant values can indicate historical population expansion, the multimodal pattern (Figure 4) for the forest elephant groups suggests that these populations encompass several subgroups as indicated in the networks. When we examined each haplogroup separately for signatures of demographic change (Table 3), a smooth and predominantly unimodal pattern was observed for HVR1 Haplogroup I, indicating a recent demographic expansion (Figure 5), while HVR1 Haplogroups II, III and IV were more complex, including the presence of some divergent haplotypes.

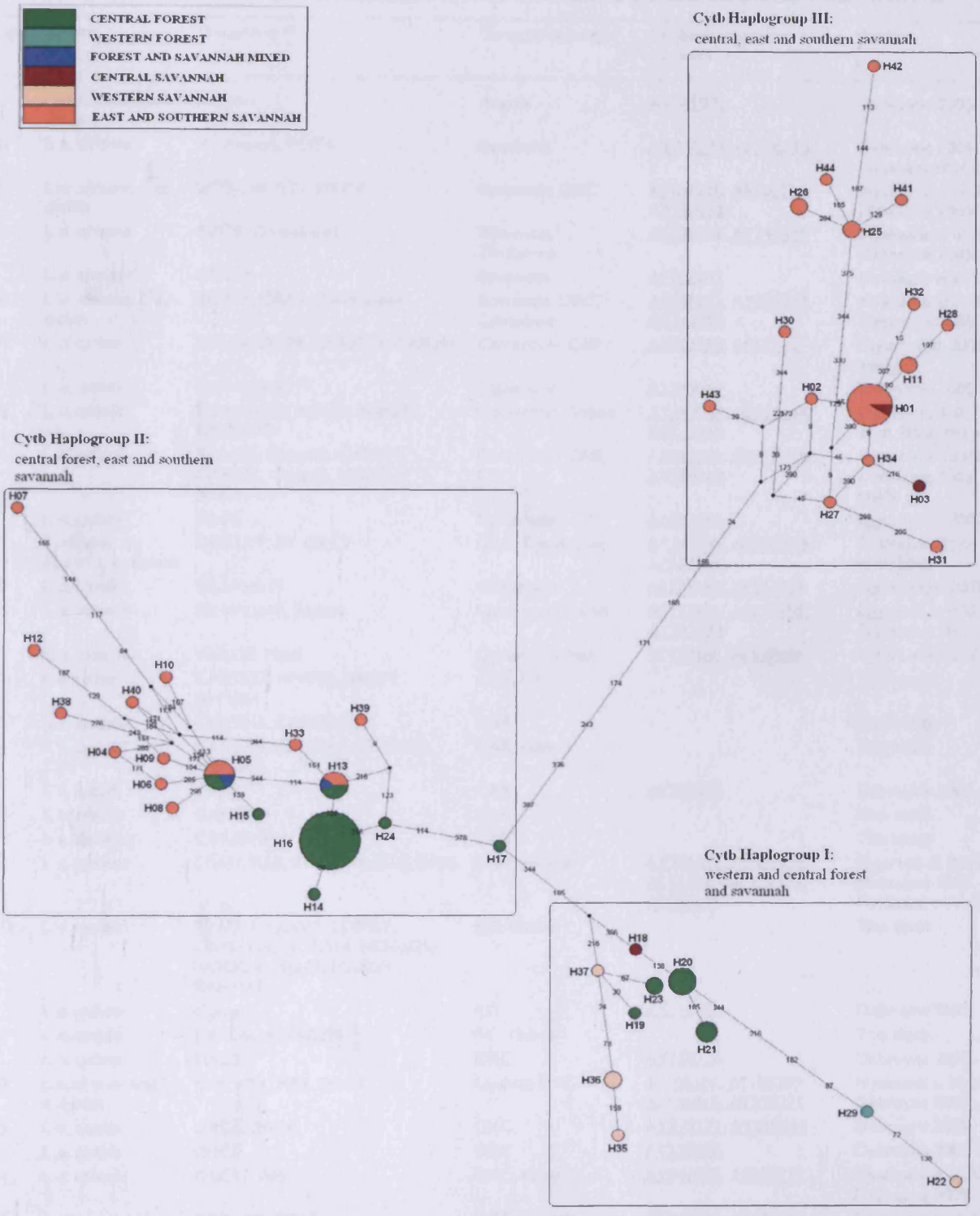

Figure 3

Median-joining networks for African elephants cytochrome *b* mtDNA haplotypes. Circle size is proportional to the haplotype frequency. The numbers on the connecting line determine the number of substitutions estimated by NETWORK V.4. 1. 1. 1. The entire list of haplotypes for cytochrome *b* MJN can be found in **Table 2**.

Table 1: HVR1 haplotypes used in the Figure 2. Haplotype frequency is indicated in brackets when there is more than one.

Haplotype	**Taxon**	Designation*	Geographic origin	Genbank accession numbers	Author
H01	*Loxodonta africana africana*	Angola1	Angola	AY741072	Debruyne 2005
H02 (2)	*L. a. africana*	Botswana1, BOT4	Botswana	AY741074, AF106230	Debruyne 2005, Nyakaana *et al.* 2002
H03 (3)	*L. a. africana, L. a. cyclotis*	BOT2, BOT21, DRC4	Botswana, DRC	AF106228, AF106234, AY359275	Nyakaana *et al.* 2002, Debruyne 2005
H04 (2)	*L. a. africana*	BOT9, Zimbabwe2	Botswana, Zimbabwe	AF106231, AY741329	Nyakaana *et al.* 2002, Debruyne 2005
H05	*L. a. africana*	BOT15	Botswana	AF106232	Nyakaana *et al.* 2002
H06 (3)	*L. a. africana, L. a. cyclotis*	BOT16, DRC1, Zimbabwe4	Botswana, DRC, Zimbabwe	AF106233, AY359277, AY742799	Nyakaana *et al.* 2002, Debruyne 2005
H07 (4)	*L. a. cyclotis*	Bmbo6, Dja39, CAR3214, CAR394	Cameroon, CAR	AF527653, AF527647	Eggert *et al.* 2002, this study
H08	*L. a. cyclotis*	Cameroon1	Cameroon	AY359267	Debruyne 2005
H09 (4)	*L. a. cyclotis*	Cameroon2, Bmbo1, Bmbo37, NYO0310	Cameroon, Gabon	AY359269, AF527646, AF527649	Debruyne 2005, Eggert *et al.* 2002, this study
H10 (7)	*L. a. cyclotis*	Bmbo16, Bmbo43, CAR274, CAR297, Congo2, NN0713, NN2911	Cameroon, CAR, CR	AF527648, AF527650, AY359268	Eggert *et al.* 2002, Debruyne 2005, this study
H11	*L. a. cyclotis*	Dja34	Cameroon	AF527651	Eggert *et al.* 2002
H12 (3)	*L africana, La. fricana, L. a. cyclotis*	DRC13**, B1, DRC9	DRC, Cameroon	AY741081, AY359279, AF527654,	Debruyne 2005, Eggert *et al.* 2002
H13 (2)	*L. a. africana*	B7, Waza15	Cameroon	AF527655, AF527659	Eggert *et al.* 2002
H14 (3)	*L. a. africana*	B8, Waza10, Sudan1	Cameroon, Sudan	AF527656, AF527658, AY741073	Eggert *et al.* 2002, Debruyne 2005
H15 (2)	*L. a. africana*	Waza27, Mali2	Cameroon, Mali	AF527660, AF527666	Eggert *et al.* 2002
H16 (4)	*L. a. cyclotis*	CAR3622, NN059, NN279, NN3014	CAR, CR		This study
H17 (2)	*L. a. cyclotis*	CAR3315, CAR381	CAR		This study
H18 (4)	*L. a. cyclotis*	CAR5712, AFE82lan, MDC012, NOG053,	CAR, Gabon		This study
H19	*L. a. cyclotis*	CAR1	CAR	AY359272	Debruyne 2005
H20	*L. a. cyclotis*	CAR309	CAR		This study
H21	*L. a. cyclotis*	CAR3519	CAR		This study
H22 (5)	*L. a. africana*	Chad1, K68, RVV15, Mole13, WA6	Chad, Ghana	AY741080, AF527643, AF527641, AF527676, AF106243	Eggert *et al.* 2002, Debruyne 2005, Nyakaana *et al.* 2002
H23 (10)	*L. a. cyclotis*	NN3218, Lan027, LOP067, LOP51a14, NOG014, NOG025, NOG026, Mpa01, Mpa028, RAB0113	RC, Gabon		This study
H24	*L. a. cyclotis*	Congo1	RC	AY359266	Debruyne 2005
H25 (2)	*L. a. cyclotis*	CKT04a14, RAB275	RC, Gabon		This study
H26	*L. a. cyclotis*	DRC2	DRC	AY359270	Debruyne 2005
H27 (4)	*L. a. africana* and *L. a. cyclotis*	KV8, MF1, MF5, DRC3	Uganda, DRC	AF106206, AF106209, AF106210, AY359271	Nyakaana *et al.* 2002, Debruyne 2005
H28 (2)	*L. a. cyclotis*	DRC6, DRC8	DRC	AY359273, AY359274	Debruyne 2005
H29	*L. a. cyclotis*	DRC5	DRC	AY359276	Debruyne 2005
H30 (2)	*L. a. africana*	DRC11, AM1	DRC, Kenya	AY741078, AF106217	Nyakaana *et al.* 2002, Debruyne 2005
H31 (2)	*L africana* and *L. a. africana*	DRC17**, QE13	DRC, Uganda	AY742802, AF106213	Nyakaana *et al.* 2002, Debruyne 2005
H32 (2)	*L. a. cyclotis*	IVI1011, RAB067	Gabon		This study
H33 (11)	*L. a. cyclotis*	Igl032, AFE85Igl, AFE86Igl, AFE88Igl, IVI1012, IVI043, LOA0310, LOP146, Mpa0319, RAB0215, WAK0410	Gabon		This study
H34 (3)	*L. a. cyclotis*	Lan015, Lan15911, RAB131	Gabon		This study

Table 1: HVR1 haplotypes used in the Figure 2. Haplotype frequency is indicated in brackets when there is more than one. *(Continued)*

H35 (5)	*L. a. cyclotis*	Lan1566, IVI05a6, IVI05b8, RAB032, WAK0817	Gabon		This study
H36	*L. a. cyclotis*	Lan16014	Gabon		This study
H37 (3)	*L. a. cyclotis*	Gabon2, LOP0710, PBA023	Gabon	AY359265	Debruyne 2005, this study
H38 (8)	*L. a. cyclotis*	IVI06b2, Kes0721, Kes0819, LOA068, AFE79LOP, PBA0510, RAB044, RAB1118	Gabon		This study
H39 (4)	*L. a. cyclotis*	Kes0211, Kes0314, Kes0517, PBA0714	Gabon		This study
H40	*L. a. cyclotis*	Gabon1	Gabon	AY359278	Debruyne 2005
H41	*L. a. cyclotis*	NOG0810	Gabon		This study
H42	*L. a. cyclotis*	PBA0612	Gabon		This study
H43	*L. a. cyclotis*	IVI05a5	Gabon		This study
H44 (3)	*L. a. cyclotis*	Bia3, Bia69, Liberia1	Ghana, Liberia	AF527677, AF527680, AY741079	Eggert *et al* 2002, Debruyne 2005
H45	*L. a. cyclotis*	Bia48	Ghana	AF527678	Eggert *et al* 2002
H46 (6)	*L. a. cyclotis* and *L. a. africana*	Bia64, RVV22, Mole9, WA3, WA14, Mali7	Ghana, Mali	AF527679, AF527642, AF527675, AF106242, AF106245, AF527667	Eggert *et al* 2002, Nyakaana *et al* 2002
H47 (2)	**L. a. africana**	Mole3, Mali14	Ghana, Mali	AF527674, AF527668	Eggert *et al* 2002
H48	*L. a. africana*	Mole33	Ghana	AF527683	Eggert *et al* 2002
H49 (2)	*L. a. cyclotis*	Tai6, Tai17	Ivory Coast	AF527670, AF527671	Eggert *et al* 2002
H50 (2)	*L. a. cyclotis*	Tai19, Tai29	Ivory Coast	AF527672, AF527673	Eggert *et al* 2002
H51	**L. a. africana**	IvoryCoast1	Ivory Coast	AY741327	Debruyne 2005
H52 (2)	*L. a. africana*	SouthAfrica3, Zimbabwe1	South Africa, Zimbabwe	AY741320, AY741321	Debruyne 2005
H53	*L. a. africana*	MM4	Kenya	AF106214	Nyakaana *et al* 2002
H54	*L. a. africana*	MM19	Kenya	AF106215	Nyakaana *et al* 2002
H55	*L. a. africana*	MM20	Kenya	AF106216	Nyakaana *et al* 2002
H56	*L. a. africana*	AM2	Kenya	AF106218	Nyakaana *et al* 2002
H57	*L. a. africana*	AM10	Kenya	AF106219	Nyakaana *et al* 2002
H58	*L. a. africana*	AM12	Kenya	AF106220	Nyakaana *et al* 2002
H59	*L. a. africana*	SA8	Kenya	AF106221	Nyakaana *et al* 2002
H60	*L. a. africana*	Mali28	Mali	AF527669	Eggert *et al* 2002
H61	*L. a. africana*	Mozambique1	Mozambic	AY741076	Debruyne 2005
H62 (5)	*L. a. africana*	Namibia1, Addo5, Uganda1, QE1, Zimbabwe10	Namibia, South Africa, Uganda, Zimbabwe	AY741325, AF527682, AF106211, AY741323, AY742800	Nyakaana *et al* 2002, Eggert *et al* 2002, Debruyne 2005
H63	*L. a. africana*	KH2	Namibia	AF106239	Nyakaana *et al* 2002
H64	*L. a. africana*	Addo1	South Africa	AF527681	Eggert *et al* 2002
H65	*L. a. africana*	KG1	South Africa	AF106240	Nyakaana *et al* 2002
H66 (3)	*L. a. africana*	KG2, Tanzania2, Zimbabwe7	South Africa, Tanzania, Zimbabwe	AF106241, AY741070, AY741067	Nyakaana *et al* 2002, Debruyne 2005
H67	*L. a. africana*	Tanzania1	Tanzania	AY742801	Debruyne 2005
H68 (4)	*L. a. africana*	QE4, Zambia1, Af9, Af10	Uganda, Zambia, Kenya	AF106212, AY741328, AF527639, AF527640	Nyakaana *et al* 2002, Eggert *et al* 2002, Debruyne 2005
H69 (2)	*L. a. africana*	Uganda2, KV1	Uganda	AY741077, AF106203	Nyakaana *et al* 2002, Debruyne 2005
H70	*L. a. africana*	KV2	Uganda	AF106204	Nyakaana *et al* 2002
H71	*L. a. africana*	KV7	Uganda	AF106205	Nyakaana *et al* 2002
H72	*L. a. africana*	KV17	Uganda	AF106207	Nyakaana *et al* 2002
H73	*L. a. africana*	KV28	Uganda	AF106208	Nyakaana *et al* 2002
H74	*L. a. africana*	WC2	Namibia	AF106235	Nyakaana *et al* 2002
H75	*L. a. africana*	WC4	Namibia	AF106236	Nyakaana *et al* 2002
H76	*L. a. africana*	WC6	Namibia	AF106237	Nyakaana *et al* 2002
H77	*L. a. africana*	WC13	Namibia	AF106238	Nyakaana *et al* 2002
H78	*L. a. africana*	WA11	Ghana	AF106244	Nyakaana *et al* 2002
H79	*L. a. africana*	AF8	Kenya	AF527638	Eggert *et al* 2002

Table 1: HVR1 haplotypes used in the Figure 2. Haplotype frequency is indicated in brackets when there is more than one. *(Continued)*

H80	*L. a. africana*	ZBE1	Zimbabwe	AF106222	Nyakaana et *al.* 2002
H81	*L. a. africana*	ZBE2	Zimbabwe	AF106223	Nyakaana et *al.* 2002
H82	*L. a. africana*	ZBE3	Zimbabwe	AF106224	Nyakaana et *al.* 2002
H83	*L. a. africana*	ZBE4	Zimbabwe	AF106225	Nyakaana et *al.* 2002
H84	*L. a. africana*	ZBE5	Zimbabwe	AF106226	Nyakaana et *al.* 2002
H85	*L. a. africana*	ZBE6	Zimbabwe	AF106227	Nyakaana et *al.* 2002
H86	*L. a. africana*	Zimbabwe3	Zimbabwe	AY741069	Debruyne 2005
H87	*L. a. africana*	Zimbabwe6	Zimbabwe	AY741071	Debruyne 2005
H88	*L. a. africana*	Zimbabwe5	Zimbabwe	AY741322	Debruyne 2005

* Original name from each author (Debruyne, 2005; Eggert et *al.* 2002; Nyakaana et *al.* 2002; and this study. ** Sample sharing both, forest and savannah haplotypes, according to the author (Debruyne, 2005).

Discussion

In the light of the results obtained with the mitochondrial sequences used here, additional interpretations of the history of African elephants become evident and suggest that the conclusions drawn in previous studies may have been hampered by incomplete sample sets. Forest elephants have been affected by cyclical climatic changes that occurred over the last 2.6 million years as the colder drier periods experienced during Pleistocene glacial maxima are believed to have led to the repeated retraction of forest cover into refugial zones followed by re-expansion, fostering allopatric divergence between isolated populations [22] and secondary contact. The forest elephant range is therefore likely to have become centred around such refugia on several occasions. The dataset presented here raises the possibility of at least two different refugia in the central African region harbouring distinct elephant populations that diverged allopatrically. If this was the case, forest elephants possessing distinct mitochondrial genotypes are likely to have come into contact relatively rapidly after the end of the last glaciation (12,000 years BP), when the forests re-expanded [23]. Such a scenario might explain not only the two haplogroups present in forest elephants but also the lower nucleotide diversity that characterises elephant populations found in forest habitat.

This scenario might also explain the high microsatellite diversity reported for forest elephants [5]. If several populations diverged in isolation, accumulating different microsatellite profiles, and subsequently became sympatric as the forest expanded, the large single population that today comprises two central African forest elephant lineages might be expected to have engendered higher microsatellite diversity. Savannah populations, especially those in the south and east, would not have been affected by forest expansion since these areas remained unforested and thus habitat would not have been lost. Those savannah populations that may have been affected are those that may have occurred in areas that subsequently became forested. One explanation for the close genetic proximity between forest and savannah genotypes in DRC could be introgression between savannah haplotypes into forest genomes as forests expanded and savannah habitat was lost. Such introgression would be in the opposite direction to that proposed by Roca *et al.* [4,6].

The results obtained for elephants in west and central Africa have strong implications for the division of elephants into forest and savannah species. These elephants are taxonomically indeterminate [24] and have been described as having an intermediate morphology [8]. Mitochondrially, West African elephants are found in the same haplogroups as the (two) forest elephant lineages of central Africa. If ancient female-mediated introgression between the two forms followed by backcrossing into savannah populations is the reason why western savannah elephants possess largely 'forest' haplotypes then nuclear markers at these loci should resemble predominantly those of southern and eastern savannah elephants today. Alternatively these elephant populations could be an example of protracted gene flow between two forms of elephant, which is ongoing (or was until recently) and that west African savannah elephants are not distinguishable at the genetic or morphological level from their forest counterparts (thus undermining the two-taxon model). A third explanation could be a 'second movement' of elephants out of the forest (from either west or central Africa) and into the savannah. There are insufficient data to determine whether there was a single movement from forest to savannah habitat or whether these were multiple events, precipitating the morphological changes observed today. Whatever the origin of the two types, our data would support continued extensive hybridisation between the two proposed forms.

Conclusion

Our mitochondrial analysis does not support the simple separation of modern African elephants into two groups. The evidence is most clear in west Africa where savannah elephants are indistinguishable at both the mitochondrial and morphological level from their forest counterparts. The two species model cannot be easily applied in this region and neither do west African elephants represent a

Table 2: cytochrome *b* haplotypes used in Figure 3. Haplotype frequency is indicated in brackets when there is more than one.

Haplotype	**Taxon**	Designation*	Geographic origin	Genbank accession numbers	Author
H01 (12)	*L. a. africana*	AM1, AM2, QE51, WC4, BO1, DRC11, MO1, NA1, TA1, UG1, UG3, ZI10	Kenya, Uganda, Namibia, Botswana, DRC, Mozambique, Tanzania, Zimbabwe	AY741074, AY741078, AY741076, AY741325, AY742801, AY741323, AY741324, AY742800	SN, Debruyne 2005
H02	*L. a. africana*	AM12	Kenya		SN
H03	*L. a. africana*	AN1	Angola	AY741072	Debruyne 2005
H04	*L. a. africana*	BOT13	Botswana		SN
H05 (6)	*L. a. cyclotis, L.a. africana, L. africana*	DRC1, DRC4, DRC17**, BOT17, ZI2, ZI4	DRC, Botswana, Zimbabwe	AY359275, AY359277, AY742802, AY741329, AY742799	Debruyne 2005, SN
H06	*L. a. africana*	BOT18	Botswana		SN
H07	*L. a. africana*	BOT1	Botswana		SN
H08	*L. a. africana*	BOT21	Botswana		SN
H09	*L. a. africana*	BOT25	Botswana		SN
H10	*L. a. africana*	BOT2	Botswana		SN
H11 (2)	*L.a. africana*	BOT4, ET1	Botswana		SN
H12	*L.a. africana*	BOT9	Botswana		SN
H13 (5)	*L. a. cyclotis, L.a. africana, L. africana*	DRC2, DRC9, DRC13**, KV8, MF5	DRC, Uganda	AY359270, AY359279, AY741081	Debruyne 2005, SN
H14	*L. a. cyclotis*	DRC3	DRC	AY359271	Debruyne 2005
H15	*L. a. cyclotis*	DRC5	DRC	AY359276	Debruyne 2005
H16 (22)	*Loxodonta africana cyclotis*	DRC6, DRC8, Cameroon2, CAR1, Congo2, CAR274, CAR297, CAR3315, CAR3417, CAR405, CAR3723, CAR4311, IVI1012, KES0819, LOP146, NN0713, NN232, NN267, NN279, NN2911, NN3116, NN3218	DRC, Cameroon, CAR, RC, Gabon	AY359268, AY359269, AY359272, AY359273, AY359274	Debruyne 2005, MJ
H17	*L. a. cyclotis*	Cameroon1	Cameroon	AY359267	Debruyne 2005
H18	*L.a. africana*	Chad1	Chad	AY741080	Debruyne 2005
H19	*L. a. cyclotis*	CKT04a14	RC		MJ
H20 (5)	*L. a. cyclotis*	Congo1, MPA01, MPA02, NOG014, NOG026	RC, Gabon	AY359266	Debruyne 2005, MJ
H21 (3)	*L. a. cyclotis*	Gabon2, Gabon1, NN255	Gabon, RC	AY359265, AY359278	Debruyne 2005, MJ
H22	*L.a. africana*	Ivory Coast1	Ivory Coast	AY741327	Debruyne 2005
H23 (2)	*L. a. cyclotis*	IVI06c4, LOPAFE79	Gabon		MJ
H24	*L. a. cyclotis*	KES0314	Gabon		MJ
H25 (2)	*L.a. africana*	Zi5, KG1	Zimbabwe, South Africa	AY741322	SN, Debruyne 2005
H26 (2)	*L.a. africana*	KG2, SouthAfrica3	South Africa	AY741320	Debruyne 2005, SN
H27	*L.a. africana*	KV19	Uganda		SN
H28	*L.a. africana*	KV2	Uganda		SN
H29	*L. a. cyclotis*	Liberia1	Liberia	AY741079	Debruyne 2005
H30	*L.a. africana*	MM19	Kenya		SN
H31	*L.a. africana*	MM20	Kenya		SN
H32	*L.a. africana*	Namibia2	Namibia	AY741326	Debruyne 2005
H33	*L.a. africana*	QE48	uganda		SN
H34	*L.a. africana*	Sudan1	Sudan	AY741073	Debruyne 2005
H35	*L.a. africana*	WA13	Ghana		SN
H36 (2)	*L.a. africana*	WA14, WA15	Ghana		SN
H37	*L.a. africana*	WA6	Ghana		SN
H38	*L.a. africana*	WC6	Namibia		SN
H39	*L.a. africana*	Zambia1	Zambia	AY741328	Debruyne 2005
H40	*L.a. africana*	ZBE1	Zimbabwe		SN
H41	*L.a. africana*	ZBE3	Zimbabwe		SN
H42	*L.a. africana*	ZBE4	Zimbabwe		SN
H43	*L.a. africana*	ZBE5	Zimbabwe		SN
H44	*L.a. africana*	Zimbabwe1	Zimbabwe	AY741321	Debruyne 2005

* Original name from each author (Debruyne, 2005; this study SN = Silvester Nyakaana and MJ = Mireille Johnson) ** Sample sharing both, forest and savannah haplotypes, according to the author (Debruyne, 2005).

third distinct entity. Central African elephant populations west of the Congo river also question the current classification. Forest elephants fall into two major groupings with mitochondrial DNA. Previous studies found two major groups for all African elephants, savannah and savannah/forest perhaps suggesting ancient introgression between forest females and savannah males in the past. However the inclusion of a larger central forest sample in this study would suggest that this explanation is too simple and that African elephants were subject to a more complex demographic history. Phylogenetic and phylogeographic reanalysis of species is important for many reasons but with the massive extinction of species in the wild in the last 50 years accurate descriptions are essential for management of wild resources. For elephants, the classification of species into savannah and forest may not reflect their evolutionary history but simply the habitat in which they currently exist. While ecotypic differentiation has been shown to be the predominant factor driving molecular divergence in one widely distributed African herbivore recently [25], this may not apply in elephants and if it does, may not conform to a simple forest *versus* savannah habitat driven divergence. To develop management strategies incorporating a simple forest/savannah model could be misleading until further lines of evidence give us a clearer picture of the origins and current conservation needs of elephants populations throughout the continent. Future studies should analyse nuclear DNA markers, including those which evolve rapidly, across the range of forest and savannah elephants and especially in transition zones to investigate this complex ongoing process further.

Methods

Sampling and laboratory procedures

Elephant sequences from 66 sites across Africa were incorporated (Figure 1). New forest elephant samples (HVR1 mtDNA: n = 71; Cyt *b* mtDNA: n = 28) were obtained using feces from 12 sites in the central African forest block (red dots, Figure 1).

Samples were stored in RNAlater (Ambion RNA *later*® and Qiagen RNA later ™) or silica gel, and DNA was extracted from these using the Qiagen DNA stool mini kit (Qiagen, Hilden, Germany) kit following the manufacturer's protocol.

An approximately 630 bp fragment of mitochondrial DNA was amplified, encompassing the 3' end of the cytochrome *b* gene, transfer RNAs (Threonine, Proline) and 358 bp of the control region. The control region section was amplified in 71 samples using primers MDL3 and MDL5 [26]. Primers AFDL1 and AFDL2 (situated 400 bp from the 3'end of the cytochrome *b* gene through to the 5' end of the control region), and AFDL3 and AFDL4 (situated 377 bp from the 3' end of tRNA proline to the 5'end of the control region) were employed to gain overlapping sequence for some degraded samples [11]. A 494 bp fragment of cytochrome *b* was analysed separately with 28 sequences using the primers L15024 and H15516 [3]. Amplifications were performed in 50 μl containing 50 mM KCl, 10 mM Tris-HCl, 1.5 mM Mg^{2+}, 200 μmol of each dNTP, 0.2 μmol of each primer, 1.5 U *Taq* DNA polymerase (Qiagen) and approx. 10 ng of genomic DNA. Thirty to 40 cycles were carried out using a Perkin-Elmer Cetus 9600 or 9700 DNA thermocycler with denaturation at 94°C for 45s, annealing at 63°C for 45s, and extension at 72°C for 45s. PCR products were purified using the Qiagen PCR purification kit and subsequently sequenced commercially (Macrogen, Korea).

Analysis of genetic diversity and differentiation

Forward and reverse sequences for each individual and the consensus sequences for all individuals were aligned using SEQUENCHER (Gene Codes Corporation 1998, version 3.1.1) and rechecked by eye. Genetic diversity for all geographic locations was estimated using haplotype *h* and nucleotide π diversities as implemented in Arlequin ver. 3.0 [27]. Paired t-tests were carried out to assess whether there was significant difference in nucleotide diversity between forest and savannah elephants. Genetic differentiation between pairs of populations was tested using the exact test using 10,000 Markov chain steps, as implemented in ARLEQUIN ver. 3.0, and this program was also employed for nested analysis of molecular variance (AMOVA) to test for patterns of spatial genetic structure. The dataset was divided in forest and savannah groupings and then four regional populations were defined (west, central, east and south). Using AMOVA the correlation among genotype distances is used as an *F*-statistic analog (Phi) at various hierarchical levels.

Weighted maximum likelihood distances [28] were used to derive a median joining network (MJN) with the program NETWORK V4.1.1.1. Haplotype networks may more effectively portray the relationships among sequences for populations than maximum likelihood or maximum par-

Table 3: Indicators of demographic change.

	Haplogroup I	Haplogroup II	Haplogroup III	Haplogroup IV
Fu's Fs	-7.30	-6.34	-4.61	-22.44
p-value	0.006	0.015	0.034	< 0.0001

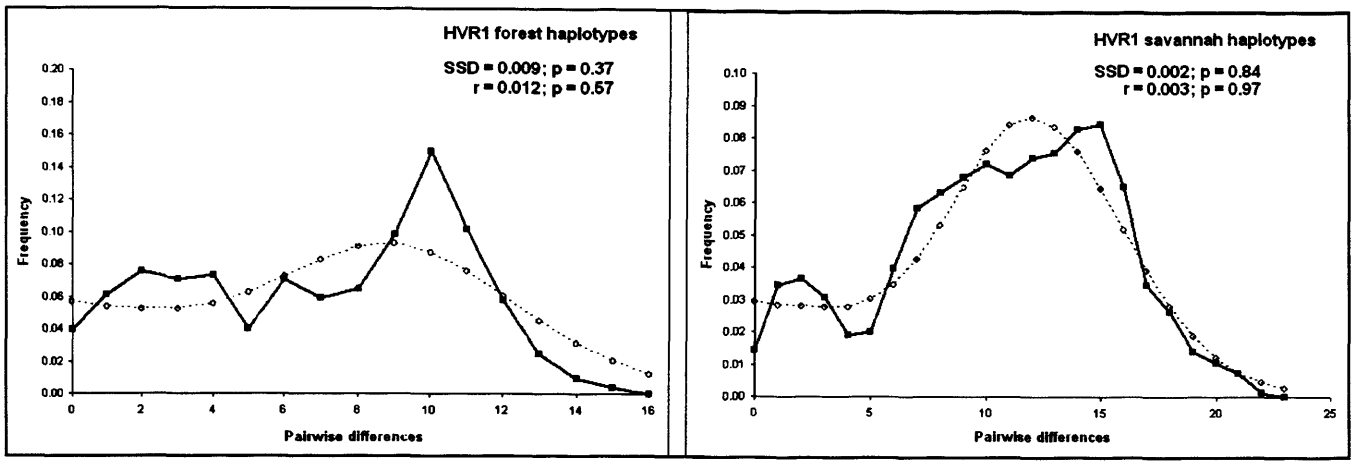

Figure 4
Mismatch distribution of the HVR1 forest and savannah African elephants haplotypes.

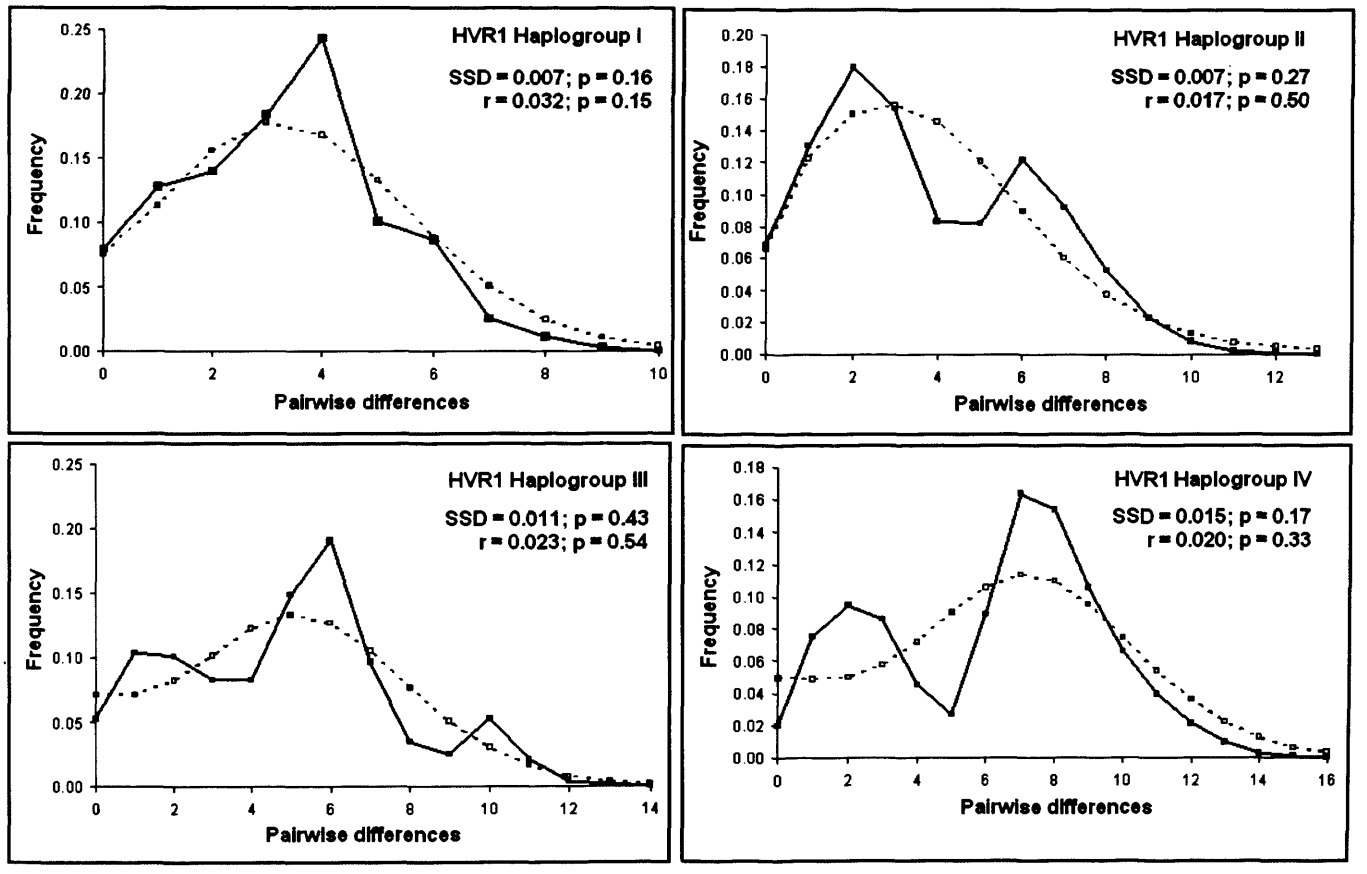

Figure 5
Mismatch distribution of the HVR1 haplogroups of African elephants.

simony which are the traditional methods developed to define interspecific relationships, leading to poor resolution at the population level [29].

Analysis of population demography

Tests were performed to detect evidence of past demographic change. We used ARLEQUIN ver.3.0 to perform a pairwise mismatch distribution, comparing the distribution of the observed pairwise nucleotide site differences with the expected distribution in an expanding population [30]. In a single origin, demographically expanding population, mismatches should follow a unimodal Poisson distribution whereas in populations at demographic equilibrium or with sub-groups, the distribution is usually multimodal. We tested the goodness-of-fit of the observed data to a simulated model of expansion with the sum of square deviations (SSD) and the Harpending's raggedness index *r*, using ARLEQUIN.

Population history was also inferred using Fu's F_S test of neutrality [31] as implemented in ARLEQUIN. We chose this test because it is the most powerful coalescent-based neutrality test for detecting population growth for larger sample sizes.

Authors' contributions

MBJ carried out the molecular genetic studies, analyzed the data and drafted the manuscript as part of her PhD dissertation.

SLC made substantive contributions data analysis and interpretation and helped to draft the manuscript.

SN provided cytochrome *b* sequences for savannah elephants from Ghana and provided comments on the manuscript.

BC and LJTW participated in the design of the study.

BG, EJW and MWB conceived and initiated the study, participated in its coordination, advised on data analysis and helped to draft the manuscript and revise it critically. MWB made substantial text contributions, especially during the review process.

All authors read and approved the final manuscript.

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