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Relatedness and Demography of African Forest Elephants: Inferences from Noninvasive Fecal DNA Analyses

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Abstract

African forest elephants (*Loxodonta cyclotis*) are genetically and morphologically distinct from their savannah counterparts, but their biology remains poorly understood. In this study, I use noninvasive fecal DNA analyses to examine the relatedness structure and historical demography of forest elephants at 2 sites in SW Gabon, central Africa. Pairwise relatedness values calculated between 162 elephant individuals genotyped at 8 microsatellite loci were significantly higher within spatially associated dung piles than between random pairings for one site. First- and second-order relatives were most commonly detected among dung piles from adult female pairs and adult females and juveniles. Pairwise relatedness estimates suggested that, like savannah elephants, forest groups are largely composed of adult females, their sisters, and juvenile offspring. Associations between males, and groups containing juveniles from multiple related females, were detected but at much lower frequency. Analysis of mitochondrial *d*-loop sequences from 70 elephant individuals identified 2 haplogroups in SW Gabon.

Key words: *demography, fecal DNA, forest elephants, Gabon, microsatellites, relatedness*

Forest elephants (*Loxodonta cyclotis*) account for a large proportion of the African elephant population (Blanc et al. 2007), but the ecology of these secretive inhabitants of central African rainforests remains obscure. Molecular systematic analyses have identified forest elephants as a third extant proboscidean species (Roca et al. 2001), although unidirectional hybridization between forest and savannah populations has created more complex phylogeographic patterns at mitochondrial loci (Eggert et al. 2002; Roca et al. 2005; Debruyne 2005; Johnson et al. 2007). Forest populations are genetically distinct at nuclear loci and in some morphological characters (Roca et al. 2007) and likely differ behaviorally and ecologically from savannah populations. The importance of patchily distributed fruits in the forest elephant diet may favor smaller group sizes (Blake and Inkamba-Nkulu 2004; Morgan and Lee 2007) and a lower degree of spatial association between individuals compared with savannah elephants that feed primarily on browse and grasses (Cerling et al. 2006). Large groups in savannah habitats may also provide enhanced protection against group-hunting lions, whereas small groups may be sufficient to thwart most predation of juvenile elephants by solitary leopards in forest habitats (Blake 2004). In this study, I use noninvasive fecal DNA analyses to examine the relatedness structure and historical demography of forest elephants in SW Gabon, central Africa.

African savannah elephants exhibit a fission–fusion social system characterized by groups of 2 or more adult females and their offspring; temporary fusing of groups leads to substantial fluid variation in aggregation sizes (Douglas-Hamilton 1972; Moss and Poole 1983). Four hierarchical tiers of social organization have been identified through behavioral association patterns: mother–calf units, families, kinship groups, and clans (Wittemyer et al. 2005). These social tiers are correlated with genetic relatedness as first-order female relatives tend to stay together during group fission, and different families led by female relatives are more likely to fuse than unrelated groups (Archie et al. 2006). Much less is known about African forest elephant relatedness patterns, and information is skewed toward observations conducted at mineral-rich forest clearings known as "bais." Adult females and their offspring have been the most commonly observed group composition at bais by a large margin (Turkalo and Fay 1995; Morgan and Lee 2007). A recent analysis from the Republic of Congo, however, has documented repeated associations between

the same individuals at bai pools, indicating that the fission–fusion system may apply in some social contexts (Fishlock et al. 2008).

The elephant social system varies dynamically throughout the year in savannah habitats, with formation of much larger groups during the wet season when food is relatively more abundant (Moss and Poole 1983). Forest elephant density may substantially increase around preferred fruiting sources or high-quality browse in riparian areas (Turkalo and Fay 1995; Morgan and Lee 2007; Buij et al. 2007), but it is unknown whether this local density results in greater association between individuals. Here, I compare genetic relatedness among individuals traveling and/or foraging together in 1) an industrial corridor dominated by oil fields where elephant groups may be nonmigratory year-round residents and 2) a riparian area in the adjacent Loango National Park that seasonally attracts a large number of elephants foraging on fruit and riparian vegetation (Buij et al. 2007; Munshi-South et al. 2008). This study is the first genetic analysis of relatedness in African forest elephants and examines relatedness patterns in both presumed mother–offspring pairs previously observed in forest elephants and potentially larger associations during periods of seasonally higher population density.

Materials and Methods

Study Sites and Sampling

Elephant dung collection surveys were conducted within the Gamba Complex of protected areas in SW Gabon (lat 1°55′S, long 9°50′E) from August–October 2006. Fecal collection areas were 1) a relatively undisturbed area in SE Loango National Park and 2) the Industrial Corridor, centered on the Rabi oil field and including areas north of the petroleum perimeter where roads have facilitated human settlement. The Industrial Corridor is an area of oil fields, roads, and associated facilities but still remains under substantial secondary forest cover. Many elephant groups in the corridor may no longer exhibit seasonal migration during the dry season but rather persist on fruits and secondary vegetation available in the corridor year-round (Munshi-South et al. 2008; Kolowski et al. 2010).

Dung surveys were conducted in 16 contiguous blocks in the Industrial Corridor (8 blocks within the oil fields and 8 blocks north of the security perimeter) and 8 contiguous blocks in Loango National Park. This uneven sampling design resulted from the needs of an earlier study (Munshi-South et al. 2008). Each block was approximately 5 × 5 km and was searched for dung on foot by 2 researchers using "recce" sampling (Hedges and Lawson 2006). Recce sampling increases dung encounters over transect methods because the search team follows paths of least resistance that are likely to be used by elephants (e.g., fresh elephant trails or abandoned roads). The team navigated across the block using a GPS receiver during the morning and then followed a different set of paths to return to the starting point in the afternoon. After one round of dung surveys, each block was searched a second time in the same order. When a dung pile was encountered, I recorded the location using GPS and measured the circumference of the 3 largest boli. I classified samples as "juvenile" or "adult" using a cutoff of 32 cm average bolus circumference as previously defined for forest elephants in Ghana (Eggert et al. 2003). Next, a sample for genetic analysis was collected from the exterior of the most-fresh bolus. Approximately 10 g of wet sticky intestinal mucus with associated fecal material was deposited in a 50-ml centrifuge tube and then mixed with 10–20 ml of Queen's College buffer (20% dimethyl sulfoxide, 0.25 M ethylenediaminetetraacetic acid, 100 mM Tris, pH 7.5, saturated with NaCl) for preservation at ambient temperature. I aimed to collect only "fresh" dung samples less than 24 h old and judged the vast majority of sampled dung piles to be <12–18 h old due to their complete covering of wet intestinal mucus on exposed surfaces. A total of 320 dung piles were sampled. Maps (Laurance et al. 2006; Buij et al. 2007) and more detailed description of the study sites and sampling regime are available elsewhere (Munshi-South et al. 2008).

Molecular Genetic Methods

I extracted fecal DNA using QIAamp DNA Stool Kits (Qiagen, Valencia, CA) with protocol modifications for elephant dung described in Archie et al. (2003). Extractions were carried out in a separate room under sterile conditions to prevent contamination. I used multilocus microsatellite genotypes to associate each dung sample with a unique elephant. Eight microsatellite loci were amplified from each sample, including the previously described loci FH67 (Comstock et al. 2000), FH126 (Comstock et al. 2002), and LafMS02 (Nyakaana and Arctander 1998). I genotyped samples at 5 other loci using primer pairs that amplify the same tandem repeat regions as published loci FH19, FH48, FH60, FH94 (Comstock et al. 2000), and LA6 (Eggert et al. 2000) but that were redesigned to amplify less of the flanking region (Eggert et al. 2007). PCR amplification conditions are described in Munshi-South et al. (2008), and primer sequences are available as supplementary material (Supplementary Table S1). I separated and visualized PCR products in an ABI 3100 automated sequencer and scored allele fragment sizes using Genotyper 2.5 (Applied Biosystems, Valencia, CA). A savannah elephant sample was included as a positive control to standardize allele scoring across PCRs in this study. In all reactions, I included a control without DNA to detect PCR contamination. Microsatellite genotypes are available on Dryad (doi:10.5061/dryad.8991).

Microsatellites amplified from dung are particularly susceptible to allelic dropout, so I controlled for data quality using approaches advocated by Paetkau (2003) and Wasser et al. (2004). First, I eliminated samples from the study if they did not produce clear repeatable genotypes for at least 5 of 8 loci within 2 attempts (2.5% of 320 were eliminated). Second, I amplified each sample at each locus at least twice for a heterozygote genotype and at least 3 times for

Table 1 Genetic diversity indices for 8 microsatellite loci genotyped in 162 African forest elephants

Locus	% Genotyped	*k*	H_O	H_E	P_{ID}	P_{SIB}	Null freq
FH19R	0.988	12	0.869	0.881	0.027	0.317	0.0055
FH48R	0.994	11	0.882	0.792	0.071	0.373	−0.0578
FH60R	0.981	12	0.881	0.868	0.033	0.326	−0.0095
FH67	0.926	8	0.807	0.805	0.067	0.366	−0.0011
FH94R	0.988	8	0.6	0.645	0.165	0.47	0.033
FH126	0.969	15	0.873	0.888	0.023	0.313	0.0074
LA6R	0.975	9	0.778	0.75	0.087	0.398	−0.0312
LafMS02	0.926	13	**0.727*****	0.871	0.031	0.324	**0.0833**
All loci	0.968	11	0.802	0.813	<0.00001	<0.001	

Percentage of 162 individuals genotyped at each locus; *k*, number of alleles; H_O, observed heterozygosity.

***, indicates that locus deviated from Hardy–Weinberg equilibrium at $P < 0.0001$); H_E, expected heterozygosity; P_{ID}, probability of 2 randomly drawn individuals having identical genotypes; P_{SIB}, probability of 2 full sibs having identical genotypes; Null freq, frequency of null alleles across populations calculated in CERVUS 3.0. Null allele frequencies >0.05 are indicated in bold.

a homozygote genotype. The high genotyping success rate and data screening described below indicate that the resulting genotypes were highly reliable. I identified multiple dung samples that came from the same individual (i.e., recaptures) by searching for matching microsatellite genotypes using the Excel Microsatellite Toolkit (Park 2001). When 2 samples differed at only one or 2 alleles across loci, I reanalyzed their fragment signatures in Genotyper to check for scoring errors. No scoring errors were detected upon these second reviews. Matching sex assignments (see below) and bolus circumference were used as additional confirmation that matching genotypes belonged to the same individual. Using these procedures, 162 unique forest elephant individuals were identified from 312 genotyped dung samples.

I established the sex of the individual that produced each dung sample using a modified version of the methods described by Fernando and Melnick (2001). First, I PCR-amplified a 141-bp fragment of the X- and Y-linked zinc-finger protein genes using the primers LaZFXYF2 (5′-CTCACACTGGGGCTTTGTTT-3′) and LaZFXYR (5′-TCTTGCTATGGACTGCCAAA-3′; Eggert, LS, M Ahlering, S Manka et al., unpublished data). PCR conditions were the same as above but were performed in 25 μl volumes and consisted of denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation at 94 °C for 1 min, annealing at 57 °C for 1 min, and primer extension at 72 °C for 1 min. I then digested 5 μl of the PCR products with *Bam*HI (New England Biolabs, Ipswich, MA) and ran out the digested products next to the original PCR products on a 3% agarose gel containing Gelstar Nucleic Acid Stain (Cambrex, San Diego, CA.). Males produce the expected 3 bands after digestion, whereas females show only the original band of the PCR product.

For mitochondrial analyses, I sequenced 323 bp of the mitochondrial DNA (mtDNA) control region and adjacent transfer RNA (tRNA)-Pro from a subset of 70 elephants using the primers Mdl3/Mdl5 (Fernando et al. 2000). These primers amplify ~600 bp of mtDNA that include portions of *cyt*B, 2 tRNAs, and the control region, but I was only able to recover good sequence from fecal DNA for the latter regions. PCR conditions consisted of denaturation at 94 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and primer extension at 72 °C for 1.5 min. Forward and reverse sequences were obtained using a Beckman-Coulter CEQ 8000 automated sequencer and then aligned and checked for accuracy using SEQUENCHER 4.8 (Gene Codes Corp., Ann Arbor, MI). Pairwise alignment of edited sequences from different individuals was performed using CLUSTALW implemented in BIOEDIT 7 (Hall 1999) and visually inspected for accuracy. Haplotypes in this data set were then aligned with central African forest elephant sequences from GenBank to identify unique haplotypes from the study population. All unique mtDNA sequences have been deposited in GenBank (accession no: GU959704–GU959708).

Microsatellite Analysis of Relatedness Structure among Dung Piles

For the microsatellite genotypes from each of the 2 sampling areas, I calculated the mean number of alleles per locus, allelic richness, observed heterozygosity, and expected heterozygosity using FSTAT 2.9 (Goudet 2002). FSTAT was also used to test for deviations from Hardy–Weinberg and linkage equilibria. For all genotypes, I estimated the observed and expected heterozygosity, the probabilities of 2 random individuals or full sibs having identical genotypes, and the frequency of null alleles, using CERVUS 3.0 (Kalinowski et al. 2007). One locus, *LafMS02*, deviated from Hardy–Weinberg equilibrium and exhibited a moderate percentage of null alleles (Table 1). Thus, I ran an identity analysis in CERVUS excluding locus *LafMS02* and using a pairwise "fuzzy matching" procedure to identify possible matching genotypes that were misclassified as unique due to genotyping error. "Fuzzy matches" were identified as 2 genotypes that have the same genotypes at 5 loci, with up to one mismatch allowed.

To examine association between dung piles, I used ARCGIS 9.1 (ESRI, Redlands, CA) to define spatial association based on proximity of genotyped dung samples. Fresh dung piles belonging to unique elephants that were sampled on the same day within 100 m of each other were assumed to constitute an observation of association between 2 or more

Table 2 Sample size and genetic diversity within each study population (n = 162 elephant samples)

Site	Industrial Corridor	Loango National Park
Overall N	94	68
No. Males	34	25
No. Females	60	43
Mean number alleles/locus	10.63	10.5
Allelic richness	10.33	10.39
H_O	0.809	0.793
H_E	0.814	0.807

individuals. This method likely overestimates the incidence of association given that dung piles near each other may have been deposited at different times. However, the vast majority of sampled dung piles were judged to have been deposited the night before collection, thus limiting this inflation. This analysis also assumes that dung piles were not in proximity due to chance encounters or attraction to the same sites.

I calculated pairwise relatedness for all possible dung pile pairs in each sampling area using MARK 2.1 (Ritland and Travis 2004). The performance of different relatedness estimators varies depending on population composition (Van de Casteele et al. 2001), so I calculated 3 different estimates for comparison (Queller and Goodnight 1989; Lynch and Ritland 1999; Wang 2002). To examine whether mean pairwise relatedness was higher in associated pairs of dung piles as defined above versus background levels of relatedness (i.e., the mean relatedness of all nonassociated pairs), I used a 2-sample randomization test for each different estimator from each study site (Manly 2006). Randomization tests were used because relatedness data were generated for dyads of individuals and thus do not represent independent observations. The one-sided P value for these tests was calculated by comparing the observed t value calculated for the difference in mean pairwise relatedness between associated and nonassociated pairs of dung piles to the distribution of t values calculated from 10 000 random draws of pairwise relatedness from the population using POPTOOLS 2.6 (Hood 2004). Randomization tests were also used to compare the mean

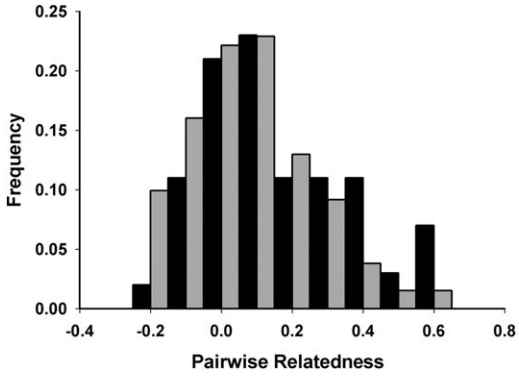

Figure 1. Histogram of Queller–Goodnight pairwise relatedness r estimated between pairs in associated groups of elephants in the Industrial Corridor (black bars) and Loango National Park (gray bars).

Table 3 Mean Queller–Goodnight relatedness estimated between associated pairs of dung piles in the Industrial Corridor and Loango National Park

	N	Mean r ± SE	Range
Industrial Corridor			
AF–AF	34	**0.22± 0.04*****	−0.27 to 0.75
AF–AM	26	0.05 ± 0.04	−0.2 to 0.68
AM–AM	3	−0.05 ± 0.04	−0.12 to 0.02
AF–J	25	**0.17± 0.04****	−0.15 to 0.59
AM–J	11	**0.02± 0.03***	−0.18 to 0.21
J–J	4	−0.06 ± 0.04	−0.15 to 0.06
All pairs	100	**0.11± 0.02*****	−0.27 to 0.58
Loango National Park			
AF–AF	27	−0.01 ± 0.03	−0.32 to 0.31
AF–AM	40	0.02 ± 0.03	−0.39 to 0.56
AM–AM	12	0.07 ± 0.05	−0.18 to 0.29
AF–J	28	0.04 ± 0.03	−0.37 to 0.47
AM–J	17	−0.09 ± 0.03	−0.36 to 0.26
J–J	7	0.2 ± 0.07	0.01 to 0.54
All pairs	131	0.02 ± 0.02	−0.39 to 0.56

AF, Adult female; AM, Adult male; J, Juvenile; Asterisks and bold type correspond to study area with significantly higher mean relatedness at the $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ level. The one-sided P value for these tests was calculated by comparing the observed mean difference between the Industrial Corridor and Loango to the mean differences calculated from 10 000 randomizations of the same sets of relatedness estimates.

relatedness of different types of associated dyads (i.e., associations between adult female–adult female, adult female–adult male, adult male–adult male, and adult female–juvenile pairs) between the 2 study sites to examine whether temporary increases in local population density in Loango National Park resulted in greater association between unrelated elephants.

Mitochondrial Sequence Diversity and Historical Demography

Diversity of mtDNA control region sequences from each sampling area was estimated using haplotype diversity, h and nucleotide diversity, π in DNASP 5.1 (Rozas et al. 2003). To visually examine the evolutionary relationships between haplotypes, I calculated a median-joining haplotype network based on weighted maximum likelihood distances in NETWORK 4.5 (Bandelt et al. 1999). To statistically test for recent population expansion in forest elephants, I calculated Tajima's D and Fu's F_S using 10 000 coalescent simulations in DNASP to calculate significance.

Results

Microsatellite Measures of Genetic Diversity and Relatedness Structure

Genotypes were obtained from 8 microsatellite loci for nearly all elephant dung samples (96.8% mean success rate across loci; Table 1). Genetic variation, as measured by the number of alleles and observed heterozygosity, was generally high in both study sites, and these 8 loci provided

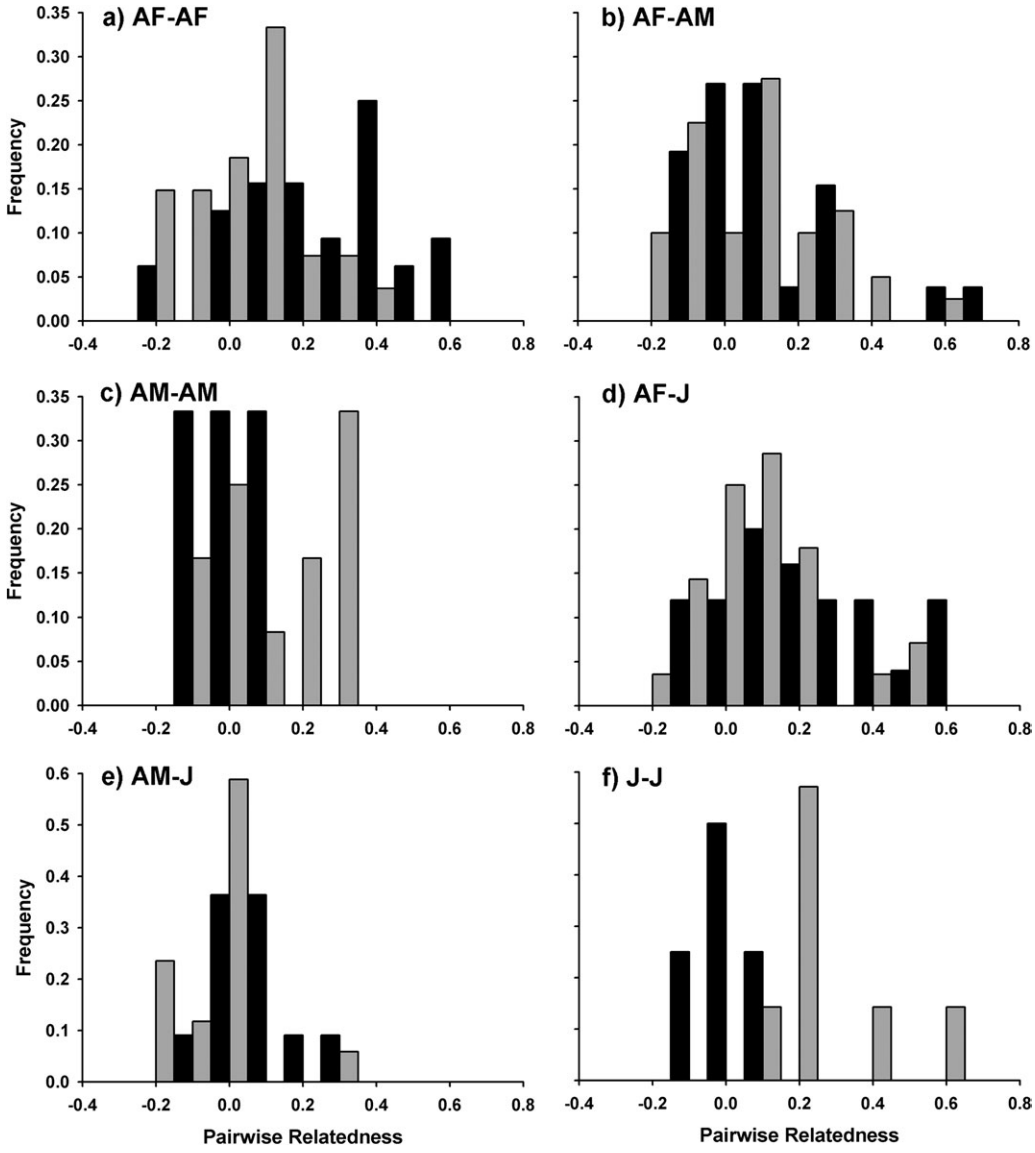

Figure 2. Histograms of Queller–Goodnight pairwise relatedness *r* estimated between pairs of associated dung piles in the Industrial Corridor (black bars) and Loango National Park (gray bars), broken down by the following age-sex dyads: (**a**) AF–AF, adult female pairs; (**b**) AF–AM, adult female–adult male; (**c**) AM–AM, adult male pairs; (**d**) AF–J, adult female–juvenile; (**e**) AM–J, adult male–juvenile; (**f**) J–J, juvenile pairs.

substantial power in distinguishing even full-sib pairs. One locus (LafMS02) exhibited a deviation from Hardy–Weinberg equilibrium and a modest percentage of estimated null alleles (Tables 1–2). When this locus was excluded and up to one mismatch allowed, the probability of 2 randomly drawn individuals having identical genotypes remained very low ($P_{ID} < 0.00001$) and only one "fuzzy match" was identified from 13 041 pairwise comparisons. The number of sampled females outnumbered males by nearly 2 to 1 (Table 2).

A total of 53 associated groups of dung piles were identified: 33 in the Industrial Corridor (mean group size ± standard error [SE] = 2.93 ± 0.21; range = 2–6) and 20 in Loango National Park (mean group size ± SE = 3.75 ± 0.21; range = 2–10; Supplementary Table S2). The distribution of relatedness estimated between associated pairs of dung piles was similar regardless of the particular relatedness estimator used (Supplementary Figure S1 and Table S2), so I report only Queller–Goodnight *r* estimates here. Mean pairwise relatedness was significantly higher ($t^2 = 51.8$, $P < 0.0001$) in associated groups of dung piles (mean relatedness ± SE = 0.114 ± 0.021) than between nonassociated dung piles in the Industrial Corridor (mean relatedness ± SE = −0.011 ± 0.003). Mean pairwise relatedness was not significantly higher ($t^2 = 1.1$, $P > 0.3$) in associated (mean relatedness ± SE = 0.019 ± 0.016) versus nonassociated (mean relatedness ± SE = 0.003 ± 0.004) dung piles in Loango. Mean relatedness within associated dung piles was higher in the Industrial Corridor than in Loango ($t^2 = 12.9$, $P < 0.001$) due to a much greater proportion of estimated

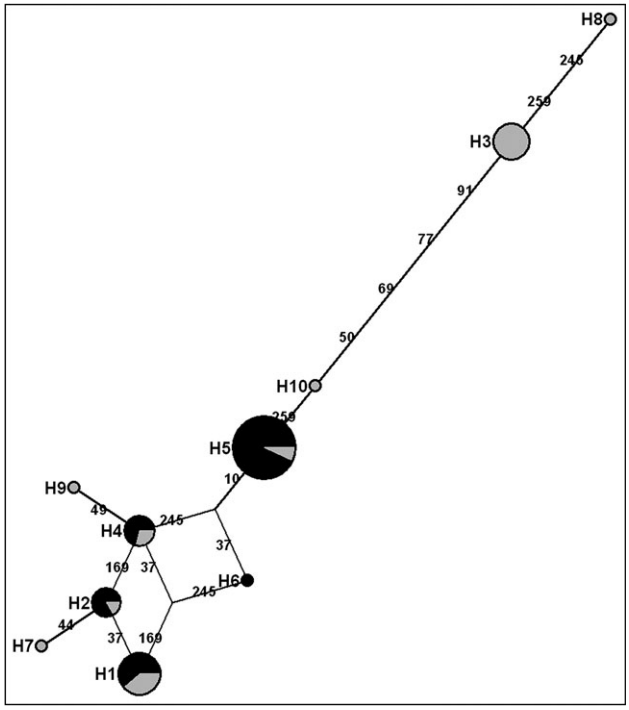

Figure 3. Median-joining network for 10 haplotypes identified from 323 bp of the mtDNA control region in 70 elephant individuals. Circle size is proportional to the haplotype frequency (range = 1–29), and color of each circle is proportional to the frequency of each haplotype in the Industrial Corridor (black) and Loango National Park (gray). Numbers on connecting lines denote mutational steps between each haplotype. Haplotypes 3 and 8 would be found in Haplogroup II, whereas all others would be found in Haplogroup I, in Johnson et al. (2007).

r = 0.3–0.5 in the Corridor (Figure 1, Table 3). Relatedness between associated dung piles from adult female pairs, and between adult females and juvenile pairs, was higher than other types of associated pairs in the Industrial Corridor (Table 3). These patterns were not statistically supported for Loango National Park. First- (QG r = 0.5) and second-order (QG r = 0.25) relatives were more commonly found among associated dung piles from adult females and female–juvenile pairs in the Industrial Corridor than in Loango. Relatives were found among dung piles from adult male pairs and juvenile pairs in Loango, but the differences between sites for these relationship categories were not statistically significant (Table 3, Figure 2).

Mitochondrial Sequence Diversity and Historical Demography

Ten mtDNA *d*-loop haplotypes were identified among the 70 elephants sequenced, 4 of which were shared between the 2 sampling sites (Figure 3). Five of these haplotypes were new for central Africa (haplotypes 6–10; Genbank accession no: GU959704–GU959708]) and five others (haplotypes 1–5 in this study) were previously sequenced from forest elephants in Gabon (haplotypes 41, 38, and 34–32, respectively, in Johnson et al. 2007). Haplotype 3 in this study belongs to a different haplogroup than all the other haplotypes shared between this study and Johnson et al. (2007). Haplotype 8 in this study thus belongs to this second haplogroup as well (Figure 3), indicating the presence of 2 distinct forest elephant haplogroups in Loango National Park. Loango elephants exhibited 5 haplotypes not recorded in the Industrial Corridor, as well as higher haplotype and nucleotide diversity, despite a small sample size (Figure 3, Table 4). A median-joining network of the 10 haplotypes indicated that one group of closely related haplotypes were shared among elephants in the Industrial Corridor. Loango elephants exhibited these haplotypes plus 5 more distantly related haplotypes not found in the Industrial Corridor (Figure 3). None of the tests for population expansion were significant in either study population (Table 4).

Discussion

I found substantial differences in patterns of relatedness between the Industrial Corridor and Loango National Park. Dung piles from adult female pairs, and adult females plus juvenile offspring traveling with or near them, in the Industrial Corridor were significantly more likely to be from first- or second-order relatives than in Loango National Park (Figure 2). In a previous analysis of fecal stress hormone levels, I unexpectedly found that elephants in the Corridor exhibited lower stress levels than elephants in Loango National Park (Munshi-South et al. 2008). These findings were interpreted as the result of one or both of the following phenomena: 1) groups of nonmigratory elephants in the relatively protected Corridor behaviorally adapting to petroleum operations and vehicle traffic and/or 2) a temporary increase in elephant density in Loango due to seasonal migration to preferred dry season foraging areas, resulting in higher stress levels from social interactions between unfamiliar individuals. A greater percentage of related individuals associating in the Corridor and a larger proportion of unrelated individuals in Loango (Figure 1) are consistent with both of these scenarios. Satellite tracking of 4 elephants in the Corridor revealed that some individuals remained in or near the area for the duration of their collar life (Kolowski et al. 2010), and one collared matriarch in a group with 2 other adult females and one juvenile was seen regularly in the area for several months after the collar stopped transmitting (Munshi-South J, personal observation). The petroleum fields of the corridor are likely inhabited year-round by a relatively small number of groups of related females and their offspring, although migratory individuals may increase the local population density during the wetter months when I did not sample. In Loango, high population density could also increase the chances of sampling dung from 2 different groups that moved through the same area at different times of the day. Poaching of matriarchs also results in the amalgamation of unrelated groups (Nyakaana et al. 2001; Gobush et al. 2009),

Table 4 Genetic diversity and statistical tests of population expansion based on 333 bp of the mtDNA control region in forest elephants from 2 study areas

	N	*H*	*h*	π	Tajima's *D*	Fu's *Fs*
Industrial Corridor	46	5	0.61	0.005	2.05	1.42
Loango National Park	24	9	0.79	0.014	2.05	1.42

H, number of haplotypes; *h*, haplotype diversity; π, nucleotide diversity. Significance of tests of population expansion was assessed using 10 000 coalescent simulations in DNASP, but no tests were statistically significant.

although no evidence exists for recent large-scale poaching in Loango.

First- and second-order relatives were detected among associated dung piles from adult male pairs and juvenile pairs in Loango (Figure 2), indicating that related males may form groups or otherwise associate with each other, and some associated groups contain more than one reproductive female and her offspring, respectively. Groups of related males have previously been reported for Asian elephants (Vidya and Sukumar 2005), suggesting that this phenomenon may be related to the behavioral ecology of proboscideans in forest habitats that forage on dispersed resources. Most groups previously observed in coastal areas of Loango were solitary males or 1–2 adult females with one offspring; groups larger than 4 individuals were particularly rare, accounting for less than 10% of total observations (Morgan and Lee 2007). Spatial proximity of multiple unrelated groups likely resulted in generally low estimates of pairwise relatedness between associated dung piles in Loango. However, results from the Industrial Corridor suggest that small female–offspring groups are composed of mothers and offspring, sisters, or half-sisters, with occasional instances of more than one reproductive female in a group. Relatedness patterns in forest elephant groups are thus similar with relatedness in core social groups of savannah elephants, although the latter may be more likely to form larger aggregations due to fusion of groups led by related females (Archie et al. 2006). Recent findings from bais indicate that fission–fusion sociality also occurs in forest elephants, at least at clearings visited by hundreds of individuals (Fishlock et al. 2008), but this study is not able to confirm or refute these observations.

The design of this study resulted in a post hoc "snapshot" of relatedness among spatially associated elephants; behavioral dynamics could not be observed in the dense forests of the sampling areas. Direct observation of association between forest elephants followed by dung collection for estimating relatedness will be extremely difficult outside of clearings such as bais. The methods used here provide preliminary estimates of relatedness among forest elephants and coarse-scale comparisons between pairwise categories of relatedness (i.e., associated adult females vs. associated adult females and juveniles) and different sites. The results here are likely an underestimate of relatedness within forest elephant social groups because 1) the study may have failed to detect relatives if they did not defecate next to each other on a given night (i.e., more than 100 m away) and 2) the study may have counted nonassociated elephants as part of the same core group if they defecated at the same location but at different times during a given night. Care was taken to collect only recently deposited dung, but these confounding factors could not be completely eliminated. The use of fecal DNA techniques was able to confirm for the first time the presence of first- and second-order relatives by sex and age class in elephant groups in central African rainforests. Genetic analysis of pairwise relatedness between spatially associated dung piles suggests that, like savannah elephants, forest groups are largely composed of adult females, their sisters, and juvenile offspring. Associations between males, and groups containing juveniles from multiple-related females, are possible but occur at much lower frequency.

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