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Effective non-invasive genetic monitoring of multiple wild western gorilla groups

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ABSTRACT

Obtaining reliable population size or abundance estimates of endangered species is critical for their conservation and management. Genotyping non-invasively collected samples is an effective way to gain insights into the number and grouping patterns of rare or elusive animals. In this study we used genetic capture–recapture estimators to obtain a precise estimate of the size of a western gorilla population inhabiting an intensely sampled 101 km^2 area in Loango National Park, Gabon. Using 394 putative gorilla samples collected opportunistically over a 3 year period, we identified 83 unique genotypes. We used a rarefaction curve, Bayesian estimator and two maximum-likelihood methods to estimate that between 87 and 107 individuals used the study area between February 2005 and September 2007. The confidence interval surrounding the genetic estimate was smaller than that obtained using traditional ape survey methods. In addition, genetic analysis showed that gorilla and chimpanzee faeces were identified with 98% and 95% accuracy in the field, respectively. Patterns of co-occurrence of individual gorillas suggest that at least 11 gorilla social groups and five lone silverback males lived in the study area and that several individuals transferred between groups during the 3-year study period. When properly designed and implemented as part of a long-term biomonitoring program, genetic capture-recapture should prove an invaluable tool for evaluating, even on a large-scale, the population size and dynamics of apes and other elusive species.

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1. Introduction

Precise and accurate methods of monitoring endangered and threatened species are difficult to develop for animals living in low visibility environments such as tropical rainforests. Effective conservation management requires species population size estimates, or at least a fairly precise baseline measure of abundance from which change can be measured. However, such data are often very expensive, and in some cases almost impossible to obtain for rare, nocturnal, or elusive species, including most wild ape populations (Kühl et al., 2008; Singleton et al., 2004; Soulé and Orians, 2001; Tutin et al., 2005). Surveying apes is difficult due to their low densities, shy nature and occurrence in remote and inaccessible areas. Ape surveys thus generally count proxies for individual apes, such as sleeping nests and/or dung piles along transects. However, extrapolation from these proxies to numbers of individual apes can yield imprecise estimates, mainly due to the unknown nest decay rate at any given site or season, unless a site-specific decay study has been carried out (Devos et al., 2008; Sanz et al., 2007; Walsh and White, 2005). In addition, careful training of field staff in data collection is required to avoid bias in transect sampling (Kühl et al., 2008). Furthermore, at sites where ape density is low, the required effort to obtain sufficient data to estimate a density is often too much for most conservation authorities in ape range states, or even for the supporting research or conservation organizations (Kühl et al., 2008). Finally, in areas where chimpanzees and gorillas occur in sympatry, differentiating between ape signs can be problematic (Furuichi et al., 1997; Sanz et al., 2007; Tutin et al., 1995). These factors are all impediments to accurate long term monitoring of apes (Bradley et al., 2008; Kühl et al., 2008; Plumptre, 2000).

By extracting DNA from non-invasively collected materials such as hair or faeces, information about the evolutionary and ecological processes affecting wild animal populations can be obtained without capturing or even observing the individuals under study (Schwartz et al., 2007; Waits and Paetkau, 2005). Non-invasive genetic monitoring and population assessment studies have been used to distinguish sympatric species, genetically distinguish and identify the number of individuals in a given area, determine trends in animal abundance, evaluate effective population sizes and infer the dispersal patterns of species (reviewed in: Schwartz et al. (2007), Waits and Paetkau (2005)). Such research thus provides the

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opportunity to study elusive species, including their spatial distribution, social structure and genetic diversity. In addition, by determining individual-specific genotypes, genetic monitoring provides a biomonitoring and population estimation alternative for when radio collaring, trapping or otherwise marking animals is unfeasible or undesirable (reviewed in: Lukacs and Burnham (2005)).

Studies evaluating genetic capture–recapture estimators via simulated data or in comparison to direct counts have found them to be generally robust under most situations (Miller et al., 2005; Petit and Valière, 2006; Puechmaille and Petit, 2007; Solberg et al., 2006). A handful of studies have compared indirect and genetic methods for counting elusive species (otters: Arrendal et al. (2007), Hájková et al. (2009); mountain gorillas: Guschanski et al. (2009); giant pandas: Zhan et al. (2006)) and showed that both over- and underestimation of population size occurs with standard indirect methods. In cases where detection of tracks or feeding remains is infrequent, genetic capture–recapture studies have doubled the previous estimated minimum population size (Arrendal et al., 2007; Hájková et al., 2009; Zhan et al., 2006). In contrast, it was found that mountain gorillas can create multiple nests at night, resulting in a 10% overestimation of population size when using indirect signs (Guschanski et al., 2009).

Disease epidemics (Bermejo et al., 2006; Köndgen et al., 2008), bushmeat hunting (Walsh et al., 2003) and habitat destruction from industrial development (logging, mining and oil extraction), agricultural encroachment and civil unrest have all caused documented declines in great ape numbers and genetic diversity (Bergl et al., 2008; Campbell et al., 2008; Goossens et al., 2006). The extent and magnitude of the declines are poorly known, adding urgency to the need to improve the accuracy and precision of ape monitoring methods. In addition to population size or density, understanding the patterns of distribution and social behavior of wild apes is also important when devising conservation management plans. For example, group living gorillas are more prone to contracting Ebola virus than solitary individuals (Caillaud et al., 2006; Walsh et al., 2007) and due to their more flexible use of space, gorillas, unlike territorial chimpanzees, are able to move away from logging when it occurs within their range (Arnhem et al., 2008; White and Tutin, 2001).

Obtaining information on the life histories and behavioral ecology of western gorillas has proven to be difficult. Researchers have observed habituated groups of gorillas at long-term research sites, or gorillas that congregate at forest clearings (locally known as "bais") (reviewed in: Robbins et al. (2004)). However, habituation requires years of intensive work and has been accomplished for only a few groups (Bermejo, 2004; Cipolletta, 2003; Doran-Sheehy et al., 2007). Multiple gorilla groups can be observed over very long periods at bais, but individuals spend only about 1% of their time at these forest clearings, meaning that most life history events are not directly observed by researchers (Parnell, 2002).

In addition to potentially providing a good alternative or supplement to traditional ape population estimation methods, non-invasive genetic sampling of apes offers the possibility to better understand gorilla society. Individual-based genetic analysis using non-invasive sampling has been carried out on largely unhabituated gorillas at several locations to estimate relative levels of genetic diversity (Bergl et al., 2008; Douadi et al., 2007), examine patterns of relatedness within and between social groups (Bradley et al., 2004, 2007) and identify population structure and the minimum number of gorillas in a given area (Bergl and Vigilant, 2007; Guschanski et al., 2008). Studies of habituated groups and known individuals have estimated male reproductive skew, group transfer patterns and the outcomes of group dissolutions (Bradley et al., 2005; Jeffery et al., 2007; Nsubuga et al., 2008) but have been limited by the number of groups under study and thus have not offered a broader scale understanding of gorilla movements. Thus, while it has been observed that females typically transfer between social groups multiple times and that males invariably emigrate from their natal group and become solitary before acquiring a group, the extent of the variation in individual transfer patterns is not well understood. However, if multiple wild gorilla groups could be repeatedly sampled over time, dispersal patterns of maturing males and females can potentially be tracked, leading to a better understanding of how gorilla groups are formed and maintained. In addition, the density of gorillas and extent of range overlap between groups is reported to vary widely among sites (reviewed in: Morgan et al. (2006)), but by genetically sampling groups over time, minimum home range size and overlap can also be inferred.

The overall goal of this study was to test the feasibility of using opportunistically collected faecal samples for genetic capture–recapture monitoring of great apes and other group-living mammals. First, we describe how we genetically distinguished the faecal remains of closely related sympatric species. Second, we applied genetic capture–recapture methods to data from opportunistically collected fecal samples from a 101 km^2 area within Loango National Park, Gabon, to obtain an estimate of gorilla population size, and compared the precision of the resulting estimate to an estimate based on nest count data collected along line transects. Finally, we estimated the number of gorilla groups in the area, their minimum membership, minimum home range size and identified cases of individual dispersal and group dissolution.

2. Methods

2.1. Study site and sample collection

The 101 km^2 Loango Ape Project research area, located in the central sector of Loango National Park, Gabon, contains several habitat types including mature, secondary and coastal forests, swamps, and savannas. The study site is bordered by the Atlantic Ocean to the west and by a lagoon to the east (Boesch et al., 2007). This region is the westernmost distribution of sympatrically living central chimpanzees (*Pan troglodytes troglodytes*) and western gorillas (*Gorilla gorilla gorilla*).

Between February 2005 and July 2008, two to four field teams conducting habituation and biomonitoring activities opportunistically collected up to 3-day-old gorilla and chimpanzee faecal samples from throughout the study site; due to the presence of dung beetles, rain and maggots, ape faeces do not persist for more than 3 days at Loango. Because both species primarily nest in trees in Loango, samples were collected from beneath night nests of both species and from where the apes had defecated as they moved through the forest during the day. The geographic coordinates of each sample were recorded using a Garmin GPSMap® 60 or 60CSx. A total of 394 putative gorilla samples (from March 2005 to September 2007) and 452 presumed chimpanzee samples (from February 2005 to July 2008) were collected using the two-step ethanol–silica procedure (Nsubuga et al., 2004), stored in the field for up to 6 months and then at 4 °C thereafter. Samples were assigned to species using the following criteria:

(1) *Form*: Gorilla dung typically has well-formed triangular "boli" while chimpanzee faeces are usually less solid and tend to be cylindrical.
(2) *Texture*: Gorilla faeces are more fibrous than chimpanzee faeces and may contain bark, which is not consumed by chimpanzees in Loango.
(3) *Seeds*: There are several fruits that are only eaten by chimpanzees (e.g. *Staudtia gabonensis, Pycnanthus angolensis, Dacyrodes normandii*) or are eaten by gorillas but wadged

(such that the seeds are not swallowed) by chimpanzees (e.g. *Manilkara lacera, Strombosia glaucesens*, *Syzigium guineense*). These seed remains help to identify the ape species in question.

(4) *Odour*: The difference is subtle, but gorilla faeces smell sweeter as a result of their greater folivory, whilst chimpanzee faeces have a more pungent, acerbic odour. Furthermore, as only chimpanzees routinely consume meat, when they do so, the resulting change in the odour of their faeces is recognizable.

(5) *Tracks/feeding signs/nests*: When distinction of faeces remained problematic, indirect signs in the vicinity known to be specific to either ape species were used to help to identify the producer. For example, a ground nest would suggest it to be a gorilla, whereas the presence of chewed fruit 'wadges' would identify a chimpanzee. Chimpanzees and gorillas also produce different looking foot and handprints which can often be found next to fecal remains.

2.2. DNA extraction, quantification and amplification

DNA was extracted from faecal samples from 1 month to 1 year after collection, using the QIAmp Stool kit (QIAGEN) with slight modifications (Nsubuga et al., 2004). DNA concentration was estimated using a 5′-nuclease assay targeting a highly conserved 81-bp portion of the c-myc proto-oncogene as described in Morin et al. (2001). Initially, three to four independent amplifications from each DNA extract were performed at 16 microsatellite loci for gorilla samples (Supplementary Table 1) and at eight microsatellite loci for chimpanzee samples (D1s2002, D3s3038, D5s1470, D6s1056, D9s910, D10s676, D14s306, D16s2624) along with a minimum of five negative controls, using a two-step multiplex polymerase chain reaction (PCR) method described in detail elsewhere (Arandjelovic et al., 2009). The sex of each individual was determined by amplifying a segment of the X–Y homologous amelogenin gene in a one-step PCR (Bradley et al., 2001).

Up to four different PCR products were combined and electrophoresed on an ABI PRISM 3100 Genetic Analyser and alleles were sized relative to an internal size standard (ROX labeled HD400) using GeneMapper Software version 3.7 (Applied Biosystems). Genotypes were confirmed with 99% certainty by observing each allele twice in two or more independent reactions for heterozygote genotypes while homozygous genotypes were ascertained by up to five independent observations depending on the quantity of DNA in the extract (Arandjelovic et al., 2009). As such, up to 14 independent PCR reactions were carried out for each DNA extract at any given locus.

DNA extracts that did not amplify at all at the amelogenin locus and contained less than 10 pg/μl DNA were not analyzed any further. Extracts were also abandoned if they produced genotypes at only four or fewer loci after the first set of PCRs at 16 loci. Some low-quality extracts which yielded confirmed alleles at five to seven loci after six independent PCR amplifications were run in quadruplicate in a 60 μl PCR reaction volume where all conditions remained the same as described above, except that all reagent volumes were tripled in the multiplex step and a 1:50 dilution of PCR products was used in the second step singleplex PCR as template.

2.3. Genetically distinguishing gorilla and chimpanzee samples

The loci used for genotyping were originally identified in humans and have proven useful for analyses in great apes (Bergl and Vigilant, 2007; Bradley et al., 2000, 2004; Guschanski et al., 2009; Langergraber et al., 2007; McGrew et al., 2004; Vigilant et al., 2001). Because there is a high degree of overlap of autosomal microsatellite allele size ranges between the two species for all loci used (Supplementary Table 1), simple examination of genotypes does not reveal whether a sample was of gorilla or chimpanzee origin. In addition, different loci were initially used for genotyping samples expected to be from chimpanzees or gorillas. Nine samples, field-identified as chimpanzee, were genotyped at 10 autosomal microsatellite loci used to characterize putative gorilla samples (D1s550, D1s2130, D2s1326, D4s1627, D5s1470, D6s1056, D7s817, D8s1106, D14s306 and D16s2624). The nine chimpanzee genotypes were then combined with the putative gorilla genotypes and analyzed using the Bayesian model-based clustering program STRUCTURE 2.1 (Pritchard et al., 2000). We varied K from 1 to 10 clusters and conducted 20 independent iterations of each run using the no admixture model, uncorrelated allele frequencies, a burn-in period of 10,000 steps and then 100,000 steps of data collection as parameters. To determine the best supported value of K we calculated the second order rate of change measure ΔK as simulation studies have shown that in most cases it corresponds to the true number of clusters present in the data set (Evanno et al., 2005).

After following the above procedure to identify any chimpanzee faeces among those originally identified in the field as gorilla faeces, the converse analysis was done to identify any gorilla faeces in the putative chimpanzee faecal data set. To do so, genotypes at the eight loci in use in chimpanzees were generated for 13 known gorilla samples (identified in the initial STRUCTURE analysis) followed by analysis in combination with the putative chimpanzees using STRUCTURE. Two field-identified chimpanzee samples that were of clear gorilla origin based on their atypical Y-chromosome haplotypes (data not shown), were also included in this analysis.

Once samples were attributed to the correct species, we added the "field-identified as chimpanzee but are genetically gorilla" samples and subtracted the "field-identified as gorilla but are genetically chimpanzee" samples to obtain a corrected value for the total number of gorilla samples extracted. We then divided the number of gorilla samples used in the final analysis by the corrected number of gorilla samples extracted to obtain the gorilla sample extraction success. We performed similar adjustments to arrive at a success rate for chimpanzee sample extractions.

The percentage of correct field identifications is the number of genetically identified gorilla samples divided by the total number of genotyped field-identified gorilla samples and the number of genetically identified chimpanzee samples divided by the total number of genotyped field-identified chimpanzee samples. To test whether misidentification of faecal remains was more common for either of the two species, we used a two-tailed Fischer's exact test.

2.4. Discrimination of individuals

We used CERVUS 3.0 to identify samples with matching genotypes. To determine with 99.9% confidence that two matching samples originated from the same individual, we determined the minimum number of loci necessary to obtain a P_{IDsibs} value of $\leqslant 0.001$ (Waits et al., 2001). Matching samples were given a consensus ID and genotype for use in subsequent analyses. Genotypes from different samples mismatching at three or fewer loci were re-examined for possible genotyping errors and in some cases additional genotyping was undertaken to resolve any ambiguities.

2.5. Gorilla population estimate by genetic analysis

Using individual genotypes as marked captures and subsequent identification of the same individual from other samples as recaptures, we employed four methods to estimate the number of gorillas that used the research area during the study period.

The rarefaction curve (RC) method using the equation of Eggert et al. (2003), $y_{(x)} = a\,(1 - e^{-bx})$, has been shown in simulations to perform well under certain circumstances and better than other accumulation curve equations overall (Miller et al., 2005; Petit and Valière, 2006). Since sampling order might affect the population estimate obtained, the sequence of samples was randomized 1000 times with replacement and the equation fitted to the data set each time using a script written in R (R Development Core Team, 2008) by R. Mundry. The population estimate a was calculated as the average asymptotic value across all randomizations and the 95% confidence intervals (CIs) were obtained from the standard deviation across the 1000 curves (Eggert et al., 2003; Petit and Valière, 2006). Using the re-sampling results, we plotted the relationship between sampling effort and population estimate, along with the associated 95% CIs surrounding each estimate.

Grouping all samples into a single-sampling session scheme, we next calculated genetic capture–recapture estimates in three ways. First, we computed maximum likelihood estimates of population size under two models of capturability as implemented in the software Capwire (www.cnr.uidaho.edu/lecg) (Miller et al., 2005). The even capture model (ECM) assumes there is no capture heterogeneity in the data set while the two innate rates model (TIRM) assigns individuals as having either a high or a low capture probability. Capwire calculates 95% CIs using the parametric bootstrap (Miller et al., 2005). As methods to detect the presence of capture heterogeneity have been shown to be inaccurate in several cases (Puechmaille and Petit, 2007) we used both the ECM and TIRM methods to estimate population size, as we could not be certain whether or not capture heterogeneity existed in our data. R. Mundry also implemented in R (R Development Core Team, 2008) the sequential Bayesian estimator method (BE) of Petit and Valière (2006) (adapted from Gazey and Staley (1986)) to estimate the number of gorillas using the study area and we used the 95% credible intervals from the estimate to reflect the highest probability density of the data as it is more representative than 95% CIs. As with the ECM model, the BE method also assumes an even capture probability for all samples (Puechmaille and Petit, 2007). All three approaches (BE, ECM and TIRM) assume a closed population and a recapture probability equaling the capture probability.

Although gorillas have very slow life histories with births, deaths, emigrations and immigrations all being rare events in any given year, it could be argued that grouping the 3 years of collected samples into a single sampling scheme may violate the assumptions of closure in our models. Therefore, we compared the estimates obtained for the entire 3-year study period to those from two shorter sampling periods. As samples were collected opportunistically and for some months no samples were collected at all, we created two sampling sessions: sampling session 1 includes samples collected between December 2005 and September 2006 ($N = 62$ samples; no samples were collected in October and November 2005) and sampling session 2 includes samples from October 2006 to September 2007 ($N = 184$ samples). These condensed temporal sampling periods represent decreased spatial sampling areas so that only 60.9 km^2 and 89.5 km^2 of the 101 km^2 study area are included in sampling sessions 1 and 2, respectively.

2.6. Comparison of genetic and nest count estimates of gorilla population size

We analyzed nest count data from two line transect surveys covering the 101 km^2 study area. Data from survey 1 were collected over six field periods between April 2006 and June 2007 using standard line transect distance sampling methods (Buckland et al., 2001; Plumptre, 2000) implemented via a systematic segmented trackline design (DISTANCE 5.0) containing 50×1 km lines, with 2 km spacing between them. Survey 2 was conducted between February and May 2007, sampling 161×0.5 km strip transects (fixed 10 m on each side) implemented via a systematic design with alternating lines and 0.5 km spacing between them. Methods followed the IUCN Best Practice standards for great ape survey data collection (Kühl et al., 2008).

As no data on nest decay rate and nest construction rate were available from the site, and because it was often not possible to differentiate between chimpanzee and gorilla nests, we compared the precision of the pooled gorilla and undefined ape nest count and genetic estimates (and not the estimates themselves). As gorilla nests could not be consistently identified, a variance estimate based on gorilla nests alone would have even lower precision than that presented here.

Transect nest data were bootstrapped to simulate datasets reflecting differing sampling effort (10 to 200 and 300 transects for surveys 1 and 2, respectively). We then used the delta method to calculate the overall coefficient of variation (CV) (Buckland et al., 2001; Seber, 1982). For analysis of survey 1 we included four terms into the estimate:

$$CV(\hat{D})^2 = [CV(n)]^2 + [CV(f(0)]^2 + [CV(t)]^2 + [CV(r)]^2$$

where n is the total number of nests encountered on all transects. Here $f(0)$ is the probability density function of perpendicular distances evaluated at zero distance, t is the mean decay time of nests, and r is the nest construction rate. The $CV(n)$ was calculated analytically from the transect data and $CV(f(0))$ was estimated from the full data set of survey 1 using DISTANCE 5.0. We used published nest decay and nest construction rate estimates (Kouakou et al., 2009) and assumed that the respective coefficients of variation (not the estimates) would approximate the site specific CVs. For analysis of survey 2 we used the same approach except that we did not include the $f(0)$ term.

We then calculated 95% CIs following Buckland et al. (2001, p77), from which we derived CI width. Since it was not possible to obtain a reliable population size estimate from the nest count data itself, due to uncertainty in identifying the nest building species and lack of a site-specific decay rate estimate, we assumed a density of 1 gorilla/km^2 (an approximation from the genetic estimate for comparison's sake) in order to estimate CI width. To determine the precision of the nest count and genetic methods, we compared the widths of the 95% confidence intervals as a percentage of the obtained estimates for varying levels of sampling effort (either number of transects walked or number of samples collected), using the nest count and genetic maximum-likelihood results.

We estimated the nest-count time-effort relationship by setting sampling effort to one 1 km line transect per day for survey 1, and to three 0.5 km strip transects per 2 days for survey 2 with monthly working time taken as 16 days. Thus, 200 transects would take 333 days to complete. To examine the effect of faecal sampling effort on the obtained genetic estimate, we divided the sampling session 2 collection period into monthly intervals and calculated the estimate and associated confidence intervals using the ECM and TIRM methods for each month cumulatively. As no samples were collected in May, July and August 2007, no cumulative estimates were calculated for these months (the estimates remain the same as the previous month). We then compared the effects of increased study effort (days of faecal sampling or transect walks) on the widths of the 95% confidence intervals obtained using the genetic maximum likelihood ECM and TIRM methods and nest count line transect survey 1 and survey 2 methods for population size estimation.

2.7. Gorilla group composition and ranging

The identity and composition of gorilla groups were established by assuming that samples from individuals collected on the same day at the same GPS location (same nest site or multiple fresh fecal remains found together) belong to individuals from the same group. Group membership was assumed to be stable throughout the study period unless evidence suggested that individuals had transferred or emigrated from the study area. Samples from males that were collected alone more than once and never in conjunction with any other individual, and whose samples were the size of silverback dung (Schaller, 1963) were considered to be lone silverback males. Group attribution could not be assigned for other males and females whose dung was sampled only once and not together with dung of other individuals.

Minimum home range size was calculated in ESRI® ArcMap™ 9.2 by using the minimum convex polygon (MCP) tool as implemented in the Hawths Analysis Tools v. 3.26 software package. MCPs were created using the GPS locations of individuals when they were identified as group members only. Because only one small area and group was frequently sampled in 2005, we included the 2005 sample locations to calculate the minimum home range size for this group alone. For all other groups minimum home range size was calculated from the 2006 and 2007 data only.

3. Results

3.1. Genetically distinguishing gorilla and chimpanzee samples

Putative chimpanzee and putative gorilla samples were genotyped using different sets of microsatellite loci, so two reciprocal analyses were necessary to identify misclassified samples. The 394 putative gorilla samples yielded 324 usable genotypes representing 88 unique individuals (Supplementary Table 1). An individual based clustering analysis (STRUCTURE) sorted the genotypes into two clusters when applied to a dataset consisting of these 88 genotypes as well as from nine field-identified chimpanzee samples. One cluster contained 82 of the 88 putative gorilla genotypes, while 6 of the putative gorilla genotypes clustered with the nine field-identified chimpanzee genotypes. After genotyping those six samples at the microsatellite loci used in the chimpanzee analysis, we found that three of the six individuals had previously been detected in the chimpanzee data set. We thus consider all six samples to represent chimpanzees and not gorillas.

The 452 putative chimpanzee samples yielded 208 usable genotypes representing 133 unique individuals. STRUCTURE analysis of these 133 genotypes, the three newly identified chimpanzee genotypes identified from the previous STRUCTURE run, and 13 known gorilla genotypes, once again suggested a division into two clusters (Supplementary Fig. 1a and b). In this case, eight genotypes from 11 putative chimpanzee samples clustered with the gorilla genotypes (Fig. 1a). After genotyping these eight individuals at the loci used in the gorilla analysis, we found that 7 of the eight individuals matched genotypes in the gorilla data set and we consider all 8 of these samples to represent gorillas.

The initial gorilla data set, the nine known chimpanzees and the additional gorilla genotype identified from the second STRUCTURE run were then reanalyzed with STRUCTURE and once again two clusters were found to be optimal (Supplementary Fig. 1c and d) and the same six individuals clustered with the known chimpanzee genotypes (Fig. 1b).

In sum, a total of 396 gorilla and 447 chimpanzee samples were extracted and 82% (326/396) of the gorilla and 46% (205/447) of

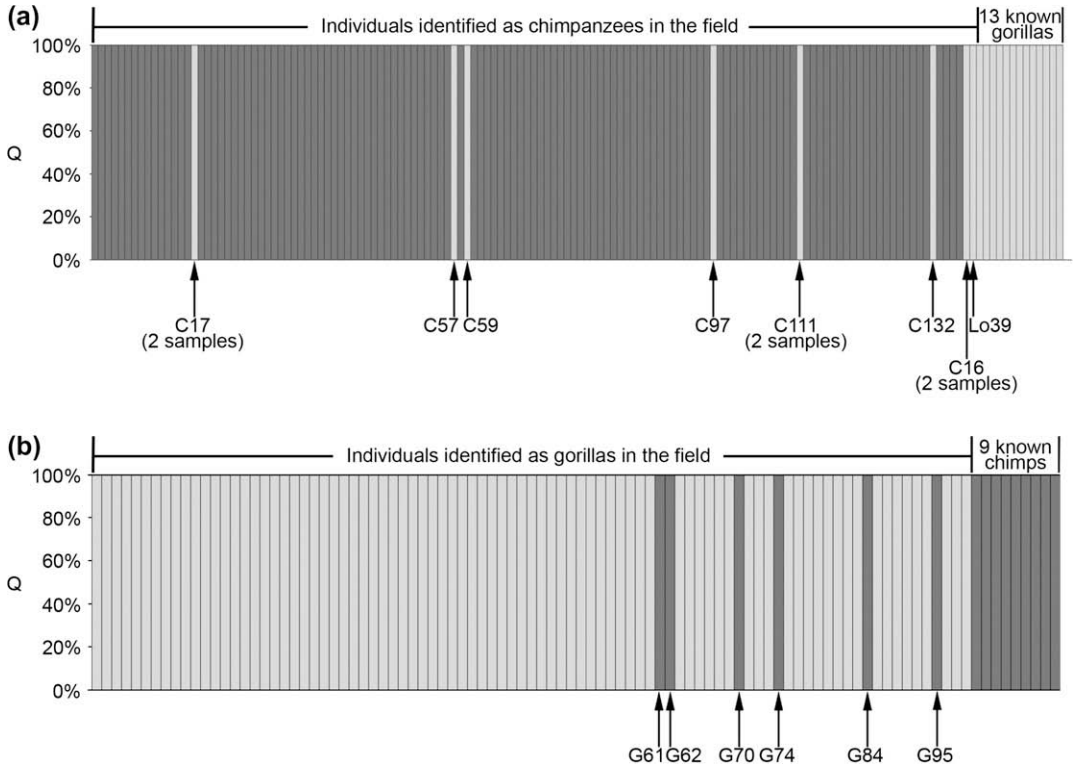

Fig. 1. Membership of unique genotypes in two clusters identified by STRUCTURE. Each bar represents an individual chimpanzee or gorilla consensus genotype. Q is the percentage membership of each individual in each cluster (in both cases, all individuals belong 100% to one of two clusters). (a) The data set consisting of all the individuals identified as chimpanzees in the field (first 133 bars) and thirteen known gorilla samples from Loango. Eight individuals (C16, C17, C57, C59, C97, C111, C132 and Lo39) were identified in the field as chimpanzees but group with the gorilla cluster. (b) The data set consisting of all the individuals identified as gorillas in the field (first 89 bars) and nine known gorilla samples from Loango. Six individuals (G61, G62, G70, G74, G84 and G95) were identified in the field as gorillas but group with the chimpanzee cluster and are thus chimpanzees.

the chimpanzee samples produced usable genotypes. Field researchers correctly identified gorilla dung (318/324 = 98%) significantly more often than chimpanzee faecal remains (197/208 = 95%) (Fischer's exact test, $p = 0.041$).

3.2. Discrimination of gorilla individuals

Genotypes from the 326 gorilla samples were on average 75.7% complete with 92.6% of extracts (301/325) genotyped at eight or more of the 16 loci. After comparing and combining individual genotypes from multiple samples, the genotypes of the 83 resulting gorillas were on average 88.5% complete. We are confident that relatives were not misclassified as the same individual because for each case in which two samples had no mismatches and were identical at a minimum of 7–9 loci, the P_{IDsibs} was $\leqslant 0.001$. One sample (G86) was genotyped at only four loci (with confirmed alleles at an additional four loci) and three samples were genotyped at only six loci (G68, G97 and G98); however, these samples mismatched all other samples at a minimum of one locus.

3.3. Estimation of population size

Of the 326 gorilla samples yielding usable genotypes, there were 40 instances where samples were collected at the same location and on the same day as other samples representing the same individuals and were thus collapsed into a single capture event as they do not represent true recaptures. An additional sample was outside the main study area and was also removed from the data set. Among the 285 resulting samples, a total of 83 unique gorilla genotypes were identified from the study area. The number of captures/recaptures per individual ranged from 1 to 5 (mean 1.51, *SD* 0.98) in sampling session 1 (December 2005–September 2006), from 1 to 9 (mean 2.59, *SD* 1.77) in sampling session 2 (October 2006–September 2007) and from 1 to 12 (mean 3.43, *SD* 2.45) for the entire 3 year sampling period (February 2005–September 2007) (Fig. 2). Across the 3-year study period, 21 individuals (7.4% of samples, 25.3% of individuals) were sampled only once (Fig. 2).

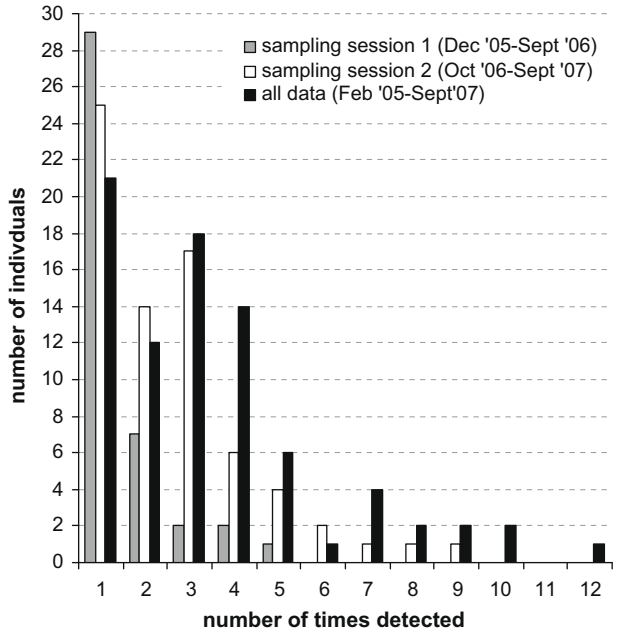

Fig. 2. Frequency of detection of individual gorilla genotypes during the study period.

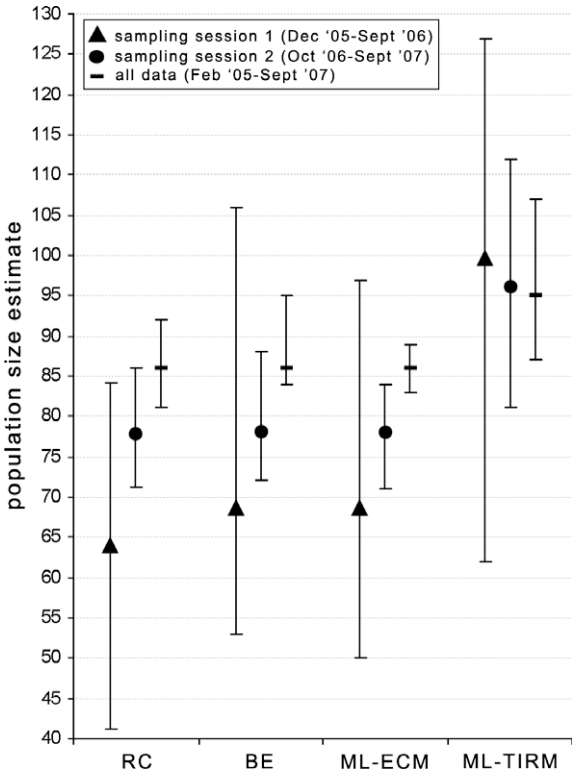

Fig. 3. Estimate of number of gorillas using the study area from February 2005 to September 2007 using four different estimators and three sampling schemes. RC – rarefaction curve method, BE – Bayesian estimator, ML-ECM – maximum likelihood, even capture model, ML-TIRM – maximum likelihood, two innate rates model. Error bars represent 95% confidence intervals (95% credible intervals for BE).

When we applied the rarefaction curve, Bayesian estimator and maximum likelihood even capture model methods, the point estimates of the number of gorillas using the study area in sampling sessions 1, 2 and the whole 3 year period were consistent (Fig. 3). In sampling session 1 the point estimate was 63 (95% CI_{RC}: 41–84) for the RC method and 68 (95% CI_{BE}: 53–106, 95% CI_{ECM}: 50–97) for the BE and ECM methods. The TIRM method, which takes heterogeneity in the data into account, gave a higher point estimate of 99 (CI_{TIRM}: 62–127) gorillas for sampling session 1. For sampling session 2, the RC, BE and ECM methods all gave the same point estimate of 78 (95% CI_{RC}: 71–86, 95% CI_{BE}: 72–88, 95% CI_{ECM}: 71–84) gorillas using the study area. Once again, the TIRM method gave a higher point estimate with larger confidence intervals (96 gorillas, 95% CI_{TIRM}: 81–112). When using the entire 3 year period in the analyses, the point estimate of the RC, BE and ECM methods was 86 in all three cases (95% CI_{RC}: 81–92, 95% CI_{BE}: 84–95, 95% CI_{ECM}: 83–89) whereas the TIRM estimate was 95 (CI_{TIRM}: 87–107). Using the results from the session 2 sampling period, these data suggest that between 81 and 112 gorillas used 89.5 km^2 of the study area from October 2006 to September 2007. Using data from all 3 years of sampling and over the entire 101 km^2 study area, we infer that between 87 and 107 gorillas used the study area.

3.4. Nest count surveys and comparison with the genetic approach for estimating population size

For 12 months of sampling effort (200 transects), the projected width of the 95% CI from nest count survey 1 was 57% of the density estimate (Fig. 4). Analysis of survey 2 provided very similar results with a CI width of 52% after 12 months of sampling. In

contrast, the width of the 95% CI obtained applying the ECM and TIRM methods to the genetic data from 12 months of sampling (in session 2) was 17% and 33% of the estimate, respectively (Fig. 4). The nest count survey 1 method always performed poorly when compared to the other methods and the genetic ECM estimator always outperformed the other methods. However the nest count survey 2 method outperformed the genetic TIRM estimator until the 4 month mark. At the 4 month mark, which is equivalent to 100 transects from survey 2 and 55 genetic samples, the survey 2 CI widths were 71% of the estimate while the TIRM estimate had CI widths that were 70% of the estimate. After 4 months, the width of the CIs surrounding the genetic estimates steeply declined while those obtained from the nest surveys leveled off.

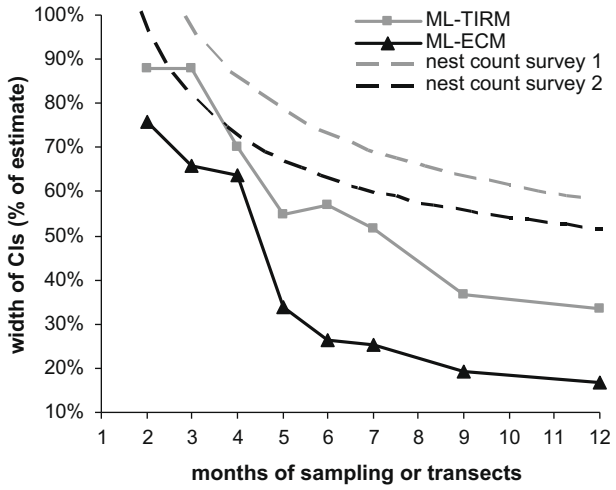

Fig. 4. Comparison of the effects of increased study effort upon the widths of the 95% confidence intervals obtained using the genetic maximum likelihood ECM (black solid line) and TIRM (grey solid line) models, nest count line transect survey 1 (grey dashed line) and survey 2 (black dashed line) methods for population size estimation. Width of 95% confidence intervals calculated as a percentage of the estimate for a given sample size. Sampling effort for line transects based on 16 1 km transects walked per month (survey 1) or 24 0.5 km transects walked per month (survey 2). ML-ECM and ML-TIRM estimates based on cumulative number of samples collected per month between October 2006 and September 2007, triangle (ML-ECM) or square (ML-TIRM) indicate number of cumulative months for which data exist (absence of shape indicates no samples collected in that month and thus no change in the estimate from the previous month).

3.5. Gorilla group composition and ranging

Groups were identified by noting which individual genotypes were found together when multiple samples were collected at a given place and time (Fig. 5). At least seven groups were present in the study area. Five of these were tracked and named by field researchers (Mandondo, Achilles, LayonA, Tonda and Indegho groups). Genotypes from multiple nest sites were obtained from these five groups allowing for a large number of individuals to be identified from each. Two additional groups (C and H) were tentatively identified by genetic analysis as samples from two nest sites were collected from each group, and no individual was found to link the two groups together or to any other group. Few samples from the individuals tentatively assigned to groups B and D were found, indicating that these individuals may actually belong to other groups.

Groups varied in size from two to 15 individuals with varied male:female sex ratios (Table 1). Group composition appeared stable during the duration of the study with the following exceptions (Fig. 5):

(1) Female G06 was first found at two nest sites in August 2005 with other members who were otherwise linked to Mandondo group. However, in August 2006 and six times between June and September 2007, she was found in nest sites with individuals from Achilles group. We consider this a case of female transfer from Mandondo to Achilles group.
(2) Female G24 was first found at an Achilles group nest site in February 2006, however, despite collection from seven nest sites from this group after August 2006, female G24 was never re-identified with any individuals from Achilles group. In November 2006 however, she was found in a nest group with male G16. We consider this a case of female transfer from Achilles group into group I. As we were only able to identify G16 and G24 as members of group I, it is unclear whether group I actually has other members or whether with the transfer of G24, a new group was formed. The latter explanation may be more parsimonious as male G16 was found alone in February 2006. Group I was never detected

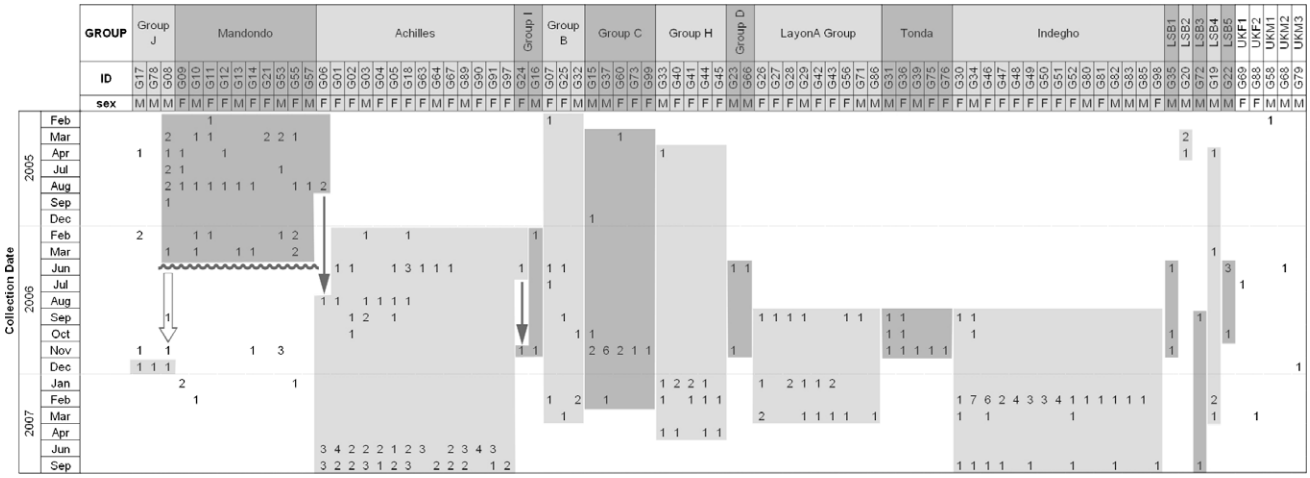

Fig. 5. The inferred composition and dynamics of the Loango gorilla groups over the 3 year study period. Number of times each gorilla was detected in each month shown. Black arrows depict female transfers (female G06 transferred from Mandondo group to Achilles group and female G24 transferred from Achilles group to group I). White arrow shows male movement (male G08 joined males G17 and G78 to form a group after the suggested dissolution of Mandondo group). Wavy line represents time at which the last nest group was found from the Mandondo group. LSB = lone silver back male, UKF = unknown female, UKM = unknown male. Unknown individuals were captured alone and once only.

Table 1
Summary of minimum group composition and minimum home range size. Groups J and I were only detected once, group D was only detected twice, as such, no minimum home range size could be calculated for these groups.

Group	Minimum # individuals	Minimum # females	Minimum # males	Minimum home range size	# of occasions group detected
Group J	3	0	3	–	1
Mandondo	10	6	4	23.1 km^2	23
Achilles	14	11	3	10.8 km^2	28
Group I	2	1	1	–	1
Group B	3	2	1	7.5 km^2	8
Group C	5	3	2	10.6 km^2	11
Group H	5	4	1	17.7 km^2	10
Group D	2	0	2	–	2
LayonA	9	6	3	3.3 km^2	9
Tonda	5	3	2	8.4 km^2	5
Indegho	15	10	5	6.5 km^2	15
LSBs	5	–	5		5
UKF/UKM	5	2	3		5
Total	83	48	35		123

again, suggesting that they may have moved out of the study area after uniting. Alternatively, individuals G16 and G24 may both have been members of Achilles and then either dispersed or died.

(3) Finally, we propose that the Mandondo group dissolved during the study period, but that some, and possibly most, individuals remained in the study area. From February 2005 to March 2006, members of Mandondo group were sampled regularly (N = 41 samples, including four nest sites and three trail sites where multiple samples were collected together, as well as 16 samples collected alone on trails), however after March 7th 2006, no nest sites from the group were ever sampled again despite continued sampling in the area. Evidence for this hypothesis also comes from the fact that during the beginning of the study, Mandondo group samples were only found along the coastal region of the field site (Supplementary Fig. 2), but when putative Mandondo males G08 and G10 were sampled in late 2006 and early 2007, respectively, they were found further inland, outside of the Mandondo group's estimated home range. Male G08 was found together with males G17 and G78 in December 2006 once again outside the previous distribution of Mandondo

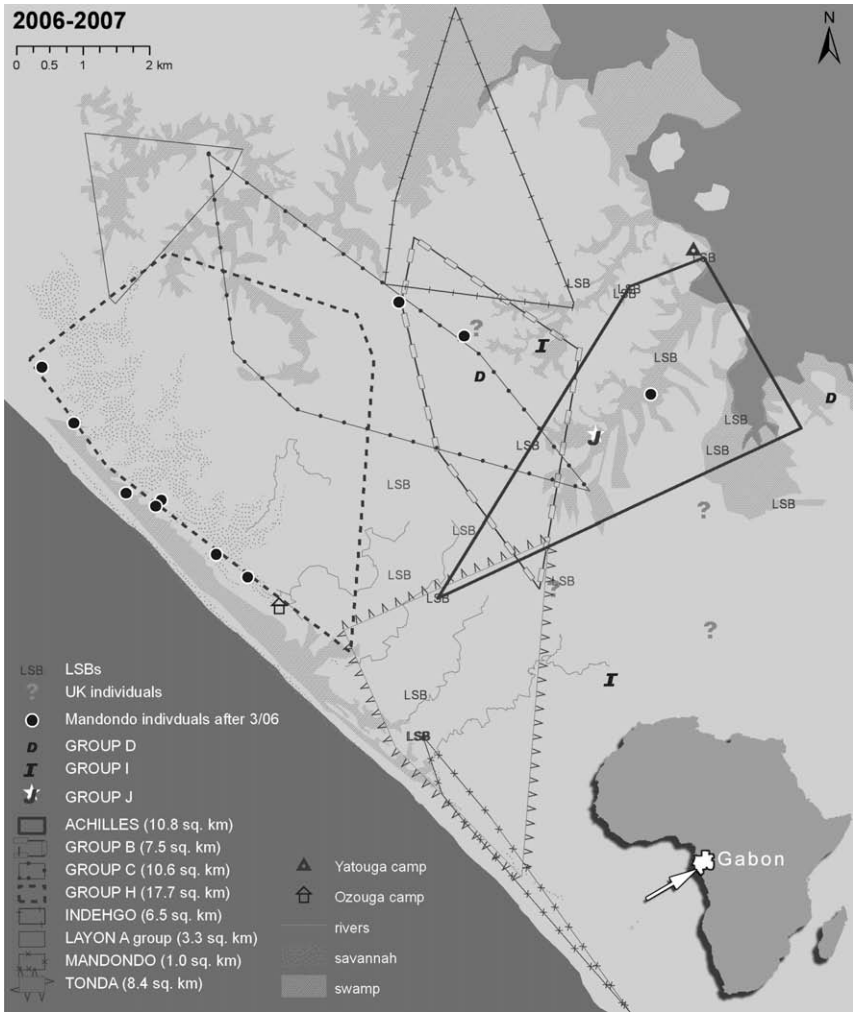

Fig. 6. Loango Ape Project study area, gorilla samples and minimum home ranges for 2006–2007 study period. Minimum home ranges (minimum convex polygons, MCPs) are delineated for the eight groups with more than two sampling locations. Area of MCPs provided in legend. Sampling locations for groups D, I (two locations, three samples each) and J (one location, three samples) are also plotted. MCP for Mandondo created only from sampling localities collected between February and March 2006. Mandondo group members are represented as black dots after March 2006. Lone silver backs ("LSB", four individuals from 14 samples) and four individuals with no group attribution ("?") are noted as well. Inset, map of Africa with Gabon highlighted in white, arrow indicates location of Loango field site.

group. Male G17 had been detected four times prior to this collection and was always alone, suggesting that these three males grouped together to possibly form a bachelor group. Another male from Mandondo group G53, remained in the coastal area after the alleged dissolution of Mandondo, however, as we do not have age class data for the samples, it is difficult to interpret whether this sample was a juvenile traveling with his kin or a young lone silverback remaining around his natal range. Furthermore, three of the six Mandondo group females were detected after the supposed dissolution of Mandondo (Supplementary Fig. 2). Females G09, G14 and G55 were all sampled in the coastal area of the study site. G14 was sampled alone, G09 was sampled alone once and with another sample a second time, however this associated sample did not contain usable amounts of DNA. G55 was sampled alone however, two samples (G40 and G41) from group H were sampled nearby on the same day, suggesting that perhaps these females transferred to group H, apparently the only other coastal group in the study area.

Once group composition was assessed, we constructed minimum convex polygons around the group member locations to estimate the minimum home range size of the gorilla groups (Fig. 6). As the Mandondo group was the only recurrently sampled group in 2005 and seemingly dissolved in 2006, we calculated its minimum home range size over the course of its identified existence. For all other groups, MCPs were only constructed for samples in the 2006–2007 study period. Minimum home range size varied from 3.3 km^2 (LayonA group) to 23.1 km^2 (Mandondo group) (Table 1) and were somewhat overlapping, especially around group B, at the center of the study area.

4. Discussion

4.1. Genetically distinguishing gorilla and chimpanzee samples

The results of this study show that field researchers could nearly always accurately differentiate dung of the two different ape species. Chimpanzee faeces were significantly more often misidentified (5% of samples) than gorilla faeces (2% of samples). Other researchers have used mitochondrial DNA (mtDNA) analysis to distinguish remains from closely related species, but here we show that use of microsatellite genotyping and STRUCTURE to differentiate sympatric species offers the opportunity to study multiple species simultaneously when amplification with the same primers is possible. This avoids the sometimes challenging amplification of large mtDNA fragments for restriction enzyme digestion, or the painstaking development of species-specific amplification primers (Fernandes et al., 2008 and references therein). Another drawback of using mtDNA is the potential presence of mitochondrial insertions in the nuclear genome (numts) which may not be identified unless long-range PCR from high quality samples is performed (Bensasson et al., 2001; Kuyl et al., 1995; Thalmann et al., 2004). Finally, analysis of mtDNA, a single maternally-transmitted genetic locus, will not distinguish species for which discordances in mitochondrial and nuclear genetic variation occur, as when insufficient time since separation or subsequent introgression exists (reviewed in: Arnold et al. (2008)).

4.2. Gorilla population estimate by genetic analysis

This study, utilizing gorilla fecal samples collected opportunistically over a 101 km^2 area, demonstrates the utility of genetic capture–recapture for estimating the size of an ape population. Sampling session 1 covered a limited part of the study area (60.9 km^2) and only 62 samples were collected during the session. Thus, the RC, BE and ECM estimators are probably underestimating the number of gorillas in the area. The three models (RC, BE and ECM) gave identical estimates in session 2 and when using the entire 3-year data set, however, simulation and empirical studies have shown that the point estimates given by the RC, BE and ECM methods tend to underestimate the true number of individuals present in the population particularly when heterogeneity is present which is likely in our case (Miller et al., 2005; Petit and Valière, 2006; Puechmaille and Petit, 2007).

Interestingly, the TIRM model gave a similar estimate in session 1 as in session 2 and when the entire 3-year data set was used, although the CI width for the TIRM estimate in session 1 was quite large (Fig. 3). The TIRM estimates appear the most robust, as this is the only estimator to incorporate heterogeneity in the data and it does not change greatly with the addition of samples. In one study in which approximately as many samples as individuals present were collected, the 95% CIs of TIRM were found to always encompass the true size of the population (Puechmaille and Petit, 2007). Using the results from the TIRM estimator, from session 2 between 81 and 112 gorillas used 89.5 km^2 of the study area for an abundance estimate of 0.91–1.25 gorillas/km^2. Using the less conservative data from all 3 years of sampling and over the entire 101 km^2 study area, between 87 and 107 gorillas used the study area, for an abundance estimate of 0.86–1.06 gorillas/km^2. At least 83 gorillas used the study area over the 3 years as that was the minimum number of unique genotypes identified in total. Furthermore, these results suggest that using the entire 3-year study period to calculate a population estimate does not grossly violate the assumptions of closure in the capture–recapture estimators in this species.

It has been shown that collecting 2.5–3 times as many samples as the number of individuals thought to exist in the study population dramatically decreases the width of the confidence intervals surrounding the obtained population estimate as well as the mean relative error when using all four estimators (Miller et al., 2005; Petit and Valière, 2006). In this study, at least two times as many samples as the number of individuals using the study site were genotyped, further suggesting that our estimates and surrounding confidence intervals are reliable. Our abundance estimates are consistent across sampling sessions and within the lower range of values found at other localities throughout the western gorilla range (0.2–10.19 individuals/km^2 (reviewed in: Morgan et al. (2006), Rainey et al. (2009))).

4.3. Comparison of genetic and nest count estimates of gorilla population size

Our results suggest that there are several benefits of the genetic sampling method in comparison to the nest count estimates traditionally used. First, traditional ape surveys require nest or dung deposition and decay rates. Estimating these may require more than a year of data collection prior to the actual survey (Kühl et al., 2008; Walsh and White, 2005), and no such data were available for the Loango field site. Thus, we cannot compare actual estimates, but we can compare the width of the confidence intervals surrounding the genetic and nest count estimates.

Applying the TIRM model to the genetic data, the 95% CI width was 33% of the final estimate (whereas the projected confidence intervals representing the best-case scenario for the nest survey method were 51% of the estimate (Fig. 3)). When using the ECM method for the genetic data, an even smaller CI (17%) was obtained around the final estimate. This shows that the precision of the genetic method can exceed that of the nest survey method, especially with moderate to high amounts of effort and in the absence of heterogeneity. In this study for instance, as of 4 months, equivalent to 100 1 km walked transects or 119 sampled dungs, the genetic

method using the TIRM estimator outperformed the nest survey method. It is important to point out that while with higher densities of transects and more information, smaller confidence intervals may be achieved with the nest survey method, it remains to be seen if the nest survey method can be as precise as the genetic method. The genetic method for gorilla population size estimation is an improvement over traditional methods in two additional ways. First, in areas with sympatric apes, only 90% of nests can be correctly identified and only when the covariates characterizing ape nest height, tree species, and habitat are used (Sanz et al., 2007), whereas with the genetic method, gorillas and chimpanzees can be unambiguously differentiated. Second, using the genetic method or other direct methods such as camera traps or direct observations, a minimum number of individuals are identified, allowing for monitoring of individuals and the population over the long term, as well as short term inferences of group membership and movement.

Notwithstanding the advantages of genetic surveys, for very rapid assessments, field-based methods may be more appropriate for estimating ape numbers (Buij et al., 2003; Kühl et al., 2008). Data on ape habitat, such as detection of poaching signs and vegetation composition, are most efficiently collected along transects, but the low encounter rate of dung on transects makes the simultaneous collection of samples for genetic capture–recapture impractical (Kühl et al., 2008; Takenoshita and Yamagiwa, 2008). This suggests that multiple complementary approaches are needed to arrive at a comprehensive understanding of ape population numbers and dynamics, ecology and threats.

4.4. Gorilla group composition and ranging

Using genetic data collected opportunistically over 3 years, we show that information regarding individual movements, group size and ranging can be obtained without habituation. Two female transfers (G06 and G24), one group formation and possible group movement out of the study site (group I), one group dissolution (Mandondo group) and subsequent formation of one non-breeding group (group J) were all identified by genotyping most of the individuals in the study population and re-sampling these individuals over time. All of these dynamics have been observed at other field sites and appear to be part of the normal behavioral repertoire of western lowland gorillas. Despite these dynamics, most of the individuals appeared to remain within the study area over the 3 year sampling period, indicating that there may not be a great deal of emigration from or immigration into the area.

As we cannot be sure that we obtained samples from all group members for any group, and because we lack age information for the collected individuals, it is difficult to draw conclusions regarding the sex ratios of the Loango groups. However, males and no females were identified in two groups (groups D and J), which may indicate the presence of two small non-reproductive groups. The case of group J particularly argues for the existence of an all male non-reproductive group as it is made up of a former lone silver back (G17), a male that had previously been found nine times in the Mandondo group (G08) and one additional male who was not identified prior to December 2006 (G78). Assemblies of previously unassociated males have been described at Lokoué, Republic of Congo (Gatti et al., 2004) but not in other western gorilla populations (Magliocca et al., 1999; Parnell, 2002; Robbins et al., 2004).

The gorilla groups using the study area ranged from two to 15 individuals, which is typical of western gorillas (group size = 2–29: Gatti et al., 2004; Robbins et al., 2004). Minimum home range sizes were estimated at 3.3–23.1 km^2. As these estimates were derived from non-homogeneous sampling and limited data they are smaller than previously reported western gorilla group home range sizes obtained as a result of direct long-term observation (11 km^2: Bermejo (2004); 18.3 km^2: Cipolletta (2003); 15.4 km^2: Doran-Sheehy et al. (2004); 22.9 km^2: Remis (1997); 21.7 km^2: Tutin (1996)). Home range overlap appears typical but its extent awaits future studies using systematic grid sampling over several seasons.

The disbanding of the Mandondo group provides observations of the dispersal decisions of group members upon group dissolution. First, after the supposed dissolution of Mandondo we observed the formation of the non-breeding group J (see above). Second, samples from former Mandondo males (G10 and G08) were found ranging far from the pre-dissolved Mandondo home range in the swampy region of the study site (samples from a fourth former Mandondo male (G53) was found alone in the coastal region of the study area). Third, former Mandondo females (G14, G09, and G55) remained in the coastal region of the Loango study zone. As there is no evidence that gorilla females range alone, we assume that these females transferred to a new group, possibly with a coastal home range. Future sampling should clarify the identity of this new group. A recent study in mountain gorillas found that females may prefer to transfer to areas in which they are familiar with the available food (Guschanski et al., 2008), but additional work is needed to elucidate the dynamics of female gorilla movement at Loango.

4.5. Recommendations for future ape genetic surveys

When used as a complement to traditional surveys of human impact and ecology of ape habitat, genetic surveys can play an important role in assessing the state of ape populations in the wild. They can provide accurate and precise data on ape numbers and some aspects of population dynamics. In our study, sample collection did not increase field expenses, as samples were collected opportunistically in the context of a long-term behavioral study. A study of similar magnitude (695 samples collected, 384 samples extracted, and the same 16 microsatellite markers) estimated collection materials and laboratory expenses at approximately 12,000 Euros (Guschanski et al., 2009) to which the cost of a student or research assistant must be added as well.

Future studies should focus on following gorillas with the intent of collecting samples so that the limitations of this study's opportunistic collection design are avoided. In areas where gorillas are regularly tracked, this implies that field assistants are trained to collect samples and that the appropriate material is always carried with the field team's scientific kit. The sampling scheme and methods should account for the fact that gorillas are group-living mammals that utilize home ranges and thus do not move homogenously through any given area. Specifically, to decrease the amount of heterogeneity in the sampling design, a virtual grid system should be created. This would allow for the use of an open population model to estimate population size, which is probably better suited to animals that can immigrate into and emigrate out of a given area and may reveal trends in the area such as survivorship. A non-opportunistic study design should also overcome many of the issues of heterogeneity in the collected data, and hence should further decrease the confidence intervals surrounding the estimates. A field team comprising at least one tracker and one field assistant should search within and collect samples from a grid square for a given amount of time before moving onto the next square, with all squares revisited at least once in a given survey. Sampling the same group in different quadrants should decrease bias in collection methods. For example, if researchers have sampled a group in a given quadrant and decide to remain in that quadrant to continue sampling, they are more likely to follow the same group they had already identified then if they were to move to another quadrant and start their search anew. Thus, to ensure that groups are resampled in an unbiased way, grid squares should be smaller than the home range size of most gorilla groups, so that the same group

can be sampled in different quadrants. By eliminating the need for determining nest or dung deposition and decay rates and habitat characteristics, this approach may reduce the amount of time and money spent in the forest.

Although the 326 gorilla samples used here were collected over a 3 year period, sufficient samples could have been collected over a 4–6 month period (assuming a conservative dung encounter rate of 2–3 samples per day) by experienced gorilla trackers. Collecting samples over a shorter period of time would also decrease the potential for miscounts arising from violations of the closed population model used in the capture–recapture analyses (no immigration, emigration, births and deaths in the study population). Given our results, we suggest that at least three times as many samples be collected than the predicted population size for apes. If additional information beyond census size is to be inferred, revisits should be carried out over several months, and preferably in different seasons, so that gorilla ranging patterns throughout the year might be determined.

In this study, five individuals were each only collected once and alone, and thus could not be attributed to any group. Two of these individuals were females and are thus highly unlikely to be ranging alone. We strongly advise collecting samples from nests whenever possible, as it is the best way to determine group membership for any given individual, as well as to detect changes in group composition.

Further studies should focus on validating the genetic capture–recapture method by implementing it in an area with a known number of apes. Furthermore, although this study was conducted over a relatively small area, a genetic capture–recapture study of brown bears using opportunistically collected faeces over a 49,000 km^2 area proved successful (Bellemain et al., 2005), indicating that with a sufficient amount of resources and planning, even large-scale DNA-based population estimates are feasible for rare and elusive species (Kindberg et al., 2009; Robinson et al., 2009). Thus, a future study could plausibly implement and evaluate a non-invasive genetic capture–recapture ape survey across an entire national park, as long as data are collected on all other species and on human impact, in order to maximize the benefit of sending teams of people into very remote areas for weeks, and sometimes months at a time.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biocon.2010.04.030.

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