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Counting elusive animals: Comparing field and genetic census of the entire mountain gorilla population of Bwindi Impenetrable National Park, Uganda

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ABSTRACT

Accurate population size estimates are an essential part of every effective management plan for conserving endangered species. However, censusing rare and elusive wild animals is challenging and often relies on counting indirect signs, such as nests or feces. Despite widespread use, the accuracy of such estimates has rarely been evaluated. Here we compare an estimate of population size derived solely from field data with that obtained from a combination of field and genetic data for the critically endangered population of mountain gorillas (*Gorilla beringei beringei*) in Bwindi Impenetrable National Park, Uganda. After genotyping DNA from 384 fecal samples at 16 microsatellite loci, the population size estimate was reduced by 10.1% to 302 individuals, compared with 336 gorillas estimated using the traditional nest-count based method alone. We found that both groups and lone silverbacks were double-counted in the field and that individuals constructed multiple nests with an overall rate of 7.8%, resulting in the overestimation of the population size in the absence of genetic data. Since the error associated with the traditional field method exceeded the estimated population growth of 5% in the last 4 years, future genetic censusing will be needed to determine how the population size is changing. This study illustrates that newly improved molecular methods allow fast, efficient and relatively affordable genotyping of several hundred samples, suggesting that genetic censusing can be widely applied to provide accurate and reliable population size estimates for a wide variety of species.

1. Introduction

Many endangered species are rare and elusive, making their direct detection difficult. For these species, counts based on direct sightings are often impossible and unreliable. This is particularly true if the species of interest lives in habitats with dense vegetation, which further reduces visibility. When trapping is not possible or poses a high risk to the animals, researchers typically use traditional census methods that evaluate indirect signs of the species' presence and abun-

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dance, such as counts of dung piles, nests, and tracks (buffalos, bushbuck, duikers, and elephants in the Virunga Volcanoes (Plumptre and Harris, 1995), orangutans (Johnson et al., 2005), elephants (Fay, 1991), Eurasian otters (Ruiz-Olmo et al., 2001), carnivores (Gese, 2001)). However, population size estimates based on these methods are often associated with very large confidence intervals that translate into high percentages of the total count (e.g., average of 36% for 13 examples of elephant dung counts (Barnes, 2001), 20–30% for nest-based orangutan and chimpanzee surveys (Johnson et al., 2005; Plumptre and Cox, 2006)). This lack of precision is especially problematic when the goal is to estimate population sizes for small populations of endangered species and it has motivated researchers to incorporate genetic analyses into survey design. Recently, techniques utilizing DNA obtained from non-invasively collected materials, such as dung and hair, have sufficiently improved to make the genotyping of even hundreds of samples feasible (Lucchini et al., 2002; Banks et al., 2003; Creel et al., 2003; Nsubuga et al., 2004; Bellemain et al., 2005; Bergl and Vigilant, 2007; Langergraber et al., 2007; Puechmaille and Petit, 2007). Individual identification using a molecular fingerprint allows a direct count of sampled individuals, and provides a means for comparison with the numbers of individuals inferred from indirect approaches.

Two recent studies compared indirect and genetic census results and found that the indirect methods substantially undercounted the number of individuals (Zhan et al., 2006; Arrendal et al., 2007). Although the sample size was small, twice as many otters were detected using genotyping than solely by tracking. Similarly, the estimate of panda numbers in one important forest reserve doubled using genetic censusing as compared to assessment from the size of bamboo bite marks. The most likely explanation for these discrepancies appears to be the inability to reliably discriminate among individuals of similar body size and overlapping ranges using indirect methods.

Since the beginning of research on wild gorillas some 40 years ago, the number of nests and the size of the dung left in nests has been used to estimate the number, ages and sexes of gorillas unhabituated to direct observation (Schaller, 1963). The critically endangered mountain gorilla (*Gorilla beringei beringei*) (Butynski, 2007) is a flagship species and occurs in only two populations: one in the Virunga Volcanoes on the borders of Rwanda, Democratic Republic of Congo and Uganda and the other in Bwindi Impenetrable National Park, Uganda. Approximately 71% of the Virunga gorillas live in habituated groups and can be directly counted. The total population was estimated to contain 380 gorillas in 2003 (Gray et al., in press). A much smaller proportion (approximately 25%) of the Bwindi gorillas can be directly observed and counted.

For mountain gorillas, the relatively small size of the protected areas (Virungas: 450 km^2; Bwindi: 331 km^2) and the ability to easily find nest sites and trails left by individuals moving through the forest led researchers to devise a 'complete sweep' census method. In this approach, multiple, closely-spaced teams systematically search the entire forest for gorilla trails and nesting sites (Weber and Vedder, 1983; Aveling and Harcourt, 1984; Sholley, 1991; McNeilage et al., 2001, 2006). Most gorillas live in social groups and the members of the group construct individual nests each night. These nests are cohesively distributed at the group's nesting site and each individual typically defecates in or next to the nest before leaving the site in the morning. During a census, team members record the location and number of nests at each nest site and the size of the associated dung for up to three consecutive nest sites per gorilla group. This information is used to infer the direction in which the group is moving, the number of gorillas and the sex/age composition of social groups at the site, as well as the number of groups and gorillas in total.

A key aspect of the complete sweep method is that it assumes that signs of essentially all population members can be detected and that each individual is counted only once. Such an approach produces a concrete number and has per definition no variance. The complete sweep method might seem superior to a sampling-based approach, such as counting animal signs along line transects, particularly for very small populations such as mountain gorillas. In a sampling-based approach, biased estimates can arise from inaccurate distance measurements and violations of the assumption concerning complete detectability of signs. Together with high variance in sign encounter rate, these lead to inaccurate population size estimates (Buckland et al., 2001). Furthermore, when counting indirect signs such as nests or dung, the counts have to be translated into the number of individuals that produced them, requiring estimates of deposition and decay rates. These, in turn require expensive and time-consuming studies and are themselves associated with high variance (Walsh and White, 2005; Kuehl et al., 2007). However, while avoiding these drawbacks, the complete sweep method will produce an erroneous result with no possibility to assess its accuracy if the underlying assumptions of the method are violated.

Using the complete sweep census method, two censuses of the Bwindi gorillas were carried out in 1997 and 2002 and suggested a 1% annual growth rate from 300 gorillas in 1997 to 320 gorillas in 2002 (McNeilage et al., 2001, 2006). However, the accuracy of the indirect complete sweep census method for gorillas has never been systematically evaluated. The intensity of the effort expended to encounter gorilla signs across the small park suggests that it is unlikely that the complete sweep census produces a substantial undercount. However, of greater concern is the possibility of double-counting gorillas. This can happen if individuals build more than one nest per night or social groups are double-counted. Both situations will violate the underlying assumption of the sweep census and inflate the population size estimate.

To assess the conservation status of the Bwindi mountain gorilla population, in 2006 we carried out a genetic census in parallel with the traditional, nest-count based census of this population. Using DNA obtained from fecal samples we aimed to: (i) determine and quantify sources of error in the traditional census method by simultaneously carrying out nest-counts and sample collection for genetic analysis and thus providing means for direct comparison between the two methods, (ii) assess the rate of population growth which is particularly important for evaluating the effectiveness of various conservation strategies, (iii) evaluate the possibility of formulating correction factors for nest-count based complete sweep census methods, and (iv) evaluate the suitability

and necessity of genetic censuses for monitoring of great ape populations.

2. Materials and methods

2.1. Study site, census procedure, and sample collection

Bwindi Impenetrable National Park is a montane forest in southwestern Uganda characterized by steep hills and narrow valleys. It is surrounded by one of the highest rural human population densities in Africa with over 300 people per km^2 (Guerrera et al., 2003). Since it was gazetted as a national park in 1991, three censuses of the gorilla population have been conducted. The census carried out between April and June 2006 employed the complete sweep method as in the 1997 and 2002 censuses. A detailed description of the method is given in McNeilage et al. (2001) and McNeilage et al. (2006). Four census teams worked simultaneously and walked a total distance of ~600 km.

The movement of one or more gorillas through the thick undergrowth leaves an easily detectable trail of flattened vegetation, discarded food items, and dung. When teams found a fresh gorilla trail (estimated at less than 5–7 days old), they followed it until up to three nest sites for each group were located. Locations for each nest site were recorded using GPS. At the nest site, we counted nests, measured dung sizes, and recorded the presence of silver hair presumably shed by a silverback male. The dung measurements (Schaller, 1963; McNeilage et al., 2006) were used to estimate the sex and age class of individuals at the nest site and infer the group composition. Once counted, each nest site was marked and GPS coordinates, date of nest construction, and size and composition of the groups were then compared to exclude double sampling of groups and to distinguish similarly sized but different groups found near one another. When signs of two similarly sized groups were found in close proximity to each other and the dates of nest sites made it possible that gorillas moved that distance, the nest sites were conservatively assumed to be from the same group (McNeilage et al., 2006). These groups were thus counted only once for the nest-based population size estimate, but treated as potentially unique groups for genetic-based population size estimate.

Several assumptions were used to derive the final nest-based count of gorillas. First, for any given group, the number of nests found usually varied among nest sites. The nest site with the largest number of individuals was assumed to best reflect the number of gorillas in that group, based on the implicit assumption that weaned gorillas build own nests each night (Schaller, 1963; Weber and Vedder, 1983) and that the lower nest site counts occur because individual nests were occasionally missed. Second, infants nest with their mothers until at least the age of 3.5 years (Sholley, 1991) and defecate in the same nest as their mother. It was assumed that because of its small size, the dung of unweaned infants is sometimes not detected in adult females' nests, particularly from infants <1 year old. Thus, the number of infant dung found was assumed to represent 2/3 of the total number of infants (McNeilage et al., 2006).

The five habituated groups in Bwindi were known from direct observation at the time of the census to contain 76 individuals of known age class and, for most adults, known sex. Though nest-counts were collected for all groups, we used the known number of animals in the habituated groups for the field census count. We do not compare nest-counts and known number of individuals for these groups, as the nest-counts were potentially biased by prior knowledge regarding the group size.

During the 2006 census we collected fecal samples for genetic analysis using the previously-described two-step collection method (Nsubuga et al., 2004). Dung was collected from all nest sites found during the census. For most of the groups, the samples obtained from at least one nest site were from the previous night (<24 h old), as inferred by the integrity and dryness of the dung. The oldest samples collected were ~4–5 days old.

2.2. Genotyping and sexing

In total, teams collected 695 fecal samples during the census and an additional 145 samples were collected from five habituated groups prior to the census. Of these, 421 samples were extracted. These samples were chosen to represent all of the groups and lone silverbacks that were found by census teams, even if additional information such as spatial and temporal distribution and group composition suggested that some of these groups might be the same (Table 1). Thus, we genotyped all nest sites found by the census teams, unless they were known from the field data to be connected by undisrupted trail and thus belong to the same gorilla group. If more than one nest site per group was available, the nest site with the highest number of individuals was chosen for sample extraction. This rule was followed even if this nest site was not the freshest. Thus, extracted samples were ~1–4 days old upon collection. Furthermore, following the assumption of the nest-based census, we treated every nest at a nest site as belonging to a unique member of the group.

Extractions were performed using the QIAamp DNA Stool Mini Kit with the following modifications. One hundred milligram of dried feces was incubated in ASL buffer overnight at room temperature, and the final elution of DNA into buffer AE occurred for 30 min. The quantity of DNA in each extract was evaluated by quantitative polymerase chain reaction (Morin et al., 2001) and its quality by attempted amplification of a sex-specific region of the amelogenin locus (Bradley et al., 2001). We found that 37 extracts contained very low (<0.5 pg/μl) amounts of DNA and did not amplify the amelogenin segment, and so no genotyping was attempted. The remaining 384 extracts, ranging in DNA concentration from 0.5 to 4430 pg/μl, were genotyped at 16 microsatellite loci that were previously used for genotyping mountain gorillas from Bwindi and the Virunga Volcanoes (Bradley et al., 2005; Nsubuga et al., 2008). To quickly obtain reliable genotypes, we established a two-step multiplexing approach (Arandjelovic et al., 2008) that increased the speed, sensitivity and accuracy of genotyping. Sexing of the samples was conducted using the amelogenin assay (Bradley et al., 2001). Depending on the quantity of DNA, up to eight repetitions per extract were conducted to ensure that a homozygous female genotype did not result from allelic dropout. PCR products were resolved using

Table 1 – Comparison of field-based and genetic counts of Bwindi mountain gorillas.

Field ID	Social unit	Total number of individuals counted at the nest site	Total nest-based count	Total 'genetic' count
KYA	hab GR	16	16	16
HAB	hab GR	21	21	21
RUS	hab GR	13	13	13
MUB	hab GR	8	8	8
NKU	hab GR	18	18	18
RUH	**GR**	**4**	**4**	**11**
I1	GR	6	6	5
I2	GR	12	12	12
I3	GR	6	6	6
J2	GR	28	28	27
L1	GR	11	11	9
N1[a]	GR	8	8	8
S1[a]	GR	7	0	0
N2[a]	GR	7	0	0
N4	GR	4	4	4
O1	GR	3	3	3
P1	GR	21	21	16
S2[b]	**GR**	**14**	**14**	**13**
M1[b]	**GR**	**12**	**12**	**0**
T1	GR	4	4	4
T2[a]	GR	9	9	9
N/T[a]	GR	5	0	0
V2	GR	4	4	4
W2	GR	17	17	16
X1[a]	GR	6	6	6
X2[a]	GR	6	0	0
X3[b]	***LSB***	**1**	**1**	**0**
Z1	GR	7	7	6
CC2	GR	6	6	6
DD5	GR	4	4	4
EE1[b]	**GR**	**17**	**17**	**14**
DD1[b]	**GR**	**10**	**10**	**0**
GG1	GR	3	3	3
HH1	GR	6	6	4
DD3	GR	8	8	8
DD7	GR	6	0	0
FF1	LSB	1	1	1
J1[b]	*LSB*	1	1	1
E1[b]	*LSB*	1	1	0
J3[c]	LSB	1	0	1
L2	LSB	1	1	1
L3	LSB	1	1	1
N3	LSB	1	1	1
U1	LSB	1	1	1
W1	LSB	1	1	1
CC1[b]	***LSB***	**1**	**1**	**1**
R1[b]	***LSB***	**1**	**1**	**0**
DD2[a]	*LSB*	1	0	0
DD6[a]	*LSB*	1	0	0
DD4[c]	LSB	1	0	1
Sum			317	284

Field ID: unique identifier for groups and lone silverbacks as used during the census. The ID is based on the sector in which the social unit was found; GR: group; hab GR: habituated group; LSB: lone silverback.

Column 3 lists all nest sites that were genotyped. Column 4 gives the number of gorillas that contributed to nest-based field count, while column 5 gives the respective number of unique gorillas in each group after genotyping, including individuals that did not produce a genotype.

Group in bold: the group was missed during the census and assumed to contain only four individuals based on a partial sampling. However, the group was found after the census and shown to contain 11 individuals.

a Groups or individuals assumed to be the same in the field and confirmed to be the same by genotyping.

b Groups or individuals assumed to be unique in the field but shown to be identical to other groups or individuals by genotyping.

c Individuals assumed to be the same as already counted individuals based on field data, but shown to be unique by genotyping.

ABI 3130XL automated sequencer, analyzed using GeneMapper v3.7 (Applied Biosystems), and scored manually.

2.3. Data analysis

We used Cervus 3.0 (Kalinowski et al., 2007) to find matching genotypes and to assess the probability of full siblings or unrelated individuals having an identical multi-locus genotype (pIDsib and pID). All genotypes mismatching at up to three loci were checked for data entry errors. In the final data set, we did not observe any cases of genotypes mismatching at a single locus. For genotypes mismatching at two or three loci, we could exclude the possibility of them being from the same individual using dung sizes, group of residence, as well as in some cases individual identification based on direct observations (habituated groups). We used Cervus 3.0 to test for deviations of used loci from Hardy–Weinberg equilibrium. Significance values were adjusted by Bonferroni correction for multiple testing, as implemented in the software.

No mark-recapture calculations were possible with either our field or genetic data, since the group tracking, nest counting, sample collection, and sample selection methods were designed to prevent multiple sampling. Although we genotyped all groups and lone silverbacks that could possibly be considered unique based on field data, which resulted in genotyping several groups up to three times, the majority of individuals were represented by single genotypes. The repeated genotyping of several nest sites of the same group allowed us to test whether genotyping of additional nest sites would lead to the detection of yet uncounted individuals.

3. Results

3.1. Genotyping success and error rates

We attempted to genotype 384 extracts at 16 loci and found that 20 extracts did not yield any genotypes, while 10 extracts yielded genotypes at fewer than four loci. For the remaining 354 extracts, the genotypes were on average 84.9% complete, with most of the extracts (342/354, or 96.6%) genotyped at eight or more loci. After identical genotypes resulting from multiple sampling of the same individual were combined, the genotypes of the 257 resulting individuals were on average 89.7% complete. In fact, only two extracts could be genotyped at four and five loci, respectively, two more at six loci, and the rest (253 genotypes, 98.4%) was genotyped at the minimum of eight loci.

We estimated allelic dropout, in which one of two heterozygous alleles is not observed at a locus, by summing the number of allelic dropouts observed over all loci and dividing by the total number of successful heterozygous reactions. Allelic dropout occurred in 6% of analyzed PCRs (weighted average of allelic dropout, Eq. (2) (Broquet and Petit, 2004), Table 2). Given that we performed at least three replicate PCRs for each extract and typed 16 loci, the overall dropout rate for a given multi-locus genotype was $0.06^3 \times 16 = 3.46 \times 10^{-3}$. In 354 extracts, we would thus expect 1.2 single-locus errors ($3.46 \times 10^{-3} \times 354 = 1.2$). Irreproducible cases of sporadic alleles, such as might arise from contamination or polymerase slippage during early stages of the PCR, were observed with low overall rate of 0.27%, resulting in 1.1×10^{-4} potentially erroneous single-locus genotypes.

Nonamplification of the Y-specific allele at the amelogenin locus can result in a male extract being scored as female. The dropout rate for the Y-chromosome allele was as high as 48.2% for extracts below 10 pg/μl. We therefore genotyped extracts until up to eight successful PCRs could be scored, to ensure highly confident ($p < 0.01$) sexing of extracts. Only twice was a male allele observed in a female extract, for an overall rate of 0.29% (total number of female extracts = 166, total number of successful amelogenin PCRs from female extracts = 685, percentage false allele = 0.29%). Both of these samples were genotyped eight times and had high DNA quantities of 176 and 815 pg/μl, suggesting that the presence of

Table 2 – Characteristics of 16 microsatellite markers in the study population.

Locus	No. of alleles	Ho	He	ADO
D1s550	6	0.692	0.666	9.90
D1s2130	6	0.749	0.722	5.53
D2s1326	6	0.734	0.699	6.99
D3s2459[a]	10	1	0.844	11.73
D4s1627	5	0.672	0.694	8.01
D5s1457	7	0.75	0.737	3.89
D5s1470	5	0.502	0.517	2.82
D6s474	5	0.757	0.729	3.89
D6s1056	5	0.531	0.529	6.14
D7s817	6	0.802	0.782	5.45
D7s2204	7	0.670	0.702	5.44
D8s1106	5	0.526	0.524	10.15
D10s1432	6	0.696	0.768	3.97
D14s306	6	0.645	0.657	4.75
D16s2624	4	0.605	0.586	3.26
vWF	8	0.796	0.755	6.37
Mean	6.1	0.676	0.671	6.14

Ho: observed heterozygosity; He: expected heterozygosity; ADO: allelic dropout (% of total number of heterozygous reactions).

a All homozygotes were manually removed from this locus, mean number of alleles as well as observed and expected heterozygosity were calculated without this locus.

male allele was a result of sporadic contamination and not due to allelic dropout of the male allele in these samples.

3.2. Microsatellite marker characteristics

A single locus (D3s2459) deviated significantly from Hardy–Weinberg equilibrium after Bonferroni correction for multiple testing by showing more homozygotes that expected. Because this locus had the highest rate of allelic dropout (Table 2) and the highest number of alleles, we manually eliminated all homozygotes, conservatively assuming that any one of them could have resulted from allelic dropout. Thus, for several individuals we artificially reduced the amount of genetic information at this locus. Overall, the microsatellite loci used were polymorphic with an average of 6.1 alleles and an average observed heterozygosity of 0.68, after excluding the D3s2459 locus (Table 2).

The set of 16 microsatellite loci used in this study was sufficiently variable to distinguish even closely related individuals by their multi-locus genotypes (Fig. 1). The theoretical probability of two full siblings sharing the same genotype at 16 loci was 2.36×10^{-6} (pIDsib) and the corresponding probability for two unrelated individuals was 2.24×10^{-14}. Although not all samples could be genotyped at 16 loci, we achieved high degree of discrimination even if the genotypes could be compared at only six loci (pIDsib $\leqslant 4.45 \times 10^{-3}$). Female gorillas transfer between groups, but infrequently (0.041 transfers per female-year; Robbins, unpublished data), and males almost never immigrate into established groups and so it is highly unlikely to find the same individual in two different groups over the short period of the census. Only in two cases in which genotypes could be compared at four and five loci, respectively, did we accept a lower pIDsib (minimum pIDsib $\leqslant 7.66 \times 10^{-2}$) and still considered these genotypes to represent the same individual using the information that the samples were collected from different nest sites of the same group. In seven cases two extracts matched at fewer than six loci with no mismatches, but information about sex, approximate age of the individual, and group of origin indicated that they were different individuals.

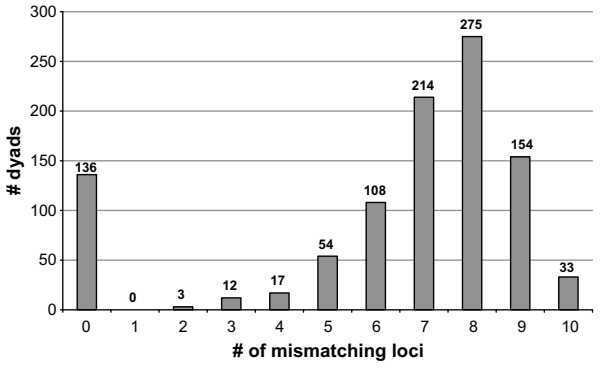

Fig. 1 – Distribution of mismatches in dyadic comparisons of 354 genotypes. The genotypes were compared at the minimum of six loci and up to 10 mismatches were allowed. All genotypes with zero mismatches are from the same individual. Numbers above the bars are counts of dyadic comparisons. No pairs of genotypes mismatched at only one locus.

3.3. Population size estimates from field and genetic methods

The field census inferred from nest-counts a total of 30 groups and 11 lone silverbacks, for an initial count of 317 gorillas. With correction for an estimated 19 undetected infants, the total is 336 (Table 3, details below). This number represents a 5% increase from the 2002 census estimate of 320 gorillas, which was itself a 7% increase upon the estimate of 300 gorillas in 1997 (McNeilage et al., 2001, 2006).

By using the same field data and incorporating genetic information, we found 28 groups and 10 lone silverbacks, comprising 257 genetically unique individuals, the vast majority of which was genotyped at a minimum of eight loci. At several group nesting sites some samples ($n = 27$) collected at an individual's nest had a very low genotyping success. Following the assumption that each individual constructs a single nest per night we included these nests into the total count, for an increase from 257 to 284 individuals. More samples could be sexed than genotyped, due to the high sensitivity of the amelogenin marker. We detected 132 males and 131 females in the population. To correct for undetected infants, we used information regarding sex and known infant to adult female ratio from habituated groups and suggest that a maximum of 17 infants was missed (detailed below). Together with other correction factors (Table 3), this results in a total of 302 individuals according to the genetic census. This number represents the maximum number of individuals for which samples were collected and genotyped. It also reflects the maximum number of unique individuals that contributed to the population size estimates using both the nest-count based and the genetic method. Comparison between the field (336) and the genetic census results (302) reveals that the counts of Bwindi mountain gorillas differed by 34 individuals, or 10.1%.

3.4. Sources of error in population size estimates and estimation of correction factors

We next examined the reasons for the discrepancy between the field and genetic census results. Several instances leading to an overcount in the field-based census were deduced (Fig. 2 and Table 1). First, genetic results showed that nests from two groups were double-counted in the field, increasing the total field count by 22 individuals. Second, lone silverbacks were misidentified in the field: three individuals that were suspected to be different were found to be identical to already counted individuals. Conversely, two nests judged in the field to be from already counted individuals were attributed to two additional different gorillas after genotyping. Thus, a net overcount was one lone silverback in the field estimate. Third, 13 individual gorillas were double-counted in the field-based estimate because they constructed and defecated in multiple nests at the nest site. Fourth, four gorillas were included in the field estimate due to data entry errors and the erroneous conclusion that two nests found in close proximity to the group nesting site were additional individuals and not ac-

Table 3 – Field-based and genetic estimates of the Bwindi mountain gorilla population.

	Nest-count based census	Genetic census
Number of groups and lone silverbacks (LSB)	30 groups, 11 LSB	28 groups, 10 LSB
Number of unique individuals	317	257
No genotyping information	–	+27
Missed infants	+19	+12–17
Missed individuals	0	+3
Estimated double-nesting of not-genotyped individuals	0	−2
Revised total	336	297–302

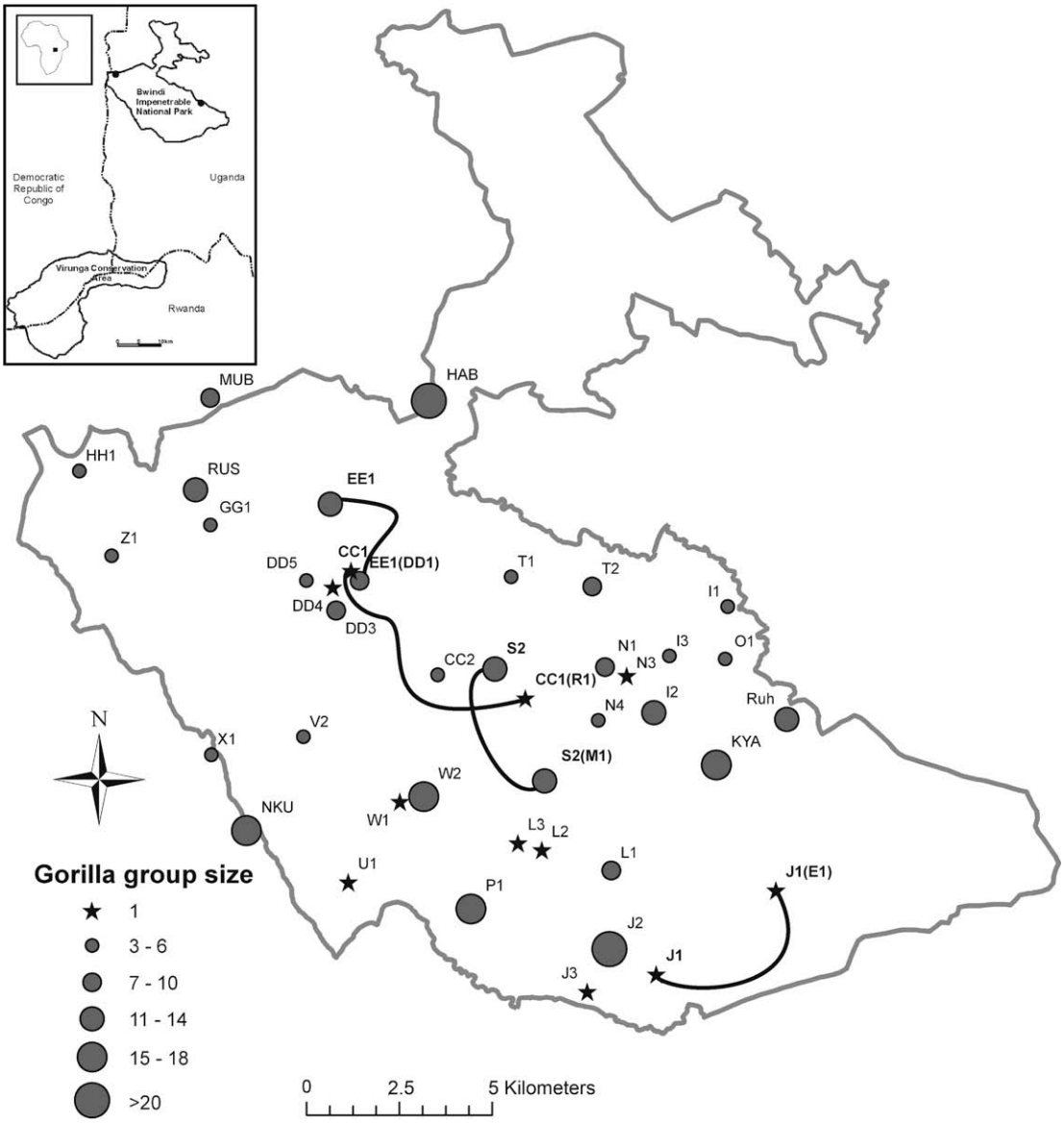

Fig. 2 – Map of the Bwindi Impenetrable National Park with the locations of gorilla groups found during the census. Stars denote lone silverbacks whereas circles represent groups. For groups sampled at several nest sites, a single nest site was chosen for depiction on the map. The size of the circle corresponds to group size. Each group and lone silverback is labeled with a unique identifier tag. Groups and silverbacks that were double-counted during the census are shown in bold and connected by a line. The identifier used in the field-based counts for the double-counted groups is given in brackets.

counted for at the group's biggest nest site. Thus, the genetic results directly show that 40 individuals should be removed from the field-based estimate.

Finally, a group with a home range overlapping one of the habituated groups was suspected to have been missed during the census due to elephant activity precluding gorilla track-

ing. After the census team left the area, there was another attempt to find this group. A nest site with four nests was collected and the total nest-based count was accordingly increased by four. However, genotyping revealed that these four individuals belonged to an already counted group. The actual missed group was subsequently collected and found to contain 11 individuals, which were added to the genetic-based population size estimate, increasing it by seven, but were not included in the nest-based census count (Table 1). Thus, before incorporating any correction factors, the nest-based and genetic-based population size estimates differed by 33 individuals (317 versus 284 individuals).

For seven groups (~1/4 of the total population), we genotyped multiple nest sites (two sites each for six groups, three sites for one group) (Table 1). We encountered four instances in which a new genotype was found at the smaller nest site. Three of these cases were infants, based on the dung size measurements. Missed infants, however, were accounted for with a correction factor (see below). In only one case did the new genotype belong to a nest-builder (adult or juvenile individual). Thus, in seven groups comprising 68 individuals, one new genotype was found after analyzing a second nest site. Extrapolating this result to the remaining 21 groups containing 179 genetically-identified individuals suggests that analyzing a further nest site for each group would have resulted in the detection of additional three individuals. Consequently, we correct our genetic estimate by adding three gorillas (Table 3).

We next assessed the rate of 'double-nesting' by individual gorillas. Out of all nest sites genetically analyzed, we found 24 cases of gorillas building more than one nest or infants defecating in more than one nest. With 306 individuals' genotypes at all these nest sites, the overall rate of individual double-nesting was 7.8%. As mentioned above, we were unsuccessful in genotyping 27 samples that we classed as new unique gorillas and added to the genetic census total (Table 3). However, some proportion of these gorillas may have built more than one nest and produced more than one dung sample. We can assume that 7.8% of the 27 untyped samples represent a duplicate sample, and reduce our genetic census estimate by two individuals (Table 3).

When looking at individual nest sites, the rate of double-nesting ranges from 0% to 43% (SD = 10.4%). This large variance is attributable to differences in group size and makes it impossible to predict the rate of double-nesting for any given nest site. Consequently, no estimations of correction factors for double-nesting at the group level can be made.

Infant dung is often difficult to detect due to its small size and it is assumed that one-third of infants is missed during the census (McNeilage et al., 2001, 2006). The field-based census counted 38 infants in unhabituated groups and estimated that another 19 were missed. By using molecular sexing information and counting the number of adult females and infants in the groups, we attempted a more precise estimate of the number of unsampled infants. The five habituated groups contained 19 infants at the time of the census. We also identified 27 adult females in these groups. Thus, the ratio of infants to adult females is 0.7. Combining dung measurement and sexing information, we estimate at least 62 adult females in the unhabituated groups and possibly as many as 69 when including female samples for which dung measurements could not be taken. Given the infant to adult female ratio and that we found 32 unique infants in unhabituated groups, the number of missed infants after incorporating genetic information is 12–17. After applying all of the described correction factors, the total estimate based on field data was 336 individuals, compared to an estimate based on field and genetic data of 302 individuals (Table 3).

4. Discussion

4.1. *Using genetic and field data to census populations*

This study demonstrates the advantages of combining genetic approaches with field methods for estimating the population size of rare and elusive species. After genotyping 384 samples at 16 microsatellite loci, the population size estimate based solely on field methods was reduced by 10.1%, to 302 individuals. We were able to directly compare field and genetic counts and find the sources of error in the nest count-based estimate. Although the traditional field method correctly identified most social units (of 41 social units found in the field, 32 were correct: 78.1%) and the genetic results confirmed five cases of double-counts of groups and two cases of double-counts of lone silverbacks in the field, the genetic analysis identified several discrepancies. The difference between the field and genetic counts can mainly be explained by the difficulty of distinguishing groups and solitary individuals based on field information alone, accounting for 6.7% of discrepancy. It proved difficult to distinguish groups of similar size that were found by different teams in neighboring sectors and thus not connected by a trail. In addition, for one of the groups, double-counting in the field was facilitated by a large discrepancy in the number of nests found in neighboring sectors (groups EE1 with 17 nests and presumed group DD1 with 10 nests (Table 1)). For lone silverbacks that sometimes travel much farther than an average group (Yamagiwa, 1986; Watts, 1994), the situation is even more challenging, since nests found far away from each other might still be from the same individual. In contrast, nests in close proximity to each other might have been constructed by different individuals (Table 1, Fig. 2). Double-nesting by individual gorillas contributed to 3.5% of the excess in the field estimate, while the rest of the discrepancy can be attributed to random events such as data entry errors and methods of calculating correction factors.

Second, we aimed to evaluate the changes in population size since the last census was carried out in Bwindi in 2002. Our results call into question the previously inferred positive trend in the population dynamics of Bwindi mountain gorillas. Over the last decade, the population was believed to show a constant, though slow, annual growth of approximately 1% (McNeilage et al., 2001, 2006). However, the imprecision of gorilla censusing via indirect signs, as detailed here, means that this trend was inferred from unreliable estimates. Genetic counts reflect the minimum number of unique individuals present in the population, since only samples from groups and individuals that were found during the complete sweep could be genotyped. Thus, they allow us to estimate a minimum population size that can be used as a benchmark for

future censuses. The error of 10.1% exceeds the estimated 5% increase in population size in the last 4 years, making it impossible to formulate any conclusions about the population size changes of Bwindi mountain gorillas. Although it seems unlikely that the population underwent a large reduction in size, the errors associated with the complete sweep method prevent any direct comparisons between the census in 2006 and those undertaken in 1997 and 2002.

Third, we found that even after genotyping almost the entire Bwindi gorilla population we cannot formulate reliable correction factors that would justify the use of the complete sweep method without concurrent genetic censusing in the future. Even though the rate of double-nesting can be expected to be the most persistent source of bias, it varies substantially between groups and we cannot predict the extent to which it has influenced previous censuses. For example, in western gorillas the rate of double-nesting can be affected by local climatic conditions that differ over the course of the year (A. Todd, personal communication). Nest-count based censuses can produce both overestimates and underestimates of the population size, and one is unable to assess the sources and extent of error. Overestimates can result from double-counting of groups and lone silverbacks. Underestimates can be produced if groups remained undetected and if groups of similar size but different identity are lumped together and considered a single group. A limitation of the complete sweep method is that the assumption that all individuals were found and counted only once cannot be evaluated. However, our study effectively shows that using molecular means of individual identification we can overcome these shortcomings.

Finally, we wanted to evaluate the suitability of molecular censusing for population size estimates of great apes. This study successfully demonstrates that genetic methods can be used to estimate population size of mountain gorillas and potentially other mammalian species. We were able to genotype over 350 non-invasively collected samples and refine our understanding of mountain gorilla population dynamics. We suggest that in the future, studies of rare and elusive species will more heavily rely on molecular census techniques. However, the prerequisite for this is the ability to collect samples from the species of interest. Several studies have shown that even if samples are collected opportunistically, reliable population size estimates can be produced for rare and elusive animals (Bellemain et al., 2005, M. Arandjelovic, personal communication). To reduce logistic effort and additional costs for coordinating sampling sessions, whenever possible sampling should be carried out in parallel to the field-based census, as was done in this study. We calculated costs for genetic census using prices in 2006. This estimate will vary depending on the method used, type of samples obtained, and the number of loci genotyped. In our case, expendable supplies for the genetic census (collection materials, DNA extraction, quantification and genotyping; but not personnel costs) added EUR 12,000 to the costs of the field census.

Bwindi Impenetrable National Park contains around half of global mountain gorilla population, while the rest is found in the Virunga Volcanoes, only ~25 km away from Bwindi. In 2003, the Virunga population numbered 380 individuals, as estimated by a census utilizing the same complete sweep method as this study (Gray et al., in press). However, in the Virunga Volcanoes, approximately 71% of the gorillas were habituated at the time of the census and the actual known composition and size of the groups was used for the population size estimates. Thus, the potential error associated with nest-counts was greatly reduced and could only have affected the estimate of 80 individuals in unhabituated groups. However, mountain gorillas still remain of high conservation concern due to the threats of poaching, habitat destruction, illegal activities, and risks inherent to small populations, including disease. Several cases of illegal gorilla killings in the Democratic Republic of Congo occurred in 2007, reducing the population size by 3% (Williamson and Fawcett, 2008). Thus, protecting the threatened mountain gorillas still remains a high conservation priority. Having accurate population size estimates is crucial for monitoring the population, raising funds and public awareness, and for assessing the effectiveness of various conservation strategies.

4.2. *Future directions*

As discussed above, genetic censusing should accompany any future complete sweep censuses of mountain gorilla populations. An alternative to the complete sweep method would be the use of a mark-recapture approach with genetic identification of groups and individuals. While we cannot prove that this method would produce more reliable estimates, the setting in Bwindi and the Virungas satisfies many of the underlying assumptions of the mark-recapture method (Otis et al., 1978; Borchers et al., 2002). The populations are closed because they are isolated from any other gorilla populations and from each other and, given the long interbirth interval of mountain gorillas of approximately 4 years and a low adult mortality rate (Robbins and Robbins, 2004), there should be no further issues of open population sampling. Various methods have been developed to control for the biases of sampling heterogeneity (Otis et al., 1978). Randomization of sampling locations between first and subsequent sampling sessions can potentially help overcome the violation of the assumption of random mixing. When the re-sampling rate is high enough (approximately three samples are collected for every assumed individual (Miller et al., 2005; Solberg et al., 2006; Puechmaille and Petit, 2007)), the confidence intervals for population size estimates provided by genetic mark-recapture approach will be very narrow (Miller et al., 2005; Petit and Valiere, 2006). For instance, the 95% confidence interval translated into 5.6% of the population size estimate for forest elephants (Kohn et al., 1999) and 1.5–15% for giant pandas (Zhan et al., 2006). Because the terrain in Bwindi and the Virungas is especially steep and densely vegetated, future work is needed to evaluate the challenges of implementing the mark-recapture method.

The study presented here highlights the advantage of using molecular techniques for censusing the populations of rare and elusive animals. Combining both field and molecular methods will allow efficient population size estimates for other mammalian populations, including the endangered great apes. Importantly, besides yielding reliable estimates of population sizes, molecular methods can provide insights

into topics such as sex ratio, relatedness and dispersal patterns. The advantage of using molecular methods for population size estimates lies in its easy standardization for use in different habitats and for different species. Compared to the most commonly used census method based on line transects, molecular censusing does not need auxiliary variables, such as estimates for nest decay and construction rate, bypasses observer and site-specific effects, and allows clear differentiation between sympatric species that might leave similar tracks. Thus, supplementary molecular censusing could result in reduced variance and produce more accurate estimates than traditionally applied census methods alone.

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