Genetic identification of elusive animals: re-evaluating tracking and nesting data for wild western gorillas

B. J. Bradley¹, D. M. Doran-Sheehy² & L. Vigilant³

¹ Department of Zoology and Christ's College, University of Cambridge, Cambridge, UK
² Department of Anthropology, Stony Brook University, Stony Brook, New York, USA
³ Max Planck Institute for Evolutionary Anthropology, Leipzig, Germany

Keywords
- genotyping; DNA; microsatellite; nests; identification; census; faeces; apes.

Abstract
Western gorillas Gorilla gorilla have been exceedingly difficult to habituate to the presence of human observers. Nevertheless, researchers have amassed a wealth of information on population densities and group structure for this ape species by locating and counting the sleeping nests of wild individuals. Such nest-count studies have suggested that western gorilla groups often have multiple silverbacks and these multimale groups occasionally divide into smaller subgroups. However, observational data from forest clearing sites and from a few recently habituated western gorilla groups show no evidence of multimale family groups or of subgrouping. This discrepancy underscores a long-standing question in ape research: How accurately do nesting sites reflect true group compositions? We evaluated these indirect measures of group composition by using DNA from faeces and hair to genetically identify individual gorillas at nesting sites. Samples were collected from unhabituated wild western gorillas ranging near Mondika Research Center in the Central African Republic and Republic of Congo. DNA extracted from these samples was genotyped at up to 10 microsatellite loci and one X–Y homologous locus for sex identification. Individuals were then identified at nesting sites by their unique multilocus genotypes, thus providing a ‘molecular census’ of individual gorillas. Results confirm that western gorillas often build more than one nest at a nesting site and, thus, nest counts can be highly inaccurate indicators of group size and composition. Indeed, we found that nest counts can overestimate group size by as much as 40%, indicating that true gorilla population numbers are probably lower than those reported from census surveys. This study demonstrates how genetic analysis can be a valuable tool for studying and conserving elusive, endangered animals.

Introduction
Wild apes are notoriously difficult to study because the process of habituating them to the presence of human observers can take years or even decades (Tutin & Fernandez, 1991; Blom et al., 2004; Doran-Sheehy et al., 2007). But by tracking ape trail signs through the forest and examining ape sleeping sites, researchers can obtain valuable information about wild ape populations, even when the apes cannot be regularly observed (Schaller, 1963). Such ‘indirect’ studies have been especially important for advancing our understanding of wild western gorillas Gorilla gorilla, an ape species that has proved particularly difficult to habituate to human presence and observation (Tutin & Fernandez, 1991; Doran-Sheehy et al., 2007).

All weaned gorillas build their own nest each night in a new location, and most individuals defecate in or beside the nest before leaving the next morning (Tutin et al., 1995; Melhman & Doran, 2002). Nests can vary from elaborate bowl-shaped supports constructed from multiple plant stems or branches to bare-earth nests characterized by a flattened-earth ‘butt print’ and the presence of faeces and shed hair. The age of the individual who slept in each nest can be estimated from the size of the associated dung lobes, and the presence of the adult male ‘silverback’ can be inferred from the presence of white hairs in the nest. By counting nests and examining the size of the associated dung, one can estimate the size and composition of the gorilla group, without necessarily observing the gorillas themselves.

Estimations of western gorilla population densities are largely based on such nest-count censuses (Tutin & Fernandez, 1984; Fay & Agnagna, 1992; Blom et al., 2001; Brugiere & Sakom, 2001; Dupain et al., 2004). By counting nests found along transect lines and taking into account an estimated age for each nesting site based on the degree of nest and dung degradation, census parties have found that western gorilla population densities can show marked regional
variation (~0.1–3.8 gorillas km⁻²). Obtaining accurate census data is increasingly important given the recent rapid decline in gorilla population numbers (Walsh et al., 2003; Caillaud et al., 2006), though there remains some debate about the best survey methods to employ (Blom et al., 2001; Walsh & White, 2005).

Gorilla nest counts have also been important for studying western gorilla social structure and ranging patterns (Remis, 1993, 1997; Tutin et al., 1995; Doran & McNeillage, 1998; Goldsmith, 1999). Indeed, until relatively recently, nest counts and gorilla tracking were our primary source of information on the daily lives of wild western gorillas (Doran & McNeillage, 1998). Gorilla tracking involves following, usually with help from local expert animal trackers, trail signs, such as feeding remains, knuckle- or foot-prints and other signs of disturbance to the forest vegetation. By tracking gorilla groups from nesting site to nesting site (day-to-day), researchers have estimated ranging patterns and monitored temporal changes in group size and composition.

Interestingly, several of these indirect studies found suggestive evidence that western gorillas, like mountain gorillas, frequently form groups with multiple silverbacks (Remis, 1997; Goldsmith, 1999). This inference came from the observation that nesting sites often have more than one nest with silverback-sized dung. In addition, nest counts sometimes vary markedly when tracking groups from day to day. For example, a group sleeping site might have 15 nests on the first and second day of tracking, but only half that number on the third day, suggesting that large multi-silverback groups are sometimes breaking into smaller subgroups. Given that western gorillas incorporate substantial quantities of fruit in their diet (Williamson et al., 1990; Doran & McNeillage, 1998), it was thought that large groups are probably breaking into smaller subgroups to avoid within-group feeding competition when resources are limited (Goldsmith, 1999).

However, observational data from recently habituated groups (Bermejo, 2004; Blom et al., 2004; Doran-Sheehy et al., 2007) and from sites using surveillance platforms at the edge of forest clearings (e.g. Magloixn, Querouil & Gautier-Hion, 1999; Parnell, 2002; Gatti et al., 2004) have found little or no evidence of western gorillas forming either multi-silverback groups or smaller subgroups that nest apart [although group spread while foraging can span several hundred metres (Doran & McNeillage, 1998)]. All of the observed family groups of western gorillas described to-date, have only a single silverback and show relatively stable size and composition. However, there are only a handful of habituated groups, and observations from forest clearings provide a limited glimpse of gorilla daily life [e.g. gorillas visiting Mbeli Bai spend only ~2% of their time there (Doran & McNeillage, 1998)]. Thus, as a recent review of gorilla sociality points out (Harcourt & Stewart, 2007), whether or not western gorillas sometimes form multimale groups that break into smaller subgroups remains an open question due to the discrepancy between the findings of the earlier, indirect studies and the more recent observational data.

Potential sources of inaccuracies in nesting and tracking data might account for this discrepancy. Because gorilla groups have overlapping home ranges, it is notionally possible that researchers sometimes switch, unknowingly, from following the trail signs of one group to following those of a different group. Similarly, nest counts might underestimate group size if some nests are unintentionally overlooked, or overestimate group size if some individuals build multiple nests (Tutin et al., 1995).

Here we examine this issue by identifying wild western gorillas at gorilla nesting sites using individually unique multilocus microsatellite genotypes. By typing the DNA from hair and faeces found at each nest, we provide a more accurate census of gorillas present at each nesting site. Specifically, we ask: How well do nest counts represent group composition? How accurate are ranging data based on tracking? And how reliable is dung size as a general indicator of sex (i.e. is silverback-sized dung always male)?

Methods

Study area, tracking and sampling

Hair and faecal samples were collected at nesting sites of wild western gorillas at Mondika Research Center, located on the border of the Central African Republic and Republic of Congo (02°21′N, 016°16′E; Fig. 2). Although there are now habituated gorilla groups at Mondika (Doran-Sheehy et al., 2007), at the time of sample collection habituation was still in progress and all samples were collected from unhabituated individuals. All nesting sites discussed here were located and sampled <10 h after they were vacated by the gorillas. Almost all nests had associated dung and/or shed hairs, including the two tree nests, which had associated dung just below the nest. All other nests were on the ground or on low-rising vegetation.

Fresh nesting sites were located by tracking with the aid of local BaAka field assistants, who are exceptionally skilled at locating and following animals through the forest (Mehlman & Doran, 2002). Note, however, that this study employed traditional tracking methods involving only short, infrequent contact with the gorillas themselves (usually ~1–2 contacts day⁻¹). In 2001, the tracking protocols at Mondika changed and contact was then maintained frequently throughout the follow (Doran-Sheehy et al., 2004, 2007).

Over the course of this study, many more nesting sites were sampled than could feasibly be analysed. Genetic analysis therefore focused on a subset of these sampled nesting sites and these were selected, to a large extent, based on the success rate of silverback samples. That is, if the sample(s) from the silverback yielded low quantities of DNA, then that nesting site was generally not included in the study. However, there is no reason to think that this selection process would bias our results. Ultimately, we examined, in detail, ‘complete’ (all or almost all nests were typed) samples from set of five independent nesting sites (i.e. thought likely to be from different groups) found
throughout the study site. In addition, we analysed a set of samples from one consecutive tracking series in which we tried to track one gorilla group from nesting site to nesting site over the course of 8 days. Each morning we located the nesting site used by that group the night before and collected samples at each nest.

Detailed maps showing the relative location of each nest and each sample were drawn at each nesting site (Fig. 3). The age class of the individual who slept in each nest was estimated by the size of the associated dung following well-established size criteria (Schaller, 1963; Williamson et al., 1990; Goldsmith, 1996). Dung was broadly assigned to one of the following categories (Fig. 1): ‘infant’ (<2 cm wide bolus), ‘juvenile’ (~2–4 cm), ‘adult’ (~5–6 cm), ‘silverback’ (>7 cm) or ‘unknown adult’ (quantity of faeces exceeded that of juvenile or infant, but dung lobs were not distinct enough to distinguish between adult and silverback). Dung categorized as adult could come from adult females or blackback males, and these were distinguished through DNA-based sex assignments. Identification of silverback nests were further aided by the presence of numerous long white hairs.

Upon finding a nesting site, the adjacent region (within a radius of c. 30 m) was carefully scanned, but we cannot exclude the possibility that a nest was occasionally overlooked.

Detailed methods of sample collection and storage are described in Bradley et al. (2004). Briefly, ~5 g of fresh faeces were placed in 50 mL tubes pre-filled with 20 g silica gel beads for desiccation. Shed hairs were stored in glassine envelopes in a dry box. Samples were stored at ambient temperatures in the field for up to 4 months, before reaching the laboratory where they were stored at +4 °C.

DNA extraction, quantification and sexing

Genomic DNA was extracted from ~100 mg of dried faeces using the QIAamp DNA Stool Kit (Qiagen, Hilden, Germany) as described in Bradley, Boesch & Vigilant (2000). Final elutions were in 200 μL of Qiagen buffer AE and were aliquoted and then stored at −20 °C. Genomic DNA was extracted from single hair follicles following Vigilant (1999). Each extraction series included multiple negative controls. The amount of amplifiable genomic DNA in each extract was quantified by a real-time PCR assay following Morin et al. (2001). This allowed for the exclusion of low-concentration DNA extracts (~<15 pg μL−1) from subsequent analyses.

The sex of each individual was determined by genotyping an X–Y homologues locus, a segment of the gene *Amelogenin*, as described in Bradley, Chambers & Vigilant (2001).

Microsatellite genotyping

Primer sequences and PCR conditions for each of the 10 microsatellite loci (vWF, D1s550, D2s1326, D4s1627, D5s1470, D10s1432, D7s817, D7s2204, D16s2624, D8s1196) used in this study are described in Bradley et al. (2000). Although primers for one locus, D8s1106, were found to amplify extraneous alleles (DNA from bacteria in the faeces) using DNA extracted from faeces of mountain gorillas (Bradley & Vigilant, 2002), such alleles were rarely amplified (~<1% of amplifications) from western gorilla DNA extracts and sufficient replications were conducted to ensure that only reproducible alleles were scored. That is, homozgyous genotypes were confirmed by multiple independent replications (usually seven) and heterozygous genotypes were confirmed by scoring each allele at least twice in two or more reactions (following Morin et al., 2001). Moreover, D4s1627 shows a size range in gorillas that does not overlap with that in humans (Bradley et al., 2000), and this locus allowed for an easy verification that gorilla, not human, DNA was being amplified. Similarly, other indicators of contamination (e.g. more than two alleles per locus; successful amplification from extraction and PCR-negative controls) were exceedingly rare.

The 5’-end of each forward primer was fluorescently labelled and PCR products were separated through capillary electrophoresis (ABI 310 and 3100, Foster City, CA, USA). Allele sizes were determined relative to a commercially available size standard (HD400 ABI).
Individual identity and group composition

Because samples were collected from unhabituated gorillas that were not directly observed at the time of sample collection, individuals were identified only by their multilocus genotypes. All genotypes were compared using the Identify Check utility of CERVUS (Marshall et al., 1998). All genotypes that mismatched at one to two loci were then examined by eye to confirm that mismatches were unlikely to be attributable to allelic drop out.

The probability of individual identity calculated for all 10 loci (following Waits, Luikart & Taberlet, 2001), P(ID), was $4.70 \times 10^{-11}$. The probability of individual identity among siblings, P(ID-sibs), was $1.66 \times 10^{-6}$ for all 10 loci, indicating that even closely related individuals are exceedingly unlikely to have the same genotype. However, because not all samples were completed at all 10 loci, a ‘worst case scenario’ P(ID) was calculated for the three least variable loci (i.e. the most conservative estimate using a subset of three loci). Results were still statistically significant ($5.98 \times 10^{-3}$), indicating that two randomly drawn individuals would have the same genotype at these three loci <1% of the time. Thus, multiple piles of faeces yielding the same genotype (and comparable at a minimum of three loci) were assigned with confidence to a single individual. The individually unique multilocus genotypes are referred to as ‘genetic profiles’. When the same individual, that is, the same genetic profile, was identified at multiple nesting sites, those nesting sites were assumed to represent the same group. In this study, matches were made only among nesting sites that were sampled within a week or two of each other and thus it is unlike that group compositions would have changed (e.g. by females transferring) during this time.

Results

Genetic identification at nesting sites

Combining genetic profiles with nesting maps allowed direct evaluations of nest count accuracy. A specific example illustrating this is given in Fig. 3. In this example, the nest counts alone suggested that this gorilla group contained: two silverbacks, 16 adults, two juveniles and one infant, giving a total of ~18 adults. When genetic identifications were combined with the nesting site data, the following individuals were inferred: one silverback, seven ‘blackback’ males, four females, two juveniles and one infant. Samples from one nest yielded insufficient DNA for genotyping. Thus, combining the genetic profiles with the nest counts indicates that this group actually contains 12–13 adults, ~30% fewer than the estimate based on nest counts alone. It is also worth noting that in this case, we would have assumed that most of the nests with adult-sized dung would have belonged to adult females, but in fact the majority belonged to blackback males.

This nesting site was not representative of all nesting sites. We examined five nesting sites at which samples from all, or almost all, nests could be genotyped (Table 1). For three of these nesting sites (Nesting sites P, B, M; Table 1), the genetic data indicated that the nest counts alone overestimated group size (by 20–40%). The other two nesting sites showed a general concordance between the nest counts and the genetic data, that is, one individual was uniquely identified at each nest.

Tracking from nesting site to nesting site

When we compared genetic profiles at nesting sites that were part of one ‘consecutive tracking series’ (Fig. 2), we found that we were not, in fact, always following the same group. Nest counts during this consecutive 8-day follow ranged from 5 to 11. We could genotype some of the samples from five of those eight nesting sites (Day 1, Day 5, Day 6, Day 7, Day 8; Table 2, Fig. 2). The silverback of Day 5 matched that of Day 7, but the silverbacks of Day 1 and Day 6 did not match Day 5/7, nor did they match each other. Unfortunately the silverback samples of Day 8 yielded insufficient DNA for accurate genotype, but one adult female and one infant (from different nests, not a mother–infant pair) could be genotyped from the Day 8 nesting site and these

Table 1 Inferred gorilla *Gorilla gorilla* group compositions based on nest counts alone versus nest counts with genetic identifications (genetic IDs)

<table>
<thead>
<tr>
<th>Inferred group composition</th>
<th>Based on nest counts alone</th>
<th>Based on nest counts + genetic IDs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adult total</td>
<td>Juvs</td>
</tr>
<tr>
<td>Nesting site P 2</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>Nesting site B 1</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Nesting site M 1</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Nesting site N 1</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Nesting site J 1</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

aDiscrepancy = per cent difference between adult total based on nest counts alone and adult total based on nest counts and genetic identifications.

SB, silverback; Juv, juvenile; Adult?, dung was adult-sized in quantity but too deteriorated to assess whether it might be silverback; M, male; F, female.
matched individuals from Day 5 and Day 7. Thus, it seems likely that the same gorilla group was sampled on Day 5, Day 7 and Day 8, but different groups were sampled on Day 1 and Day 6. Thus, between Day 1 and Day 5, we unknowingly switched to the trail of different group. Between Day 5 and Day 6 we again picked up the trail of a different group, although by Day 7 we returned to the same group we were with on Day 5 and remained with that group until Day 8.

**Dung size accuracy**

Of the 21 silverback-sized dung samples typed using the X–Y homologous marker for assigning sex, 19 typed as male and two typed as female. Of the two samples that typed as female, one was found in a nest with no hair and one was found while tracking (i.e. was not associated with a nest). Therefore, overall, at least 10% of these samples were incorrectly assigned to the silverback category. However, the nests categorized as silverback based on both dung size and the presence of numerous long, white hairs were always assigned correctly. In addition, it is worth noting that there was a single case where an adult-sized sample yielded the same genetic profile as the silverback-sized sample at the same nesting site.

**Discussion**

Here we have conclusively demonstrated that western gorillas often build more than one nest at a nesting site and thus nest counts can be highly inaccurate indicators of group size and composition. Thus, the discrepancy between earlier indirect studies of western gorillas (i.e. based on nest counts and tracking), which found evidence of multimale groups and subgrouping, and more recent observations at forest clearing platforms, which find only one-male groups with generally stable size and composition, are likely due to the misleading nature of western gorilla nesting sites. Moreover,
population numbers for western gorillas, which are based on
nest count censuses, are likely overestimates.

Nesting sites that seemingly represented multimale (>1 silverback) groups in earlier indirect studies, were in fact probably cases where the single silverback built more than one nest, as was the case here for Nesting site P (Table 1, Fig. 3), or cases where female or blackback dung was mistakenly categorized as silverback. We found that nest counts can overestimate group size by as much as 40%, a figure that is worrisome given that this species is already highly threatened and population numbers are plummeting (Walsh et al., 2003). Moreover, although dung size is generally a good estimator of age/sex class, female dung was occasionally categorized as silverback (10% of silverback samples), suggesting that dung size itself can on occasion be misleading. A current study examining dung collected from the same individuals on numerous occasions indicates that dung size can indeed vary (by as much at 2+ cm) from day to day (K. Guschanski, pers. comm.). However, in our study silverback nest sites identified by both faeces size and the presence of white shed hair were always correctly typed as male and nest counts did accurately reflect group composition in two of five cases.

Unfortunately, it is difficult to calculate an ‘adjustment factor’ for estimating the true size of any one gorilla group based on nest counts alone. Environmental factors, such as rainfall and temperature, are known to influence nest building behaviours in western gorillas (Tutin et al., 1995; Mehlman & Doran, 2002), but we found no clear association between nest count accuracy and these environmental factors. That is, the three inaccurate nesting sites were not associated with more rainfall or lower temperatures compared with the two accurate nesting sites, making it difficult to identify factors that might indicate which nesting sites are likely to be misleading. In addition, although painstaking efforts were made to find all nests at each nesting site, nests might have occasionally been overlooked, possibly causing nest counts to be underestimates of group size in some cases. On balance, it seems likely that nesting counts alone overestimate the size and composition of western gorilla groups. But providing a precise value for this overestimate is not possible.

We also found that successfully tracking a single gorilla group over several consecutive days is difficult, even when working with highly skilled local trackers. As our analysis of one such consecutive tracking series shows, researchers can unknowingly track several different groups over the course of a few days. Because western gorilla groups often have overlapping home ranges and relatively frequent intergroup encounters (Doran-Sheehy et al., 2004), and many different groups can regularly visit the same shared resources (e.g. fruiting trees, forest clearings), it is not surprising that the paths of multiple groups often criss-cross over the course of a day. For this reason, estimates of day journey length (mean: 2 km, range: 0.4–4.9 km) and home-range size (15.4 km²) at Mondika are based on consecutive follows of semi-habituated or habituated groups where contacts with groups occurred frequently while tracking (Doran-Sheehey et al., 2004). These estimates of day journey length are slightly less than those based on traditional tracking and nest counting of unhabituated groups at a nearby site [mean: 2.6 km, range: 0.3–5.2 km at Bai Hokou (Goldsmith, 1996, 1999)].

The inaccuracies of the nest counts and the difficulties of following the path of a single group have probably both contributed to the earlier suggestions that western gorilla groups were temporally dividing into subgroups that would nest apart. Because our results confirm that nest count variation can be caused by inaccuracies in nest counts and tracking, and subgrouping has never been observed, we posit that western gorillas do not form subgroups that nest apart, but instead live in one-male groups that are generally cohesive when nesting.

These results have important implications for conservation. It is unclear whether the regional variation in gorilla density estimates might, in part, reflect the inherent difficulties in obtaining accurate census data via nest counts. In any case, due to ebola outbreaks and commercial hunting (Walsh et al., 2003; Caillaud et al., 2006), population numbers are declining rapidly. Genetic identification of individuals on a large scale would aid efforts to calculate densities and monitors groups over time. Here it is worth noting that a gorilla group identified at Mondika in 1999 was genetically identified (matching silverback and adult
female genotypes) again in the same general region 2 years later.

The costs of genotyping DNA from noninvasive samples, in terms of both time and money, have dropped rapidly in recent years (Puechmaille, Mathy & Petit, 2007), and we expect that genetic censusing studies, like the one presented here, will continue to gain broad application in behavioural ecology and conservation biology. This study, along with others (Zhan et al., 2006; Arrendal, Vila & Bjorklund, 2007; Jeffery et al., 2007), demonstrates that molecular tagging and censusing of wild, cryptic animal populations can, not only resolve long-standing questions about the behaviour and social dynamics of elusive animals, but can also highlight concerns and priorities for their conservation.

Acknowledgements

This project would not have been possible without the expert skills of the BaAka trackers working at Mondika and the assistance of numerous people in the field, including N. Shah, M. Wakefield, A. Lilly, P. Mehlman, D. Greer and C. Hicks. We also thank the WWF and WCS, and the Ministries of Scientific Research and Forest & Water in the Central African Republic and Republic of Congo. P. Braun, T. Otto, C. Tietze, C. Richter, K. Simonov, C. Köhler, S. Seifert and T. Biedermann provided expert lab assistance. We thank K. Guschanski for helpful comments on the paper and we thank E. Stokes and D. Schwindt for sharing the map of the study site used in Fig. 2. This research was funded by the Max Planck Gesellschaft, the National Science Foundation (SBR-9910399, SBR-9729126), Wenner-Gren Foundation, and the L.S.B. Leakey Foundation.

References


