*Journal of Heredity* 2005:96(6):679–687
doi:10.1093/jhered/esi117
Advance Access publication October 26, 2005



# Noninvasive Genotyping and Mendelian Analysis of Microsatellites in African Savannah Elephants

J. B. A. Okello, G. Wittemyer, H. B. Rasmussen, I. Douglas-Hamilton, S. Nyakaana, P. Arctander, and H. R. Siegismund

From the Molecular Biology Laboratory, Makerere University Institute of Environment and Natural Resources, P. O. Box 7298, Kampala, Uganda (Okello, Nyakaana); the Department of Environmental Science, Policy and Management, 201 Wellman Hall, University of California at Berkeley, CA 94720–3112 (Wittemyer); the Animal Behaviour Research Group, Department of Zoology, University of Oxford, South Park Road, Oxford OX1 3PS, UK (Rasmussen); Save the Elephants, PO Box 54667, Nairobi 00200, Kenya (Wittemyer, Rasmussen, Douglas-Hamilton); and the Department of Evolutionary Biology, University of Copenhagen, Universitetsparken 15 DK-2100 Copenhagen Ø, Denmark (Arctander, Siegismund).

Address correspondence to J. B. A. Okello at the address above, or e-mail: jbaokello@muienr.mak.ac.ug.

## Abstract

We obtained fresh dung samples from 202 (133 mother-offspring pairs) savannah elephants (*Loxodonta africana*) in Samburu, Kenya, and genotyped them at 20 microsatellite loci to assess genotyping success and errors. A total of 98.6% consensus genotypes was successfully obtained, with allelic dropout and false allele rates at 1.6% ($n = 46$) and 0.9% ($n = 37$) of heterozygous and total consensus genotypes, respectively, and an overall genotyping error rate of 2.5% based on repeat typing. Mendelian analysis revealed consistent inheritance in all but 38 allelic pairs from mother-offspring, giving an average mismatch error rate of 2.06%, a possible result of null alleles, mutations, genotyping errors, or inaccuracy in maternity assignment. We detected no evidence for large allele dropout, stuttering, or scoring error in the dataset and significant Hardy-Weinberg deviations at only two loci due to heterozygosity deficiency. Across loci, null allele frequencies were low (range: 0.000–0.042) and below the 0.20 threshold that would significantly bias individual-based studies. The high genotyping success and low errors observed in this study demonstrate reliability of the method employed and underscore the application of simple pedigrees in noninvasive studies. Since none of the sires were included in this study, the error rates presented are just estimates.

In species that are rare, sensitive, at risk of extinction, or under intensive behavioral study, noninvasive genotyping is preferred because it avoids disrupting the animals under observation. Techniques for genotyping samples with low DNA quantity have become widely employed in molecular ecological studies in the last decade, particularly in analyses of samples obtained noninvasively (Broquet and Petit 2004). Noninvasive DNA analyses have been implemented in population censuses (Creel et al. 2003; Eggert et al. 2003; Ernest et al. 2000; Lucchini et al. 2002; Mills et al. 2000), parentage assignment (Constable et al. 2001), and mating systems and social structure analysis (Morin et al. 1994). However, concern over the accuracy of amplifying nuclear DNA markers, especially highly polymorphic microsatellites, from such samples is still prevalent (Bayes et al. 2000; Broquet and Petit 2004; Fernando et al. 2003; Maudet et al. 2004; Roon et al. 2005). Of particular interest are genotyping errors and null alleles, which can compromise conclusions drawn from such data when present in high frequencies (Broquet and Petit 2004; Dakin and Avise 2004; Hoffman and Amos 2005).

In the context of noninvasive sampling, genotyping errors can be generated during sampling (especially for animals with common toilets), DNA extraction, molecular analysis, scoring, data analysis, or by a variety of other factors, such as chance, human errors, and technical artifacts (Bonin et al. 2004). Microsatellite null alleles, however, can result from poor primer annealing due to mutations within flanking regions (Kwok et al. 1990), differential amplification of size-variant alleles (partial nulls; Wattier et al. 1998), or polymerase chain reaction (PCR) failure due to low template DNA quantity (Gagneux et al. 1997). Total eradication of genotyping errors is difficult because molecular techniques and sample handling are not perfect (Fernando et al. 2003; Flagstad et al. 1999; Wehausen et al. 2004). Therefore, systematic tracking of the origin and frequencies of genotyping errors are necessary for obtaining clean data sets (Bayes et al. 2000; Maudet

et al. 2004; Miller et al. 2002; Taberlet et al. 1996, 1999) and validating final results (Bonin et al. 2004; Hoffman and Amos 2005).

Quantification and reporting of error rates do not discredit final conclusions drawn from the data but provide a measure of the quality and trustworthiness of the results (Bonin et al. 2004). It is therefore essential to report error rates in molecular studies. Strategies for identifying, quantifying, and reducing genotyping errors include comparison of multiple samples (Frantzen et al. 1998; Taberlet et al. 1996, 1999), quantification of target DNA in extracts (Morin et al. 2001), independent calling of alleles and verifying genotypes derived noninvasively with those from more reliable sources (Fernando et al. 2003; Launhardt et al. 1998; Parsons 2001; Taberlet et al. 1999; Wasser et al. 1997), and estimation of null allele frequencies (Brookfield 1996; Chakraborty et al. 1992; Van Oosterhout et al. 2004). The quality of a microsatellite data set can also be inferred by testing deviations for Hardy-Weinberg proportions (Gomes et al. 1999; Hosking et al. 2004; Shaw et al. 1999). The inclusion of family data offers one of the most robust methods to estimate genotyping errors and validate final results (Hoffman and Amos 2005; Marshall et al. 1998). However, only a few studies have performed inheritance analysis of alleles for parent-offspring pairs (Bayes et al. 2000; Ewen et al. 2000; Launhardt et al. 1998; Smith et al. 2000). Achieving accurate and reliable genotypes (Bonin et al. 2004) and quantifying genotyping error rates for each species or population is recommended for any widescale genotyping study (Maudet et al. 2004).

Using dung samples collected from a free-ranging elephant population, we analyzed for variation at 20 microsatellite loci while applying repeat procedure to estimate genotyping error rates (Taberlet et al. 1996), and we assessed for mismatches of alleles among mother-offspring pairs (Marshall et al. 1998). Since maternity was based on field observations and behavioral association, mismatches across some loci may be rooted in inaccuracies in assignment of maternity (Marshall et al. 1998). High frequencies of null alleles are expected to leave a characteristic signature of repeated homozygote-homozygote mismatches between parent-offspring dyads (Pemberton et al. 1995). Genotyping errors would be expected to cause significant deviations from Hardy-Weinberg, particularly because of excess homozygotes (Gomes et al. 1999) and deficiencies and excesses of particular genotypes (Van Oosterhout et al. 2004). We present the analysis protocols conducted and make suggestions on techniques to minimize problems with noninvasively collected genetic resources. The results of this study validate our genotyping method, data set, and the conclusions to be drawn from it in forthcoming genetic analyses.

## Materials and Methods

### Study Area and Characterization of Individual Elephants

The study area is composed of Samburu, Buffalo Springs, and Shaba National Reserves in northern Kenya. It is part of the greater Samburu-Laikipia elephant population that once covered northern Kenya in the early 1970s but was reduced because of excessive poaching (Omondi et al. 2002). Save the Elephants, a Kenyan-based nongovernmental organization, has assembled detailed data on population demography, movement, and behavioral patterns of approximately 900 elephants roaming these three reserves and surrounding areas (Wittemyer et al. 2005a). Each elephant has been identified based on sex, age, and unique body features, enabling accurate sampling (Wittemyer 2001). All calves born after 1997 were observed shortly after birth, enabling definitive assessment of mother-calf pairs. Calves born before 1997 were assigned a mother based on close association with a specific female and may therefore include mother-calf assignment errors. Overall, sampled breeding females and their calves were estimated as being born from 1942 to 1994 and from 1987 to 2003, respectively.

### Sampling

Samples from 202 identified elephants were collected between 2001 and 2004, comprising 133 calves and 76 breeding females (7 individuals serve as both mother and calf, and most breeding females had more than one calf). Fresh dung samples were obtained using sterilized equipment soon after the animal defecated, minimizing potential misidentification of the target animal's dung and enabling collection of the mucosal layer of dung prior to desiccation. Epithelial cells sloughed from the gut lining and contained in the mucosal lining of the dung bolus yield undegraded DNA and low concentration of PCR inhibitors (Fernando et al. 2003; Flagstad et al. 1999). All samples were immediately preserved in sample vials containing either 25% dimethyl sulphoxide saturated with 5 M NaCl (Amos and Hoelzel 1991) or 70% ethanol and stored at ambient temperature during day of collection, 0°C in the field station, and −80°C in the laboratory. Because previous studies have shown that the aforementioned two samples' storage media perform equally well for fecal DNA (Frantzen et al. 1998), we used any of them whenever available to preserve samples analyzed in this study.

### DNA Extraction

Total genomic DNA was extracted from the samples following standard procedures for animal tissues in DNeasy tissue kit (QIAGEN GmbH, Germany), with a slight modification. From each dung sample, approximately 250 μL of the mucous-laden dung was placed in a 1.5 mL microcentrifuge tube with 180 μL lysis buffer and 20 μL proteinase K (20 mg/μL) solutions. The mixture was incubated overnight at 70°C in a 75 × g rotor. Digests were centrifuged at 6000 × g for 1 min in a microcentrifuge and the supernatants separated from the undigested plant materials in the tubes, after which the protocol from step 3 of the DNeasy procedure for animal tissue was followed. To minimize cross contaminations, all the steps from DNA extractions to amplifications included negative controls, and pre- and post-PCR work were conducted at separate locations with dedicated instruments and reagents.

**Table 1.** Loci analyzed on 202 individual elephants from Samburu, Kenya

| Locus | AT (°C) | PCR | A | Mo-Cf | M-M (N) | Null F | Errors | H-W (*P*) | $F_{IS}$ |
|---|---|---|---|---|---|---|---|---|---|
| LaT05 | 58 | 28 | 13 | 133 | 4 (1) | 0.011 | 0.028 | .037 | 0.027 |
| LaT06 | 52 | 27 | 19 | 130 | 0 (0) | 0.000 | 0.000 | .243 | 0.000 |
| LaT07 | 58 | 28 | 19 | 130 | 2 (0) | 0.033 | 0.012 | .002* | 0.069 |
| LaT08 | 58 | 28 | 11 | 131 | 2 (0) | 0.000 | 0.016 | .400 | 0.003 |
| LaT13 | 56 | 33 | 11 | 133 | 1 (0) | 0.000 | 0.009 | .244 | 0.000 |
| LaT16 | 54 | 33 | 10 | 122 | 2 (1) | 0.019 | 0.019 | .193 | 0.043 |
| LaT17 | 56 | 28 | 12 | 132 | 3 (0) | 0.006 | 0.025 | .704 | 0.017 |
| LaT18 | 56 | 32 | 11 | 132 | 2 (0) | 0.025 | 0.017 | .374 | 0.050 |
| LaT24 | 52 | 28 | 11 | 132 | 3 (0) | 0.020 | 0.021 | .002* | 0.043 |
| LaT25 | 52 | 38 | 9 | 113 | 3 (0) | 0.017 | 0.026 | .061 | 0.037 |
| LaT26 | 54 | 40 | 13 | 125 | 2 (0) | 0.042 | 0.015 | .231 | 0.084 |
| FH1 | 52 | 28 | 5 | 132 | 1 (1) | 0.000 | 0.024 | .125 | −0.006 |
| FH39 | 58 | 34 | 10 | 133 | 3 (1) | 0.018 | 0.028 | .334 | 0.037 |
| FH40 | 57 | 30 | 6 | 132 | 0 (0) | 0.000 | 0.000 | .224 | −0.089 |
| FH67 | 60 | 25 | 8 | 131 | 2 (0) | 0.000 | 0.030 | .884 | −0.001 |
| FH103 | 57 | 38 | 6 | 131 | 1 (0) | 0.000 | 0.024 | .263 | −0.038 |
| La4 | 55 | 40 | 5 | 132 | 3 (0) | 0.000 | 0.049 | .044 | −0.089 |
| La6 | 59 | 25 | 4 | 133 | 1 (1) | 0.037 | 0.027 | .022 | 0.080 |
| LafMS02 | 56 | 28 | 7 | 133 | 0 (0) | 0.000 | 0.000 | .661 | −0.047 |
| LafMS06 | 58 | 28 | 8 | 133 | 3 (0) | 0.000 | 0.043 | .426 | −0.022 |

AT = annealing temperature; PCR = number of polymerase chain reaction cycles; A = number of alleles detected; Mo-Cf = mother-offspring pairs; M-M (N) = genotypic mismatches (due to null alleles); Null F = null allele frequency based on Van Oosterhout et al. (2004); Errors = estimated error rate; H-W = probability for Hardy-Weinberg proportions; and $F_{IS}$ = Weir and Cockerham's analog (1984) of Wright's fixation index.

* Significant at 5% level after Bonferroni correction.

### Microsatellite Screening and Optimization

For a pilot study, we tested 40 polymorphic microsatellite loci previously isolated from the elephant genome on DNA extracted from fresh elephant dung samples. These loci are LaT05, LaT06, LaT07, LaT08, LaT13, LaT16, LaT17, LaT18, LaT24, LaT25, LaT26 (Archie et al. 2003); FH1, FH19, FH39, FH40, FH48, FH60, FH67, FH71, FH94, FH102, FH103 (Comstock et al. 2000); FH126, FH127, FH153 (Comstock et al. 2002); LA1, LA2, LA3, LA4, LA5, LA6 (Eggert et al. 2000); LafMS01, LafMS02, LafMS03, LafMS04, LafMS05 (Nyakaana and Arctander 1998); and LafMS06, LafMS07, LafMS08, LafMS09 (Nyakaana et al. 2005).

Although the original annealing temperatures of microsatellite primers used were already published by their developers, we determined the optimum annealing temperature at each locus by using the PCR gradient in a Hybaid Thermo-Express (Thermo Hybaid, UK). After optimization, we selected a panel of 11 tetra-nucleotide and 9 di-nucleotide repeat microsatellite loci (see Table1), based on clear banding patterns and high polymorphisms that they exhibited in a preliminary investigation involving 15 randomly selected individuals sampled from distinct family units (as defined in Wittemyer et al. 2005b). Amplification success rates and frequency of false alleles were estimated based on repeated genotyping of those individuals in order to find the number of replications that would be needed to get reliable genotypes from each noninvasive sample (Broquet and Petit 2004; Taberlet et al. 1996). All amplifications were done on a Hybaid Thermo-Express thermocycler, each in approximately 10 μL reaction volume containing 1–2 μL undiluted DNA extract solution, 1.5 mM $MgCl_2$, 10 mM Tris buffer, 50 mM KCl, 5 ng Bovine Serum Albumin, 200 μM each dNTP, 2 pmol each primer, and 0.5U AmpliTaq Gold DNA polymerase (Boehringer Mannheim GmbH). PCR products were visualized after electrophoresis on agarose gels to check for visible signs of contamination and spurious amplification profiles.

We developed an automated system of multiplexing based on the expected allelic fragment sizes of each locus, and we applied it throughout the analysis to reduce the cost of genotyping. Loci LaT08(FAM) + LaT05(FAM) + LaT17(FAM) + LaT26(JOE); FH67(FAM) + LaT06(FAM) + LafMS06 (JOE) + LaT24(JOE); FH40(FAM) + FH103(JOE) + LaT16(JOE) + La4(TAMRA); FH39(FAM) + LaT07(FAM) + LafMSO2(JOE) + LaT18(JOE); La6(FAM) + LaT25 (FAM) + FH1(JOE) + LaT13(JOE) were post-PCR multiplexed and separated on 4% polyacrylamide gels. Polyacrylamide gels were prerun for 1 hr to overcome electrophoresis artifacts, and multiplexed samples electrophoresed using a positive control to check gel consistency (Fernando et al. 2001). Alleles were separated on an ABI 377-automated DNA sequencer with Rox-500 as an internal size standard and analyzed using the programs GENESCAN 3.0 and GENOTYPER 2.1 (Applied Biosystems, Foster City, CA). In each gel analysis, electrophoresis lanes were manually tracked and checked, and a standard curve was generated by the "Local Southern Standard" to correct for any minor gel variation. Individual signal strength varied both within and between gels throughout the run, ranging from 50 to 6,000 electrophoresis peak height, but as matter of principle we only scored individual peaks with a height of $\geq 100$.

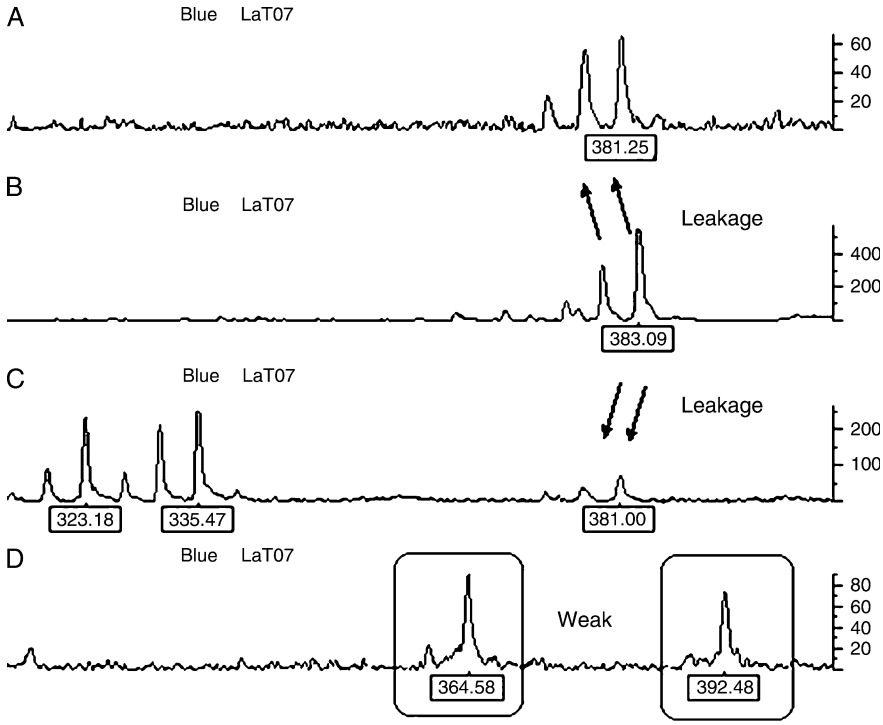


**Figure 1.** ABI-377 electropherogram showing characteristics of two potential error sources in microsatellite allele–scoring process, including false peaks due to leakage from a true amplification loaded in the neighboring lane (peaks of lanes A and C leaked from B) and weak peaks with < 100 fluorescent units that cannot be reliably scored (D).

### Genotyping Criteria

We used rigid criteria in scoring and accepting consensus genotypes in order to minimize potential genotyping errors (Schlötterer and Tautz 1992), including the scoring of alleles by two individuals experienced in microsatellite genotyping and the validation by a third person. False peaks that result from leakages of strongly amplified products in the neighboring lanes were not scored (e.g., peaks in lanes A and C are leaked from lane B; Figure 1A–C). Such peaks are approximately two base pairs shorter than their true peaks if lanes were loaded alternately with 2 min of short electrophoresis run in between. Weaker peaks (less than 100 fluorescent units of electrophoresis peak heights) were considered failed because they could not be reliably scored (Figure 1D).

Following a preliminary amplification success rate of 92.2% (70 failures out of 900 PCRs) and false allele frequency of 0.36% (3 false genotypes), we adopted recommendations from Bayes et al. (2000), Ernest et al. (2000), and Parsons (2001) and genotyped each individual locus at least twice to obtain a reliable genotype. Where an inconsistency or amplification failure occurred, that individual was regenotyped at least twice at the locus in question to verify alleles, and a majority consensus was taken (Taberlet et al. 1996). A genotype at a particular locus that consistently yielded ambiguous or indistinguishable stutter peaks would not be scored, and those successfully amplified in only a few of the repeat typing were considered failed. A consensus genotype was assessed and designated (1) as having had allelic dropout if it was typed homozygous once and subsequently reproduced as heterozygous of the same allele in replicate typing and (2) as having had a false allele if one or more alleles were detected in any of the amplifications at that locus (either homozygous or heterozygous) and not observed consistently in other repeat typing.

### Genotyping and Mendelian Error Analysis

We computed the rate of genotyping error as the proportion of cases in which errors were detected to the total consensus genotypes, as in Creel et al. (2003). Assuming all positive PCRs giving correct or consensus genotypes as being successful, we estimated the rate of allelic dropout ($p$) as the ratio of the number of consensus genotypes with allelic dropout cases to the total number of heterozygous genotypes obtained. The frequency of false alleles was estimated as the ratio of the number of consensus genotypes with one or more false alleles to the total number of consensus genotypes. The rate of allelic dropout was estimated based on heterozygous genotypes only, because truly homozygous genotypes do not lead to erroneous estimates (Broquet and Petit 2004). This approach enables the sum of allelic dropout and false allele rates to give an overall genotyping error rate in the study.

We also estimated Mendelian error rate across all loci using the program CERVUS 2.0 (Marshall et al. 1998), assuming

parent-offspring pairs as being equally independent and correct. Here, an error is defined as the replacement of the true genotype at a given locus with a genotype selected randomly under Hardy-Weinberg's assumption. If mother-offspring pairs are known from field data, the average genotyping error rate can be statistically estimated from the frequency of mismatches (Mendelian allelic incompatibility) between mothers and their offspring. Mendelian compatibility of alleles and associated genotyping error rate were therefore assessed for the overall 133 pedigree (mother-calf) relationships and separately for two partitions based on the age and thus relative confidence of the mother-calf pair. Because the individual monitoring of the study population did not begin until 1997, mother-calf pairs in which the calf was born before 1997 are estimated using association data, whereas calves from 1998 to 2003 are known from observations.

### Data Quality Assessment

Microsatellite data quality can be statistically assessed by testing deviations from Hardy-Weinberg proportions, allowing identification of either probable null alleles or allelic dropout, which leads to an excess of homozygotes (Gomes et al. 1999; Hosking et al. 2004; Shaw et al. 1999). An exact test for deviation from Hardy-Weinberg proportions at each locus was conducted based on a Markov chain algorithm (Guo and Thompson 1992) in the computer program GENEPOP 3.4 (Raymond and Rousset 1995). We performed sequential Bonferroni corrections (Holm 1979; Rice 1989) on $P$ values obtained at 5% significance levels to correct for type 1 error due to multiple comparisons. The potential presence of other genotyping errors, such as null alleles, short allele dominance (large allele dropout), or scoring or typographic errors in the microsatellite data set, was assessed using the computer program MICRO-CHECKER 2.2 (Van Oosterhout 2004).

## Results

### Genotyping Success and Mendelian Consistency

Of the 202 individual elephant samples genotyped at 20 microsatellite loci (4,040 genotypes), 98.6% achieved consensus genotypes. Of the 56 genotypes that failed to yield consensus genotypes, 17 (30%) consistently failed in repeat typing and 26 (46%) produced nonreproducible genotypes. The other errors detected and resolved by repeat genotyping were caused by (1) allelic dropouts leading to false homozygotes in 46 (1.6%) of all consensus heterozygous genotypes and (2) false alleles in 34 (0.9%) of all homozygous and heterozygous consensus genotypes combined.

Mendelian analysis of alleles in the 133 mother-offspring pairs showed consistent inheritance patterns in all but 38 of 2,660 total genotype pairs compared, with 5 of them having homozygote-homozygote mismatches and hence suggesting the presence of null alleles. The mean genotypic mismatch error due to the Mendelian incompatibilities observed was 2.06%. When the mother-calf elephant relationships were categorized into two groups (known and assumed pairs), we observed a slightly greater proportion of mismatched genotypes in the assumed group (calves born in 1987–1997; $n$ = 22, error rate = 2.44%) compared to the known group (calves born in 1998–2003; $n$ = 16, error rate = 1.68%). Although most of the Mendelian incompatible mother-offspring pairs had single mismatches, only two pairs had three genotypic mismatches and an additional three had two genotypic mismatches observed.

### Data Quality

On sequential Bonferroni correction, we observed significant deviations from Hardy-Weinberg proportions in 2 of the 20 microsatellite loci analyzed (Table 1), all due to heterozygote deficiency (LaT07, $P = .002$; LaT24, $P = .002$). Allelic dropouts in the data set would be expected to cause Hardy-Weinberg deviation due to excess of false homozygotes; however, no evidence of short allele dominance or large allele dropout was detected at any of the 20 polymorphic microsatellite loci analyzed. Microsatellite loci LaT07 ($r = 0.033$, $P > .05$) and LaT26 ($r = 0.042$, $P > .05$) showed evidence of null alleles as suggested by the general excess of most allele size classes, though not at a significant level (Figure 2). Across loci, null allele frequencies were low ($r < 0.05$) but with a relatively higher error rate observed in the tetra-nucleotide than di-nucleotide microsatellite loci analyzed (Table 1).

## Discussion

### Noninvasive Genotyping

We successfully obtained consensus genotypes in 98.6% of the total genotypes and the overall estimated genotyping error rate of 2.06%. Out of the overall genotyping errors estimated from consensus genotyping, 1.60% was attributed to allelic dropout, identified as false homozygotes in heterozygous genotypes, and 0.92% was attributed to false alleles, cases where one or more wrong alleles were observed at some loci in both homozygous and heterozygous genotypes. Contrary to the general assumption that genotyping errors are greater in di- than in tri- and tetra-nucleotide loci (Morin et al. 2001; Schlötterer and Tautz 2002; Taberlet et al. 1999), our findings included a relatively uniform level of null allele frequencies in the tetra-nucleotides and a somewhat lower level in di-nucleotides (Table 1). Other studies have reported similar trends (Fernando et al. 2003; Gagneux et al. 1997). Microsatellite null alleles lower than a frequency of $f < 0.2$ cause only a slight underestimation of average exclusion probability at that particular locus (Dakin and Avise 2004). The levels observed in this study will not substantially affect individual-based studies. Such a relatively low frequency of microsatellite null alleles and error rates that we observed in this study are similar in magnitude to levels from other quality DNA sources, such as blood and tissue (Jeffery et al. 2001), although genotyping errors in the latter are not always reported (Dakin and Avise 2004).

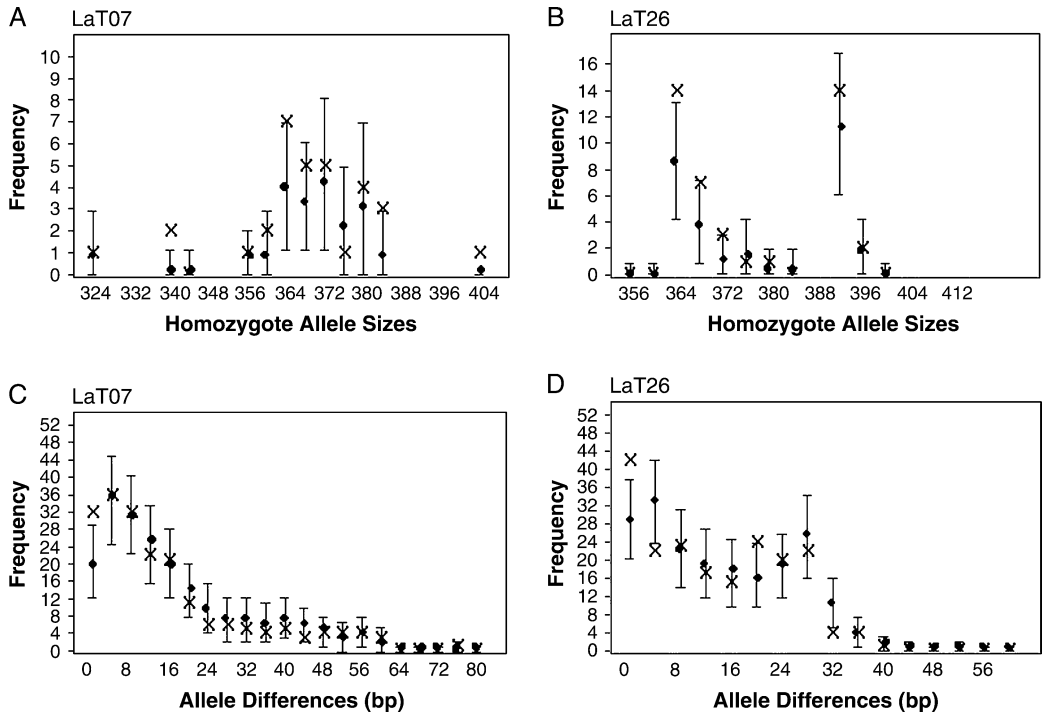


**Figure 2.** Graphs showing homozygote frequencies by class size (A, B) and allele size differences (C, D) for microsatellite loci LaT07 and LaT26, respectively. The observed frequencies of homozygote classes were compared to values generated after 10,000 Monte Carlo simulations. Bars in each graph represent the range of simulated values within 95% confidence interval; the mean values are shown as black circles and the observed values as black crosses. No statistically significant evidence for null alleles was suggested by the general excess of homozygotes for most allele size classes ($P > .05$).

### Mendelian Analysis

Mendelian analysis on known mother-offspring showed that 38 (0.95%) of all genotype pairs were incompatible, yielding a Mendelian-based genotyping error rate of 2.06%. We believe that many of the mismatches observed in the post-1997 mother-calf category (error rate = 1.68%) arose from genotyping errors and possibly mutations rather than inaccuracy in field maternity assignment. The reason is that direct observations were used to classify mother-calf pairs in the 1998–2003 group while conducting behavioral studies on these elephants.

Although we anticipated a somewhat higher level of ambiguous parentage in the pre-1998 sample category, the relatively low mismatch error rate (2.44%) observed in this study strengthened our assumption that closely associating female elephants and young calves were actually true biological relatives. A Mendelian mismatch can result from different paternal relations and erroneously typed parental or offspring genotypes, null alleles (Marshall et al. 1998), or marker mutation (Smith et al. 2000). Out of the 38 mismatches, 5 could be ascribed to null alleles (mother and offspring homozygous for different alleles), whereas the other combinations were heterozygous in at least mother or offspring. Of these, 23 differed by one repeat, 4 by two repeats, 4 by three repeats, 1 by five repeats, and 1 by seven repeats. If all of these were ascribed to mutations, they would amount to an average mutation rate of $33 / (133 \times 20) = 0.012$, which seems somewhat within the upper limit for microsatellite mutation rates (Jarne and Lagoda 1996; Schug et al. 1997; Webster et al. 2002; Whittaker et al. 2003). Although mutations alone would account for the observed Mendelian mismatches, we attribute them to both genotyping errors and, to a limited extent, inaccuracy in maternity assignment awaiting a thorough genotyping of additional offspring, which are to be sampled. It is therefore reasonable to say that offspring-Dam pairs with single mismatches represent mutation or mistyping, whereas those with mismatches at multiple loci are likely due to false maternity.

### Reasons for Genotyping Success

This study suggests that fresh dung sampling provides a reliable source of DNA with error rates low enough for individual-based investigations. In comparison to that of other studies based on noninvasively obtained samples (Table 2), fecal DNA extracted from elephants yielded reliable results with low genotyping error rates. This study demonstrates that noninvasive genotyping of elephant dung can be done with high accuracy. Furthermore, fecal sample material in this species is not limiting, thus enabling ample quantities of mucosal-laden portions to be easily sampled.

Technically, we attribute the relatively high genotyping success and reliability achieved in this study to the following

**Table 2.** Percentage of genotyping success and error rates observed in this study in comparison to other noninvasive studies conducted on other mammals

| Mammal | Genotyping success | Allelic dropouts | False alleles | Reference |
|---|---|---|---|---|
| *Loxodonta africana* | 98.6 | 1.6 | 0.9 | Current study |
| *Gulo gulo* | 77.0 | 9.8 | 0.5 | Flagstad et al. (2003) |
| *Elephas maximus* | 99.0 | 0.4 | 0.8 | Fernando et al. (2003) |
| *Papio cynocephalus* | 53.0 | 48.0 | 14.2 | Smith et al. (2000) |
| *Papio cynocephalus* | 70.0 | 8.0 | 9.1 | Bayes et al. (2000) |

facts. First, all dung samples analyzed were obtained fresh, emphasizing intestinal mucosal laden portions. Second, all microsatellite loci isolated from the *Loxodonta africana* genome were screened on a sample of individuals from the study population, and the most polymorphic and easiest-to-amplify loci were employed in this study. Third, the extent of PCR failures due to *Taq* DNA polymerase inhibitors, such as polysaccharides, alkaloids, RNA, and other plant substances abundant in dung, were potentially minimized in our study by using the DNA polymerase anti-inhibitor Bovine Serum Albumin in all our PCR master mixes. Fourth, amplification artifacts due to polymerase slippage during PCR (leading to false heterozygosity or multiple alleles) were substantially minimized or eliminated by following a gel-running protocol proposed by Fernando et al. (2001). Fifth, negative and positive controls from the extraction step to gel analysis were employed to minimize incidences of cross-contaminations and inconsistencies that would have compromised the genotyping success. Last, we attempted to minimize potential causes of contamination by separating extraction and amplification activities from those associated with handling postamplification products and by using dedicated instruments, reagents, and lab consumables for each activity.

### Recommendations

To successfully carry out a noninvasively based study, we suggest full observance of set criteria for accepting consensus genotypes. Selection of hypervariable loci should be conducted in a pilot study to enhance variability in results. Simple pedigree (parent-offspring) relationships, if available, should be utilized in genotyping process and data validation, respectively, to obtain accurate, reliable, and low error-containing microsatellite genotypes. Genotyping error rates should be used to detect odd genotypes, discard unrealistic markers that are difficult to score, and clean up the data sets. As noted by Bonin et al. (2004), we also suggest that reporting error rates should be incorporated in all genetic research. It is important to recognize that the assumption of an error-free data set is unrealistic; even genetic data from tissue or blood can still harbor considerable errors (Bonin et al. 2004; Dakin and Avise 2004). Because complete eradication of genotyping errors is difficult to achieve (Fernando et al. 2003; Flagstad et al. 1999; Wehausen et al. 2004), we emphasize the need to develop statistics or data-analysis packages with provision for errors in their parameter estimates, such as in CERVUS (Marshall et al. 1998).

## Acknowledgments

This study is part of collaborative genetic research among Makerere University Institute of Environment and Natural Resources, Department of Evolutionary Biology at the University of Copenhagen, and Save the Elephants (Kenya). It was funded by Danish International Development Agency, Danish Natural Science Research Council, International Elephant Foundation, and National Science Foundation of the United States of America. We thank our technician Pernille Selmer Olsen and other staff at the partner institutions, the Samburu and Buffalo Springs county councils, wardens, rangers, and Samburu community for their respective support. We are so grateful to the Office of the President of Kenya and Kenya Wildlife Service for permission to carry out this research.

## References

Amos W and Hoelzel AR, 1991. Long term preservation of whale skin for DNA analysis. In: Genetic ecology of whales and dolphins (Hoelzel AR, ed). Report of the International Whaling Commission (special issue 13); 99–104.

Archie EA, Moss CJ, and Alberts SC, 2003. Characterization of tetranucleotide microsatellite loci in the African savannah elephant (*Loxodonta africana africana*). Mol Ecol Notes 3:244–246.

Bayes MK, Smith KL, Alberts SC, Altmann J, and Bruford MW, 2000. Testing the reliability of microsatellite typing from faecal DNA in the savannah baboon. Conserv Genet 1:173–176.

Bonin A, Bellemain E, Eidesen PB, Popanon F, Brochmann C, and Taberlet P, 2004. How to track and assess genotyping errors in population genetic studies. Mol Ecol 13:3261–3273.

Brookfield JFY, 1996. A simple new method for estimating null allele frequency from heterozygote deficiency. Mol Ecol 5:453–455.

Broquet T and Petit E, 2004. Quantifying genotyping errors in non-invasive population genetics. Mol Ecol 13:3601–3608.

Chakraborty R, De Andrade M, Daiger SP, and Budowle B, 1992. Apparent heterozygote deficiencies observed in DNA typing data and their implications in forensic applications. Annal Human Genet 56:45–57.

Comstock KE, Georgiadis N, Pecon-Slattery J, Roca AL, Ostrander EA, O'Brien SJ, and Wasser SK, 2002. Patterns of molecular genetic variation among African elephant populations. Mol Ecol 11:2489–2498.

Comstock KE, Wasser SK, and Ostrander EA, 2000. Polymorphic microsatellite DNA loci identified in the African elephant (*Loxodonta africana*). Mol Ecol 9:1004–1006.

Constable JL, Ashley MV, Goodall J, and Pusey A, 2001. Noninvasive paternity assignment in Gombe chimpanzees. Mol Ecol 10:1279–1300.

Creel S, Spong G, Sands JL, Rotella J, Zeigle J, Joe L, Murphy KM, and Smith D, 2003. Population size estimation in Yellowstone wolves with error-prone non-invasive microsatellite genotypes. Mol Ecol 12:2003–2009.

Dakin EE and Avise JC, 2004. Microsatellite null alleles in parentage analysis. Heredity 93:504–509.

Eggert LS, Eggert JA, and Woodruff DS, 2003. Estimating population sizes for elusive animals: the forest elephants of Kakum National Park, Ghana. Mol Ecol 12:1389–1402.

Eggert LS, Ramakrishnan U, Mundy NI, and Woodruff DS, 2000. Polymorphic microsatellite DNA markers in the African elephant (*Loxondonta africana*) and their use in the Asian elephant (*Elephas maximus*). Mol Ecol 9:2223–2225.

Ernest HB, Penedo MCT, May BP, Syvanen M, and Boyce WM, 2000. Molecular tracking of mountain lions in the Yosemite Valley region of California: genetic analysis using microsatellites and faecal DNA. Mol Ecol 9:433–441.

Ewen KR, Bahlo M, Treloar SA, Levinson DF, Mowry B, Barlow JW, and Foote SJ, 2000. Identification and analysis of error types in high-throughput genotyping. Am J Human Genet 67:727–736.

Fernando P, Evans J, Morales JC, and Melnick DJ, 2001. Electrophoresis artefacts—a previously unrecognized cause of error in microsatellite analysis. Mol Ecol Notes 1:325–328.

Fernando P, Vidya TNC, Rajapakse C, Dangolla A, and Melnick DJ, 2003. Reliable noninvasive genotyping: fantasy or reality? J Hered 94:115–123.

Flagstad Ø, Hedmark E, Landa A, Brøseth H, Persson J, Andersen A, Segerstrom P, and Ellegren H, 2003. Colonization history and noninvasive monitoring of a re-established wolverine population. Conserv Biol 18: 676–688.

Flagstad Ø, Røed K, Stacy JE, and Jakobsen KS, 1999. Reliable noninvasive genotyping based on excremental PCR nuclear DNA purified with a magnetic bead protocol. Mol Ecol 8:979–883.

Frantzen MAJ, Silk JB, Ferguson JWH, Wayne RK, and Khon MH, 1998. Empirical evaluation of preservation methods for faecal DNA. Mol Ecol 7:123–1428.

Gagneux P, Boesch C, and Woodruff DS, 1997. Microsatellite scoring errors associated with non-invasive genotyping based on nuclear DNA amplified from shed hair. Mol Ecol 6:861–868.

Gomes I, Collins A, Lonjou C, Thomas NS, Wilkinson J, Watson M, and Morton N, 1999. Hardy-Weinberg quality control. Ann Human Genet 63:535–538.

Guo SW and Thompson EA, 1992. Performing the exact test of Hardy-Weinberg proportions for multiple alleles. Biometrics 48:361–372.

Hoffman JI and Amos W, 2005. Microsatellite genotyping errors: detection approaches, common sources and consequences for paternal exclusion. Mol Ecol 14:599–612.

Holm S, 1979. A simple sequentially rejective multiple test procedure. Scan J Stat 6:65–70.

Hosking L, Lumsden S, Lewis K, Yeo A, McCarthy L, Bansal A, Riley J, Purvis I, and Xu CF, 2004. Detection of genotyping errors by Hardy-Weinberg equilibrium testing. Eur J Human Genet 12:395–399.

Jarne P and Lagoda PJL, 1996. Microsatellites form molecules to populations and back. Trend Evol Ecol 11:424–429.

Jeffery KJ, Keller LF, Arcese P, and Bruford MW, 2001. The development of microsatellite loci for song sparrow, *Melospiza melodia* (Aves) and genotyping errors associated with good quality DNA. Mol Ecol Notes 1:11–13.

Kwok S, Kellog DE, McKinney N, Spasic D, Goda L, Levenson C, and Sninsky JJ, 1990. Effects of primer-template mismatches on the polymerase chain reaction: human immunodeficiency virus 1 model studies. Nucleic Acids Res 18:999–1005.

Launhardt K, Epplen C, Epplen JT, and Winkler P, 1998. Amplification of microsatellites adapted from human systems in faecal DNA of wild Hanuman langurs, *Presbytis entellus*. Electrophoresis 19:1356–1361.

Lucchini V, Fabbri E, Marucco F, Ricci S, Biotani L, and Randi E, 2002. Noninvasive molecular tracking of colonizing wolf (*Canis lupus*) packs in the western Italian Alps. Mol Ecol 11:857–868.

Marshall TC, Slate J, Kruuk LEB, and Pemberton JM, 1998. Statistical confidence for likelihood-based paternity inference in natural populations. Mol Ecol 7:639–655.

Maudet C, Luikart G, Durray D, Von Hardensberg A, and Taberlet P, 2004. Low genotyping error rates in wild ungulate faeces sampled in winter. Mol Ecol Notes 4:772–775.

Miller CR, Joyce P, and Waits LP, 2002. Assessing allelic dropout and genotype reliability using maximum likelihood. Genetics 160:357–366.

Mills LS, Citta JJ, Lair KP, Scwartz MK, and Tallman DA, 2000. Estimating animal abundance using noninvasive DNA sampling: promises and pitfalls. Ecol App 10:1835–1844.

Morin PA, Chambers KE, Boesch C, and Vigilant L, 2001. Quantitative polymerase chain reaction analysis of DNA from non-invasive samples for accurate microsatellite genotyping of wild chimpanzees (*Pan troglodytes verus*). Mol Ecol 10:1835–1844.

Morin PA, Moore JJ, Chakraborty R, Jin L, Goodall J, and Woodruff DS, 1994. Kin selection, social structure, geneflow, and evolution of cimpanzees. Science 265:1193–1201.

Nyakaana S and Arctander P, 1998. Isolation and characterisation of microsatellite loci in the African elephant (*Loxodonta africana*). Mol Ecol 7:1436–1437.

Nyakaana S, Okello JBA, Muwanika V, and Siegismund HR, 2005. Six new polymorphic microsatellite loci isolated and characterized from the African savannah elephant genome. Mol Ecol Notes 5:223–225.

Omondi P, Bitok E, Kahindi O, and Mayiendi R, 2002. Total aerial count of elephants in Samburu/Laikipia, Kenya. Nairobi: Kenya Wildlife Service Report.

Parsons KM, 2001. Reliable microsatellite genotyping of dolphin DNA from faeces. Mol Ecol Notes 1:341–344.

Pemberton JM, Slate J, Bancroft DR, and Barrett JA, 1995. Non-amplifying alleles at microstaellite loci: a caution for parentage and population studies. Mol Ecol 4:249–252.

Raymond M and Rousset F, 1995. GENEPOP: population genetics software for exact tests and ecumenicism. JHered 86:284–249.

Rice WR, 1989. Analysing tables of statistical tests. Evolution 43:223–225.

Roon DA, Thomas ME, Kendall KC, and Waits LP, 2005. Evaluating mixed samples as a source of error in non-invasive genetic studies using microsatellites. Mol Ecol 14:195–201.

Schlötterer C and Tautz D, 1992. Slippage synthesis of simple sequence DNA. Nucleic Acids Res 20:211–215.

Schug MD, Mackay TFC, and Aquadro CF, 1997. Low mutation rates of microsatellite loci in *Drosophila melanogaster*. Nat Genet 15:99–102.

Shaw PW, Pierce GJ, and Boyle PR, 1999. Subtle population structuring within a highly vagile marine invertebrate, the veined squid *Loligo forbesi*, demonstrated with microsatellite DNA markers. Mol Ecol 8:407–417.

Smith KL, Alberts SC, Bayes MK, Bruford MW, and Altmann J, 2000. Cross-species amplification, non-invasive genotyping, and non-Mendelian inheritance of human STRPS in savannah baboons. Am J Primat 51: 219–227.

Taberlet P, Griffin S, Goossens B, Questiau S, Manceau V, Escaravage N, Waits LP, and Bouvet J, 1996. Reliable genotyping of samples with very low DNA quantities using PCR. Nucleic Acids Res 24:3189–3194.

Taberlet P, Waits LP, and Luikart G, 1999. Noninvasive genetic sampling: look before you leap. Trends Ecol Evol 14:323–327.

Van Oosterhout C, Hutchinson WF, Wills DPM, and Shipley P, 2004. MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. Mol Ecol Notes 4:535–538.

Wasser SK, Houston CS, Koehler GM, Cadd GG, and Fain SR, 1997. Techniques for application of faecal DNA methods to field studies of ursids. Mol Ecol 6:1091–1097.

Wattier R, Engel CR, Saumitou-Laprade P, and Valero M, 1998. Short allele dominance as a source of heterozygote deficiency at microsatellite

loci: experimental evidence at the dinucleotide locus Gv1CT in *Gracilaria gracilis* (Rhodophyta). Mol Ecol 7:1569–1573.

Webster MT, Smith NGC, and Ellegren H, 2002. Microsatellite evolution inferred from human- chimpanzee genomic sequence alignments. PNAS 99:8748–8753.

Wehausen JD, Ramey RR II, and Epps CW, 2004. Experiments in DNA extraction and PCR amplification from bighorn sheep feces: the importance of DNA extraction method. J Hered 95:503–509.

Weir BS and Cockerham CC, 1984. Estimating *F*-statistics for the analysis of population structure. Evolution 38:1358–1370.

Whittaker JC, Harbord RM, Boxall N, Mackay I, Dawson G, and Sibly RM, 2003. Likelihood-based estimation of microsatellite mutation rates. Genetics 164:781–787.

Wittemyer G, 2001. The elephant population of Samburu and Buffalo Springs National Reserves. Afr J Ecol 39:357–365.

Wittemyer G, Daballen D, Rasmussen H, Kahindi O, and Douglas-Hamilton I, 2005a. Demographic status of elephants in the Samburu and Buffalo Springs National Reserves, Kenya. Afri JEcol 43:44–47.

Wittemyer G, Douglas-Hamilton I, and Getz WM, 2005b. The socio-ecology of elephants: analysis of the processes creating multi-tiered social structures. Ani Behav 69:1357–1371.

Received April 18, 2005
Accepted July 20, 2005

Corresponding Editor: Stephen O'Brien