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TECHNICAL NOTE

# Reliable microsatellite genotyping of dolphin DNA from faeces

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**Abstract**

**Noninvasive samples have proved useful in genotyping studies of free-ranging mammals. However, potential genotyping errors associated with such samples dictate the need for validation studies. This pilot study demonstrates the use of dolphin faeces in multilocus microsatellite genotyping studies. An empirical approach to calculating the rate of genotyping error was applied to data from matched pairs of blood or tissue and faecal samples from both captive and wild bottlenose dolphins. Microsatellite genotypes were assigned to dolphin faecal extracts with greater than 95% confidence by using a multiple tube approach, and at least two independent replicate genotypings.**

*Keywords*: cetaceans, faeces, microsatellite, noninvasive genotyping, *Tursiops truncatus*

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The amplification of DNA from noninvasive samples of hair and faeces promises to revolutionize studies of natural populations (e.g. Taberlet *et al*. 1997). This approach offers the opportunity to obtain genetic samples in situations where traditional methods for tissue or blood sampling are impractical (e.g. Morin *et al*. 1994; Gerloff *et al*. 1995). Furthermore, noninvasive sampling techniques can increase sample sizes (Taberlet *et al*. 1999), thereby facilitating estimation of important population parameters. Nonetheless, complications inherent in the genetic typing of noninvasive samples have raised concerns about the reliability of multilocus genotypes obtained from faecal DNA sources (Taberlet *et al*. 1999).

Quantifying the rate of genotyping error in noninvasive genetic analyses is imperative (Taberlet & Luikart 1999; Taberlet *et al*. 1999). Substantial differences in the amount and quality of DNA extracted from faeces can exist among species (Taberlet & Luikart 1999). Therefore, it is important to examine species-specific biases in molecular scatology (Wasser *et al*. 1997; Taberlet *et al*. 1999) through pilot studies that allow the empirical evaluation of genotyping errors for each new species studied. Direct comparison of microsatellite genotypes obtained for blood or tissue samples paired with faecal samples from the same individual animal provides a useful method for examining the utility and reliability of noninvasive samples.

Water-borne dolphin faeces can provide noninvasive samples suitable for sequencing mitochondrial DNA fragments (Parsons *et al*. 1999). However, to the best of my knowledge, such samples have not yet been used for multilocus microsatellite genotyping. Here I examine the feasibility and estimate error rates of microsatellite genotyping DNA extracts of faeces from bottlenose dolphins (*Tursiops truncatus*). A 'multiple-tubes' approach (Taberlet *et al*. 1996) was employed to quantify the occurrence of such polymerase chain reaction (PCR) artefacts as 'false' alleles and 'allelic drop-out', and to estimate the number of replicate PCR reactions required to obtain a reliable genotype.

Matched samples of blood or tissue and faeces were obtained from 12 captive and three wild bottlenose dolphins. Samples from the captive dolphins were collected by veterinary personnel employing routine husbandry practices. Blood samples were collected in EDTA vacutainers and faecal samples were collected in sterile tubes. All samples were stored frozen at –20 °C.

Samples of water-borne faeces were collected from wild bottlenose dolphins as part of an ecological study in southeast Abaco, Bahamas (26°00′ N 077°25′ W), as previously described in Parsons *et al*. 1999. Skin samples were collected from free-swimming dolphins using a nontethered remote biopsy system, following Barrett-Lennard *et al*. (1996), then stored in 20% DMSO, 5 M NaCl (Amos & Hoelzel 1991).

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**Table 1** Information on the microsatellite loci amplified in this study

| Locus | $T_a$ (°C) | Repeat motif | Allele size range | No. alleles | Reference |
|---|---|---|---|---|---|
| EV37 | 58 | AC | 207–215 | 6 | Valsecchi & Amos (1996) |
| KWM1b | 49 | AC | 188–190 | 2 | Hoelzel *et al*. (1998) |
| GATA098 | TD60-50* | GATA | 71–99 | 4 | Palsboll *et al*. (1997) |

$T_a$, annealing temperature.

*TD60-50 refers to a 'touchdown' PCR program consisting of 20 annealing cycles starting at 60 °C for 30 s and dropping by 0.5 °C per cycle, followed by 15 cycles of 30 s denaturation at 92 °C and 30 s annealing at 50 °C.

Total genomic DNA was extracted from blood and tissue samples using standard phenol/chloroform extraction protocols (Sambrook *et al*. 1989). DNA was extracted from faecal samples as previously described (Parsons *et al*. 1999) using 1 mL of thawed faecal matter in each extraction. A single extraction was performed on each faecal sample owing to the small quantity of faeces available. Negative controls were included in each set of extractions.

Each faecal DNA extract was typed seven times for each of three microsatellite loci (Table 1) and the corresponding blood or tissue DNA extracts were typed twice for each locus. Faecal genotypes were compared to the genotype obtained for blood or tissue DNA from the same individual dolphin.

PCR reactions (10 μL) contained 1.5 mM $MgCl_2$, 1× $NH_4$ buffer, 0.2 mM of each nucleotide, 0.25 μM of each primer and 0.25 units of *Taq* polymerase (Bioline). Forward primers were end-labelled with [$\gamma^{32}$P]-dATP. PCR profiles consisted of 35 cycles of 30 s denaturation at 94 °C, 30 s annealing, and 30 s extension at 72 °C. The final cycle was followed by 5 min extension at 72 °C. Annealing temperatures were optimized for each locus (Table 1). PCR fragments were resolved by electrophoresis on 6% denaturing polyacrylamide gels (Sambrook *et al*. 1989), and detected by autoradiography. Allele sizes were determined by reference to an M13mp8 DNA sequencing reaction. All series included both positive and negative control reactions.

The number of replicate PCRs needed to obtain the correct genotype from faecal DNA extracts was estimated from the microsatellite genotype data for the 15 matched pairs of samples. A three-locus microsatellite genotype was obtained for each replicate faecal PCR. These genotypes were compared to the 'correct' genotype and the numbers of correct and incorrect faecal genotypes per sample were recorded for each locus. Reactions that failed to yield an amplification product were recorded as 'missing' data.

The probability that the correct genotype was the most frequent genotype obtained was calculated for two or more replicate PCRs using the binomial probability distribution conditioned on the individual. It was assumed that replicate PCRs yielded either a correct or an incorrect genotype, and missing data were grouped with correct genotypes as such data would not result in the erroneous typing of a sample.

Extraction of all 15 faecal samples yielded amplifiable DNA. Faecal samples from captive dolphins and the waterborne faeces from wild dolphins did not appear to differ. For all individuals, the two blood or tissue DNA genotypes were identical. Only six cases (1.90%) of amplification failure, or missing data, were observed. These six reactions represented six different dolphins, and were therefore unlikely to result from poor quality DNA from one particular sample, but rather stochastic sampling error (Taberlet *et al*. 1996). Three incorrect genotypes out of 309 positive PCRs (0.97%) were observed, all of which represented faecal DNA from one captive sample (Dolph11) at one locus (EV37). All of the incorrect genotypes observed were due to extra alleles created by an inability to distinguish between the true allele and stutter bands. Genotyping errors due to allele dropout, the nonamplification of one allele in a heterozygous individual, were absent.

The 315 faecal genotypes were categorized as either correct or incorrect. These data were used to calculate the probability of obtaining the correct genotype at least twice for a given number of PCR replicates (Fig. 1). The underlying assumptions were: (i) a multiple tubes approach is used; (ii) obtaining the same genotype more than twice determines the correct genotype; and (iii) the probability of obtaining the same incorrect genotype in two or more replications is ignored due to the very low probability of occurrence. The probability of assigning the correct genotype to a dolphin faecal sample is greater than 0.95 for two replicates, and greater than 0.99 for eight replicates.

Noninvasive sampling techniques provide new opportunities for molecular genetic analyses of free-ranging mammals (e.g. Gerloff *et al*. 1995; Valsecchi *et al*. 1998) but potential genotyping errors necessitate pilot studies that examine the validity of such results (Taberlet & Luikart 1999; Taberlet *et al*. 1999). This study demonstrates the reliable genotyping of DNA extracted from the faeces of free-swimming bottlenose dolphins, and presents a straightforward method for estimating the optimum number of replicate PCRs using a multitube approach.

Previous studies of faecal DNA genotyping have identified genotyping errors when using dilute template DNA (Taberlet *et al*. 1996). However, in this pilot study, dolphin

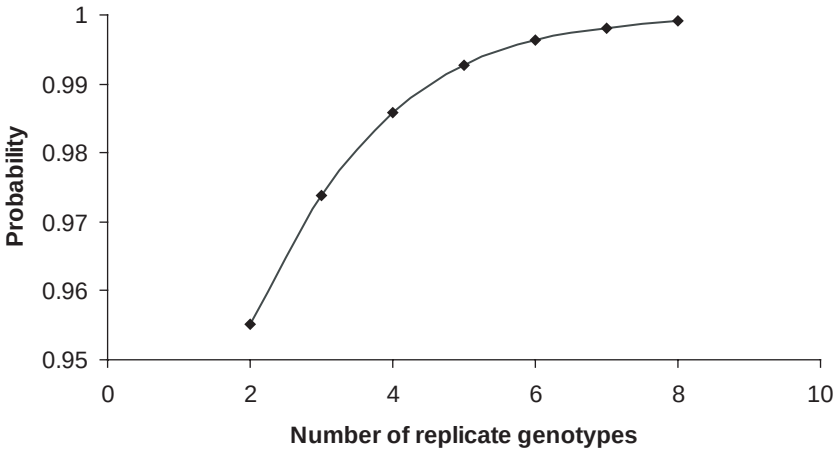


**Fig. 1** The probability of obtaining at least two correct microsatellite genotypes for a given number of replicate genotypes of faecal DNA.

faecal samples were typed at both dinucleotide and tetranucleotide loci with a high level of confidence. This may be explained by the immediacy of collection of cetacean faeces. As collection occurs immediately following defecation, there is little or no opportunity for the DNA degradation that probably occurs in faeces from terrestrial mammals (Taberlet & Luikart 1999). The only PCR errors observed were false alleles at one particular dinucleotide repeat locus (EV37) in one sample. This finding is consistent with other studies that indicate the characteristic 'stuttering' of dinucleotide microsatellites can make scoring of alleles difficult (Taberlet *et al*. 1996; Taberlet & Luikart 1999). Further optimization of PCR conditions and screening of a greater number of loci, facilitating preferential selection of tri- or tetranucleotide loci, could further reduce this source of error.

The multiple-tubes approach is recommended for examining PCR reproducibility and calculating genotyping errors for noninvasive samples (Taberlet *et al*. 1996). Nonetheless, this method is constrained because the limited amount of template DNA results in a decrease in the number of loci typed with an increase in the number of replicate PCRs (Taberlet *et al*. 1996). In addition, multiplexing PCR primers to maximize the number of loci amplified in a single reaction may increase nonamplification due to the limited availability of template DNA (Ernest *et al*. 2000). This trade-off between accurate genotyping and maximizing the number of loci can constrain many analyses. To jointly maximize the use of noninvasive samples and genotype accuracy, one must determine the optimum number of replicate PCRs that satisfy both criteria.

The approach adopted here to empirically calculate the reliability of faecal genotypes using matched tissue and faecal sample pairs could prove useful in other noninvasive studies for calculating the required number of replicate genotypes. This method permits the user to define the desired level of confidence in the genotypes, and thereby assess the feasibility of noninvasive genetic typing prior to committing costly resources. Furthermore, this study has demonstrated the use of dolphin faeces as a valuable supplemental source of DNA for individual-based investigations and presents a novel approach for examining the social organization and population structure of dolphin species.

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