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).
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 MgCl₂, 1× NH₄ 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 [³²P]-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 (Dolphin11) 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 non-amplification 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 multivariate 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
faecal samples were typed at both dinucleotide and tetra-
ucleotide 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 exam-
ing PCR reproducibility and calculating genotyping
errors for noninvasive samples (Taberlet et al. 1996). None-
theless, 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, multiplex-
ing 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 investi-
gations and presents a novel approach for examining the
social organization and population structure of dolphin
species.

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


