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PRIMER NOTE

Improved noninvasive genotyping method: application to brown bear (*Ursus arctos*) faeces

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Abstract

We redesigned new microsatellite primers and one sex-specific primer for amplification of faecal DNA from brown bears (*Ursus arctos*). We also combined a semi-nested polymerase chain reaction (PCR) with a newly developed multiplex preamplification method in order to increase the quality of the amplified DNA fragments. In comparison with a conventional PCR approach, the genotyping error rate was substantially reduced and the amplification rate was increased. This new approach could be transposed to other species where conventional PCR methods experience low success due to limited DNA concentration and/or quality.

Keywords: microsatellites, multiplex preamplification method, noninvasive samples, sex-specific primer, *Ursus arctos*

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Noninvasive genetic techniques have received much interest in the last few years. The use of DNA from faeces, hairs, urine or feathers as an individual genetic tag can provide useful information for population monitoring and the estimation of important genetic parameters. However, the amplification of DNA extracted from noninvasive samples is often problematic because the DNA is usually degraded and present in low quantity. This can lead to scoring errors such as allelic dropout or false alleles and will often produce incorrect genotypes (Taberlet *et al*. 1996; Taberlet & Luikart 1999; Smith *et al*. 2000). These problems are now well understood, and different methods have been proposed to limit genotyping errors and their impacts on the subsequent analyses (Schwartz *et al*. 1999; Mills *et al*. 2000; Miller *et al*. 2002; Paetkau 2003; Piggott *et al*. 2004).

Here, we combined a two-step PCR approach, or 'semi-nested PCR' with the newly defined 'multiplex preamplification method' (Piggott *et al*. 2004) to amplify DNA from brown bear (*Ursus arctos*) faecal samples, with the aim to increase the quality of the multilocus genotypes.

For all collected bear faeces, DNA was extracted using the Qiamp DNA Stool® kit (Qiagen), especially designed for this type of material. All extractions were carried out in a room dedicated only to processing hairs and faeces. The final volume of the DNA extract was 150 μL. Negative controls were included in all extractions batches in order to check for exogenous DNA contamination. Each DNA extract was first screened with one microsatellite (G10P; Paetkau & Strobeck 1994) acting as a species-diagnostic marker. PCR products were separated on an agarose gel and viewed, using ethidium bromide, under UV light. Only the DNA samples that showed a fluorescent DNA band were kept for subsequent analyses.

Based on genetic data from more than 700 individuals typed for 18 or 19 microsatellite loci (Waits *et al*. 2000; E. Bellemain, unpublished data), we selected the six most informative for identifying individuals, i.e. those with the lowest probability of identity between unrelated individuals (PI; Paetkau & Strobeck 1994) and between siblings (PIsibs; Waits *et al*. 2000) (Table 1). Most of the original primers were redesigned in order to obtain smaller amplified fragments and more similar annealing temperatures. We also redesigned new sex-identification primers (Table 1), amplifying an 80 bp fragment more specific to carnivores (i.e. avoiding amplifications from most of the prey DNA co-extracted from a bear faeces).

We combined the multiplex preamplification method (Piggott *et al*. 2004) with a semi-nested PCR (Fig. 1; see Ulrich *et al*. 1993). The first step involved a multiplex

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Table 1 External and internal primers for *Ursus arctos* used for the two steps of the multiplex preamplification method

Locus	Microsatellite sequence†	Fluorescence (internal primer)	Primer's final concentration (μM)	Allele size range (bp)	PI (Paetkau & Strobeck 1994)	PIsibs (Waits *et al*. 2001)	GenBank Accession no.	Redesigned from or reference
Mu10	F*: ATTCAGATTTCATCAGTTTGACA R*: CCATAAATGGTAGCATGAGC RI*: TCAGCATAGTTACACAAATCTCC	FAM	0.5	113–129	6.757e-02	3.652e-01	Y09642	Taberlet *et al*. 1997
Mu23	F: GCCTGTGTGCTATTTTATCC R: TTGCTTGCCTAGACCACC RI*: TAGACCACCAAGGCATCAG	NED	0.6	141–153	1.247e-01	4.364e-01	Y09645	Taberlet *et al*. 1997
Mu50	F*: GTCTCTGTCATTTCCCCATC R*: GAGCAGGAAACATGTAAGATG RI*: AACCTGGAACAAAAATTAACAC	FAM	0.4	92–102	1.313e-01	4.292e-01	Y09647	Taberlet *et al*. 1997
Mu51	F*: AGCCAGAATCCTAAGAGACCT R: GAAAGGTTAGATGGAAGAGATG RI*: AAAGAGAAGGGACAGGAGGTA	HEX	0.6	105–125	8.138e-02	3.787e-01	Y09648	Taberlet *et al*. 1997
Mu59	F: GCTCCTTTGGGACATTGTAA R: AGTGTTTGTGGTGCCTGTG RI*: TGACTGTCACCAGCAGGAG	NED	0.5	96–120	1.220e-01	4.183e-01	Y09649	Taberlet *et al*. 1997
G10L	F*: CAGGACAGGATATTGACATTGA R*: GATACAGAAACCTACCCATGCG FI: ACTGATTTTATTCACATTTCCC	HEX	0.5	138–156	1.204e-01	4.178e-01	U22088	Paetkau & Strobeck 1994
SRY	F: GAACGCATTCTTGGTGTGGTC R: CGGTATTTATAGTCCGGGTAGTT RI: TGATCTCTGAGTTTTGCATTTG	HEX	0.5	75 for males only	—	—	X74007	Taberlet *et al*. 1997

†F and R represent forward and reverse external primers. FI and RI represent forward and reverse internal primers. *Indicates the microsatellite sequences that were redefined.
PI: probability of identity between unrelated individuals.
PIsibs: probability of identity between siblings.

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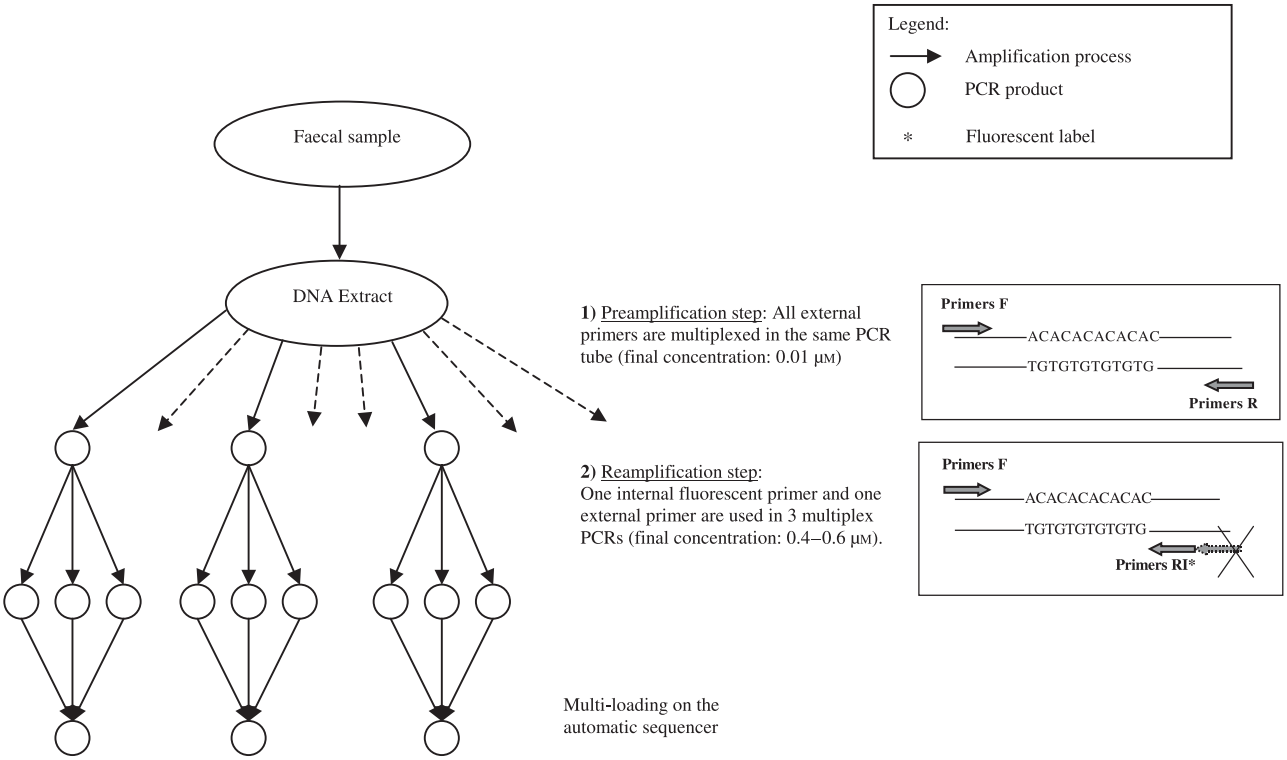

Fig. 1 General principle of the multiplex preamplification method combined with a semi-nested PCR (the figure shows three replicates out of eight.

preamplification that simultaneously amplified all loci with external primers (Fig. 1). Each amplification was repeated eight times (multi-tubes approach; Taberlet *et al*. 1996). Aliquots from the preamplification were then used as templates in another round of PCRs. In this second step, we simultaneously amplified two or three loci (Mu10/G10L; Mu23/Mu50; SRY/Mu51/Mu59) using the internal fluorescent primers together with the appropriate external primers. The fluorescent PCR products can be electrophoresed together on the single lane.

PCR preamplifications were prepared in a 25 µL volume containing 5 µL template DNA, 0.1 mM of each dNTP, 0.01 µM of each primer, 2 mM $MgCl_2$,*0.5 U AmpliTaq Gold Polymerase (Applied Biosystems) and 1 × Taq buffer (containing 100 mM Tris-HCl, pH 8.3, 500 mM KCl, according to the manufacturer's specifications; Applied Biosystems). For the second step, PCR amplifications were prepared in a 13 µL volume containing 3 µL preamplified product, 0.1 mM of each dNTP, 0.4–0.6 µM of each primer (depending on the primer, see Table 1), 2 mM $MgCl_2$,*0.5 U AmpliTaq Gold Polymerase (Applied Biosystems) and 1 × Taq buffer (as described above). Amplifications were performed in a GeneAmp PCR system 9600 (Applied Biosystems) with the following conditions: 10 min at 95 °C, 25 cycles for the preamplification step and 35 cycles for the second amplification step, composed of 30 s denaturing at 95 °C, 30 s annealing at 60 °C, 1-min extension at 72 °C, and as a final extension step, 7 min at 72 °C. The PCR products from the second step were mixed (3 µL of Mu10/G10L; 3 µL of Mu23/Mu50 and 4 µL of SRY/Mu51/Mu59). One microlitre of this multiplex was added to a 10 µL mix of formamide and ROX 350 (10:0.2), and then loaded on an automatic sequencer ABI3100 (Applied Biosystems). Electrophoresis was run for 3 h with a POP4 polymer. Microsatellite patterns were examined with GENEMAPPER version 3.0 (Applied Biosystems).

The PI for the six microsatellites was low: $PI = 1.38 \times 10^{-6}$; $PIsibs = 4.52 \times 10^{-3}$, which allowed a reliable individual identification. We compared the results obtained from this multiplex approach with a conventional approach (same amplification conditions and primers as described for the second step of the multiplex approach, with 55 cycles) for eight samples and eight replicates for each locus. The amplification rate was improved substantially (Wilcoxon ranked test; $P = 0.058$) and the genotyping error (false alleles or allelic dropouts) rate was much reduced (Wilcoxon ranked test; $P = 0.063$). In addition, the DNA typing profiles were much clarified using the new multiplex method (data not shown). Next, we diluted the DNA extract from four to 12 times to simulate more limiting conditions. The genotyping error rate was from two to 10 times lower with the mutiplex preamplification method than with the conventional method.

* 0.25µL BSA per tube,

We applied this multiplex approach to more than 1900 faecal samples collected in central Sweden for estimating brown bears population size (Bellemain *et al*. 2004). A preliminary study showed that the reliability of the genotyping was not significantly different when using four or eight replicates, probably because template DNA was not in too limiting concentration in the extracts. This might be due to good field climatic conditions leading to good DNA preservation in the field before the sampling. As a consequence, we used only four replicates for this study and were able to successfully genotype 75% of the samples. To ensure the reliability of the results, we randomly repeated 5% of the genotyping and obtained an error rate of less than 2%, including the sex identification (Bellemain *et al*. 2004).

The newly defined primers, together with the PCR strategy described here, have the potential to substantially improve genotyping success rate and reduce genotyping errors. Moreover, the strategy of combining the multiplex preamplification method and a semi-nested PCR could be transposed to other species where conventional PCR approaches yield low success due to limiting DNA concentration and/or quality.

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