Conservation Genetics **5:** 417–420, 2004.

A multiplex pre-amplification method that significantly improves microsatellite amplification and error rates for faecal DNA in limiting conditions

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Received 23 May 2003; accepted 27 July 2003

Key words: faecal DNA, non-invasive genetic sampling, PCR

DNA recovered from non-invasive samples, in particular faeces, is usually degraded and/or in small quantities. This often leads to PCR errors during microsatellite genotyping (due to allelic dropout or false alleles), resulting in the identification of incorrect genotypes (Taberlet et al. 1996, 1999; Smith et al. 2000). This is a major concern because microsatellite genotypes are commonly used for individual identification, parentage, relatedness, and population genetics (e.g., Taberlet et al. 1997; Constable et al. 2001; Garnier et al. 2001). PCR methods to overcome genotyping errors when the template concentration is low have to date relied on replications (e.g., Navidi et al. 1992; Taberlet et al. 1997, 1999; Morin et al. 2001). However, high degrees of replication can exhaust a sample very quickly as well as increasing the consumables, costs and time required (Morin et al. 2001).

To avoid the need for numerous replicates, and to maximise the proportion of faecal samples that contains the critical threshold amount of DNA for accurate genotyping (Taberlet et al. 1996; Morin et al. 2001), we devised a two-step PCR method with the aim of increasing the quality and quantity of the desired DNA template. The basis of this approach is that an initial large-volume (50 μl) PCR is carried out containing primers for the entire panel of loci to be genotyped. Two microlitres of the amplified product from this first PCR are then used as template in separate PCRs for each locus. We compared this new method with conventional single-step PCR on the same faecal DNA extracts from three Australian marsupials (the brush-tailed rock-wallaby, *Petrogale penicillata*; the spotted-tailed quoll, *Dasyurus maculatus*; and eastern quoll, *D. viverrinus*). These species were chosen because preliminary results using single-step microsatellite PCR had produced results ranging from good to bad.

A fresh faecal sample (<12 h old) was collected from each of 10 *D. viverrinus*, *P. penicillata* and *D. maculatus* individuals. All faeces were dried in paper bags and DNA was extracted 1 week following collection. Tissue samples (ear biopsies) were collected from five of each of the *P. penicillata* and *D. maculatus* and DNA was extracted following Sunnucks and Hales (1996).

Faecal DNA extraction was performed using the surface wash protocol outlined in Banks et al. (2002) with a final elution volume of 400 μl. Each DNA extract was divided into 200 μl aliquots for use in conventional single-step and multiplex PCRs. PCR reaction mixtures for each method are described in Table 1. Eight replicate PCRs per sample per locus were carried out for each method. *Petrogale penicillata* samples were genotyped for six loci (Pa 385, Pa 55, Pa 297, Pa 593; Spencer et al. 1995 and Me 17, Me 14; Taylor and Cooper 1998) using annealing temperatures given in Spencer et al. (1995) and Taylor and Cooper (1998), respectively. For the two *Dasyurus* spp., six quoll microsatellites (Firestone 1999) were used

Table 1. PCR reaction mixtures used for the single-step conventional PCR and two-step multiplex pre-amplification PCR methods

PCR reaction mixture	Single-step PCR	Multiplex PCR: first step	Multiplex PCR: second step
Available volume of source template	200 μl DNA sample	200 μl DNA sample	50 μl PCR Product from Step 1
DNA template	4 μl	12 μl	2 μl
Tris–HCl (pH 8.8)	75 mm	75 mm	75 mm
$(NH_4)_2SO_4$	20 mm	20 mm	20 mm
Tween 20 (%)	0.01	0.01	0.01
$MgCl_2$ (25 mm)	2.0	2.0	2.0
dGTP, dTTP and dCTP	200 μm	200 μm	200 μm
dATP	20 μm	200 μm	20 μm
[α^{33}P]- dATP at 1000 Ci/mmol	0.05 μl	–	0.05 μl
BSA (MBI) (%)	0.5	0.5	0.5
Primer	0.5 μm	0.01 μm of all primers to be used	0.5 μm
Taq polmerase (MBI) (units)	0.5	1	0.5
Reaction volume	20 μl	50 μl	20 μl
Annealing temperature	Spencer et al. (1995), Taylor and Cooper (1998), Firestone (1999)	50°C	Spencer et al. (1995), Taylor and Cooper (1998), Firestone (1999)
No. of cycles	40	25	40

with annealing temperatures as described. PCR products were electrophoresed through a 6% polyacrylamide sequencing gel and visualised by autoradiography.

For the conventional single-step and two-step multiplex PCR techniques we compared three parameters: amplification rate (proportion of PCR replicates that yielded at least one scoreable allele), genotyping error rates (false alleles and allelic dropout) and the proportion of samples yielding a reliable genotype (using the criterion that an allele had to be present in five of eight replicates). Genotyping error rates were assessed as the proportion of all PCR replicates that did not match the consensus from other replicates and, where possible, by comparing profiles obtained from faecal DNA with tissue samples collected from the same individuals. Wilcoxon Signed Rank tests were used to compare the difference between these three parameters for single-step and two-step multiplex PCRs using Systat version 10 (SPSS Inc.).

The multiplex pre-amplification PCR method proved to be significantly better for all three species compared to the single-step approach (Tables 2 and 3). For the two *Dasyurus spp*. significant improvements were seen in amplification rates (Table 2), genotyping error rates (particularly false alleles) (Table 3) and the number of samples that could be reliably genotyped (Table 2), compared to the conventional approach. For *P. penicillata* there was a significant improvement in genotyping error rates (Table 2), although one *P. penicillata* sample contributed a large percentage of the genotyping error rate in the conventional PCR method and failed to amplify for any locus using the multiplex approach. Thus, only nine *P. penicillata* samples could be reliably scored for both PCR techniques (Table 2). Both Taberlet et al. (1996) and Morin et al. (2001) suggested that for conventional PCR genotyping there is a critical threshold value (56 and 25 pg, respectively) below which allelic dropout could be so high as to result in samples being unusable. The greater improvement for quolls compared to rock-wallabies may be due to faecal DNA quantity and/or quality, which may be sufficient for reliable genotyping in *P. penicillata*, such that the multiplex pre-amplification technique offers less improvement over single-step PCR.

The multiplex technique allows more loci to be analysed than the single-step approach because it uses substantially less of the DNA extract: only 12% required to perform eight replicates at each of six loci, compared with 96%. This major benefit may be vital for genotypic applications such as individual identification and parentage/related-

Table 2. Comparison of amplification, genotyping error rates and number of correct genotypes (using the criterion that an allele had to be present in five of eight replicates) from faecal DNA extracts from *Dasyurus maculatus*, *D. viverrinus* and *Petrogale penicillata*

Species	Locus	Amplification (%)		Genotyping Error (%)		Matching Genotypes	
		Conventional	Multiplex	Conventional	Multiplex	Conventional	Multiplex
Dasyurus	3.1.2	81.25	77.50	26.15	0	5	8
maculatus	1.3	62.50	100	12.0	0	3	9
(n = 10)	3.3.2	FA	80	FA	0	0	8
	3.3.1	78.75	100	19.05	0	3	8
	4.4.10	13.75	50	9.09	0	0	5
	4.4.2	FA	86.25	FA	0	0	9
	Average	39.37	82.29	11.04	0	1.83	7.83
	P-value[a]	<0.001 ($Z = 4.99$)		<0.001 ($Z = -3.73$)		<0.0001 ($Z = 5.84$)	
Dasyurus	3.1.2	7.50	90	0	0	1	9
viverrinus	1.3	62.5	72.5	7.5	0	5	8
(n = 10)	3.3.2	13.3	50	0	1.25	1	5
	3.3.1	87.5	98.75	12.08	0	8	10
	4.4.10	10	65	0	0	1	6
	4.4.2	10	80.0	0	0	1	8
	Average	43.05	75.18	3.26	0.21	2.83	7.67
	P-value[a]	<0.001 ($Z = 4.95$)		<0.01 ($Z = -2.34$)		<0.0001 ($Z = 5.21$)	
Petrogale	Pa593	98.75	90	5	0	9	9
Penicillata	Pa385	98.75	90	0	0	9	9
(n = 10)	Pa297	98.75	85	8.03	0	9	9
	Pa55	100	90	5	0	9	9
	Me17	98.75	93.75	16.40	0	9	9
	Me14	90	80	0	1.25	9	8
	Average	97.5	88.125	5.73	0.25	9	9
	P-value[a]	0.074 ($Z = -1.78$)		<0.05 ($Z = -2.52$)		0.317 ($Z = -1.00$)	

Values are averaged over 10 individuals and 8 replicate PCRs at each of 6 microsatellite loci, using conventional single-step PCR and the multiplex pre-amplification PCR method. n indicates the number of individuals sampled and FA indicates the locus failed to amplify for any sample or replicate.

[a] Wilcoxon Signed-Rank Test.

ness, particularly in species with low levels of genetic diversity (e.g., Taylor et al. 1994; Eldridge et al. 1999). Samples that amplified by the conventional approach generally amplified as well by the multiplex method so there may be no disadvantage in routine use of the technique. The multiplex pre-amplification method has also been successfully trialled on European brown bears

Table 3. Comparison of the average percentage of false alleles and allelic dropout for conventional PCR and two-step multiplex pre-amplification PCR methods for *Petrogale penicillata*, *Dasyurus viverrinus* and *Dasyurus maculatus*

Species	Conventional false alleles (%)	Multiplex false alleles (%)	Conventional allelic dropout (%)	Multiplex allelic dropout (%)
P. penicillata	3.72	0.02	0.92	0
D. maculatus	7.59	0**	2.77	0*
D. viverrinus	2.56	0*	0.41	0.21

P is the probability that there is no significant difference between the number of false alleles and allelic dropout for each sample for conventional PCR and two-step multiplex pre-amplification PCR using Wilcoxon Signed-Rank Test.

* $P < 0.05$, ** $P < 0.001$.

(*Ursus arctos*), although there was no significant difference in amplification rates or genotyping error rates compared to a conventional single-step PCR (E. Bellemain, data not shown).

In conclusion, the multiplex pre-amplification PCR method worked well for three different species, representing both carnivores and herbivores. We envisage that this method will markedly improve amplification and genotyping error rates for a range of species where conventional single-step PCR approaches experience low success due to limiting conditions. For other species where conventional PCR does work well, the multiplex method may still be a useful alternative if a larger number of loci are to be analysed.

Acknowledgements

Thanks to the staff at Healesville Sanctuary for their assistance with collecting faeces from *D. maculatus* and *D. viverrinus*. Dr. Gerhardt Kortner and Shaan Gresser are thanked for providing faecal and tissue samples from wild *D. maculatus* individuals. Natasha Czarny is also thanked for obtaining samples from *D. viverrinus* individuals from Pearcedale Conservation Park. Thanks to Geoff Underwood at Tidbinbilla Nature Reserve for his assistance in supplying faecal and tissue samples from *P. penicillata* individuals. This research was funded by an Australian Research Council IREX grant and was carried out under Monash University Animal Ethics Permit No. BSCI/2000/09. M. Piggott is supported by an APAI Scholarship from an Australian Research Council SPIRT grant.

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