

## TECHNICAL NOTES

### A differential DNA extraction method for sperm on the perivitelline membrane of avian eggs

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Sperm competition is common in birds, and arises when sperm from different males compete to fertilize eggs (Birkhead 1998). Sperm could compete at several locations within the female reproductive tract up to the moment of gamete fusion, and the tract itself could influence the outcome. Molecular techniques have been used extensively to elucidate offspring paternity, but also hold great promise to further our understanding of sperm competition mechanisms.

In birds, sperm are stored in tubules at the uterovaginal junction, but fertilization takes place in the infundibulum. Just after fertilization, sperm present near the egg are trapped between the ovum's perivitelline layers, providing an allelic record of the males competing to fertilize the egg. Comparing this information with putative paternal and maternal alleles, we can examine the existence of sperm competition close to the time and place of fertilization. A hurdle to this endeavour is the presence of maternal and/or embryonic DNA attached to the perivitelline membrane (PVM), which — due to its potentially higher concentration — might out-compete sperm DNA in polymerase chain reaction (PCR) amplifications. Here we present a two-step differential extraction method used in human forensics to restrict PCR competition between sperm DNA and vaginal epithelial cell DNA (Gill *et al.* 1985; Yoshida *et al.* 1995).

We collected two clutches from the socially monogamous tree swallow (*Tachycineta bicolor*), a species with high levels of sperm competition (Kempnaers *et al.* 1999). Eggs were removed at the start of incubation and stored at 4 °C. Within 9 days, we counted sperm on the PVM according to the method described by Birkhead *et al.* (1994). The tissue was then stored in 70% ethanol at 4 °C.

Sperm nuclei resist SDS/proteinase K lysis, but can be lysed in a dithiothreitol (DTT)/proteinase K mixture (Gill *et al.* 1985). This allows purification of DNA from sperm collected with other tissues. After removing ethanol from the PVM by centrifugation at 7000 g, we lysed non-sperm tissue at 60 °C for 1–2 h in 600 µL extraction buffer A (100 mM Tris, 100 mM NaCl, 2 mM EDTA, 1% SDS, 100 µg/mL proteinase K). We pelleted sperm nuclei (SP fraction) from maternal/embryo DNA (EM fraction) by 5 min centrifugation at 13 000 g. We

then lysed the SP fraction at 55 °C for 2–3 h in 300 µL of extraction buffer B (10 mM Tris, 10 mM NaCl, 20 mM EDTA, 1% SDS, 0.04% proteinase K, 1% DTT). Both fractions were subsequently purified with a standard phenol/chloroform extraction and concentrated using Centricon YM100 tubes.

We performed PCR amplifications of both fractions separately using two microsatellite loci (HrU6 and HrU7; Table 1). The 10 µL PCR reactions contained 1 µL DNA, 0.5 µM of each primer (one fluorescently labelled), 100 µM each dNTP, 1.8 mM (HrU6) or 2.5 mM (HrU7) MgCl<sub>2</sub>, 10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.1% Triton®X-100 and 0.5 units Taq DNA polymerase (Promega). The thermal profile was one 5 min cycle at 94 °C, 45 cycles of 30 s at 94 °C, 30 s at 62 °C and 30 s at 72 °C, and one 30 min cycle at 60 °C. Products were electrophoresed on an ABI310 Genetic Analyser and scored using Genotyper 1.1 (Perkin-Elmer Applied, Biosystems). All necessary precautions and controls were used to prevent PCR contamination.

For most eggs, non-parental alleles were amplified (Table 1). Excluding mutation as a source of novel alleles (unlikely), sperm from multiple males were present on the PVM. This suggests that, in tree swallows, sperm competition continues up to the site of fertilization.

The differential extraction method appears to have been successful in separating maternal/embryo DNA from the SP fraction (Table 1). No female alleles were amplified in the SP fraction for HrU6. As HrU7 shows mainly common alleles (Kempnaers *et al.* 1999), the alleles in the SP fraction matching those of the social female might well be from extra-pair male sperm. Alternately, this could reflect a failure to totally exclude maternal/embryo DNA from the sperm (Yoshida *et al.* 1995). Regardless of the extent to which female alleles were removed, reduction of maternal/embryo tissue resulted in amplification of novel alleles in the SP fraction (Table 1).

Our method was not as successful at separating sperm from the EM fraction, which might be due to sperm nuclei lysis prior to the differential extraction. Also, some sperm alleles were only found in the EM fraction. Thus, when searching for male alleles, both fractions should be examined. There was also a suggestion of preferential amplification of smaller (< 200 bp) HrU6 alleles (Table 1), highlighting the importance of selecting appropriate loci (i.e. polymorphic and narrow allele size range).

Currently, little is known about the mechanism of sperm competition within the avian female reproductive tract. Our findings indicate that sperm from multiple males are present at the fertilization site. By using the method described here, we foresee several promising avenues for research on sperm competition. For example, storage patterns of sperm within individual sperm storage tubules or differential release of sperm from sperm storage tubules could be investigated.

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**Table 1** Alleles amplified from the perivitelline membrane of tree swallow *Tachycineta bicolor* eggs using the differential extraction method

	Number of sperm†	Locus			
		HrU7		HrU6	
		EM‡	SP§	EM	SP
<b>Clutch 1</b>					
Female alleles			A		G
Social male alleles			AB		HQ
Egg 1	312	<b>A B C D</b>	<b>A D E</b>	G H	<b>H I</b>
Egg 2	259	<b>A D E</b>	<b>A D</b>	<b>G I J</b>	<b>H I</b>
Egg 3	224	A	<b>A D</b>		<b>I K</b>
Egg 4	191	<b>A B C D F</b>	<b>A D F</b>	<b>H I J L</b>	
Egg 5	85	<b>A B C D F</b>	<b>A C D</b>	G H	I
<b>Clutch 2</b>					
Female alleles			BE		MN
Social male alleles			AF		IO
Egg 1	249	<b>A B D E</b>	A F	<b>H I</b>	<b>G I</b>
Egg 2	324	<b>A B D E</b>	A B E	<b>G H J</b>	<b>G I</b>
Egg 3	125	A B E	<b>D</b>	<b>G I P</b>	I
Egg 4	80		A B D E	<b>I J N</b>	<b>I J</b>
Egg 5	257	B E	<b>A D</b>	<b>J K M N</b>	

Letters (A–Q) refer to alleles of increasing size in the following size ranges: HrU7: 101–147 bp, HrU6: 150–347 bp (total number of alleles found in the population: HrU7: eight alleles, 101–147 bp; HrU6: 91 alleles, 150–430 bp; Kempnaers *et al.* 1999); non-parental alleles are in bold; alleles >200 bp are in italics. †Sperm numbers on the perivitelline membrane. ‡EM, embryonic/maternal DNA fraction. §SP, sperm DNA fraction.

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## Urine collected in the field as a source of DNA for species and individual identification

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The development of noninvasive genetic sampling is increasingly important to field biologists as the methods offer new ways to answer elusive questions in conservation biology and behavioural ecology. Mitochondrial DNA (mtDNA) sequence variation allows for species identification, and nuclear DNA (microsatellites) polymorphism can be used to identify individuals. Hairs and faeces are the most commonly used source of DNA for noninvasive mammal studies. mtDNA is relatively easy to amplify via the polymerase chain reaction (PCR) from such samples, but the very low DNA content of the extracts makes the analysis of nuclear DNA difficult and can lead to genotyping errors due to allelic dropout and/or PCR artifacts (Taberlet *et al.* 1996; Goossens *et al.* 1998).

Some studies have shown that urine samples might represent a potentially valuable source of DNA (e.g. Boom *et al.* 1990; Marklund *et al.* 1996; Yokota *et al.* 1998; Vu *et al.* 1999). However, these studies used urine samples directly obtained from individuals, but never used samples dropped in the field. In this note, we assess the potential of urine samples collected in snow for species and individual identification in Canids.

A total of five urine samples (U1 to U5) were collected in snow along canid tracks in the Mercantour National Park (Maritime Alps, France) and preserved at –20 °C during one

**Table 1** Alleles observed in each sample over the six microsatellite loci (eight PCR per locus) using urine collected in the field as a source of DNA. The species (wolf or dog) identified by mtDNA sequences is indicated in brackets. The per cent of successful amplified loci is shown in the last line of the table. Underlined alleles correspond to alleles that appear more than three times among the eight repetitions of the multiple-tubes protocol

	U1 (wolf)	U2 (wolf)	U3 (wolf)	U4 (wolf)	U5 (wolf)	U6 (dog)
FH2140	<u>132</u>	132	<u>132</u>	—	<u>132, 154</u>	132, 140, 148
PEZ17	<u>196, 200</u>	<u>196, 200</u>	<u>196, 200, 204</u>	—	<u>196, 200, 204</u>	200, <u>204</u> , 208, <u>212</u>
FH2161	<u>267</u>	263, 267	<u>267, 271</u>	—	<u>239, 267, 271</u>	<u>247, 250</u>
FH2050	<u>249, 259</u>	249, 259	249, 259	—	<u>249, 259</u>	249
FH2137	<u>160, 166</u>	<u>160, 166</u>	<u>160, 166, 174, 179</u>	—	<u>160, 166, 174, 179</u>	<u>166, 174, 177, 185, 189</u>
FH2096	<u>96, 100</u>	<u>96, 100</u>	<u>96, 100</u>	—	<u>96, 100</u>	<u>100, 104</u>
% positive PCR	85.4%	52.1%	79.1%	0.0%	91.7%	75.0%

Locus	U1		U2	
	undiluted	diluted (0.1×)	undiluted	diluted (0.1×)
FH2096	0% (100%)	75% (100%)	66.7% (75%)	— (0%)
PEZ17	12.5% (100%)	100% (75%)	71.4% (87.5%)	100% (37.5%)
FH2137	0% (100%)	71.4% (87.5%)	85.7% (87.5%)	100% (25%)
Mean	4.2% (100%)	82.1% (87.5%)	74.6% (83.3%)	100% (20.8%)

**Table 2** Percentage of allelic dropout and of successful PCR (in brackets) using urine collected in the field as a source of DNA. Allelic dropout percentage is calculated over the successful PCR

to 12 months before DNA extraction. A last sample (U6), preserved at ambient temperature, corresponds to a piece of larch bark on which the presence of canid urine was suspected.

To precipitate DNA and/or to pellet cellular remains from 15 mL of melted snow samples, we added 1.5 mL of sodium acetate 3 M (pH 5.2) and 33 mL of absolute ethanol. After incubation overnight at  $-20^{\circ}\text{C}$ , we centrifuged the mixture (5500 g, 35 min,  $6^{\circ}\text{C}$ ) and discarded the supernatant. The pellet was then subjected to a classical DNA extraction using QIAamp Tissue Extraction Kit (Qiagen). For urine on tree bark, we used the QIAamp DNA Stool Extraction Kit (Qiagen). Bark pieces were vigorously vortexed in the buffer ASL and subsequently centrifuged. The supernatant was subjected to DNA extraction following the suppliers' instructions. To detect whether contamination of samples with exogenous DNA had occurred during the extraction procedure, tubes without samples (extraction negative control) were treated identically through both the DNA extraction and amplification.

A part of the mitochondrial control region (about 360 bp) was amplified by the PCR method using the primers L15995 (called 'forward' in Taberlet & Bouvet 1994) and H16498 (Fumagalli *et al.* 1996). Six microsatellite loci (Mellersh *et al.* 1997; Neff *et al.* 1999; see Table 1) were used to test individual identification. Because we suspect that urine samples have low DNA quantities (like scats or hairs), we may expect allelic dropout during the microsatellite analysis. Eight repetitions (multiple-tubes approach; Taberlet *et al.* 1996) were performed for each individual and for each locus to estimate the rates of successful PCR and allelic dropout. For two individuals, we performed additional experiments using a 10× diluted DNA extract as template for three out of the six loci (individuals are heterozygous for these loci).

Amplification was carried out in a 25- $\mu\text{L}$  reaction volume containing 1 U/tube AmpliTaq® Gold DNA Polymerase (Perkin Elmer), 10 mM Tris-HCl, 50 mM KCl, 2.5 mM  $\text{MgCl}_2$ , 0.1  $\mu\text{M}$  each dNTP and 200  $\mu\text{g}/\text{mL}$  bovine serum albumin (BSA, Boehringer-Mannheim). Template volumes were 5  $\mu\text{L}$  for mtDNA and 2  $\mu\text{L}$  for microsatellites amplifications. After 10 min at  $95^{\circ}\text{C}$  (*Taq* activation), the PCR cycles were as follows: 55 cycles of 30 s at  $95^{\circ}\text{C}$ , 30 s at  $55^{\circ}\text{C}$  (for mtDNA and for FH2050, FH2096, FH2161 loci) or  $50^{\circ}\text{C}$  (for FH2140, FH2137, PEZ17 loci) and 1 minute at  $72^{\circ}\text{C}$ .

For all samples, mtDNA was successfully amplified and sequenced. Five of the samples were identified as wolf (U1 to U5) and one sample as domestic dog (U6). For microsatellites, the screening of six microsatellite loci has revealed that nuclear DNA can be amplified with good success (see Table 1) except for one sample. However, the eight repetitions clearly showed that some samples contained more than two alleles (Table 1). This indicates the mixing of urine from more than one individual, which is consistent with field observations showing that several canids could deposit urine at the same place. Concerning the rate of successful PCR (successful amplification rate, SAR) and of allelic dropout (allelic dropout rate, ADR), they greatly varied between the two samples (Table 2). For the first one (U1), the SAR was 100% and the ADR was very low (4.2%). However, when we diluted 10× the extract products, the reliability of individual identification decreased greatly (SAR decreased to 87.5% and ADR increased to 82.1%). For the second sample (U2), SAR and ADR are, respectively, drastically lower and higher than for the first sample (both undiluted and diluted extraction product; see Table 2).

This pilot study clearly demonstrated that urine collected in snow represents a valuable source of DNA. However, the total

amount of DNA is highly variable and can be very low. Thus, we highly recommend the multiple-tubes approach (Taberlet *et al.* 1996). Another potential difficulty comes from the fact that several individuals might urinate in the same place, so we also recommend collecting a snow sample only when it comes from a single individual according to snow tracks.

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## Microsatellite isolation using amplified fragment length polymorphism markers: no cloning, no screening

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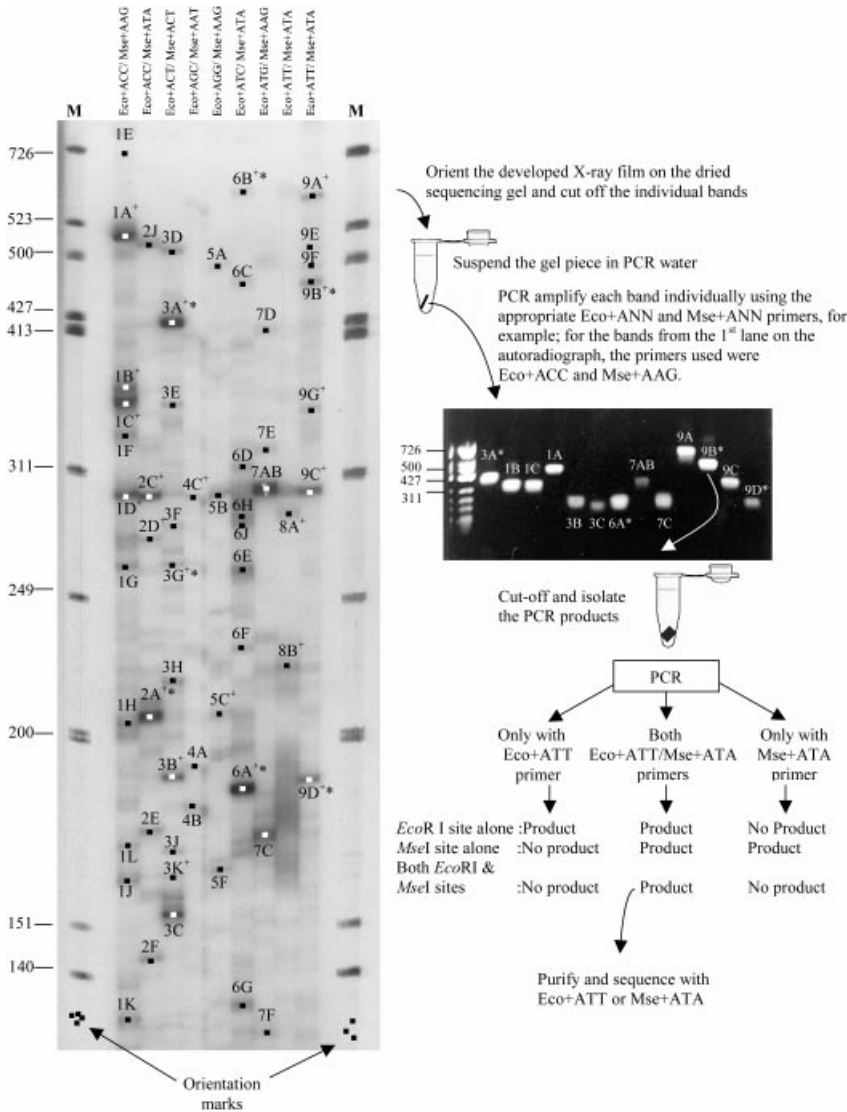
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Although microsatellites are very informative type of markers (Tautz 1989), the need for prior sequence information to produce locus specific primer sets is a major limitation. The method presented here provides an alternative approach for the isolation of microsatellite loci in any organism and eliminates labour-intensive library constructions, screening of libraries and the maintenance of the colonies. The approach combines amplified fragment length polymorphism (AFLP) marker generation together with an enrichment step and applicable to all organisms. AFLP fragments were generated with Eco + 3 and Mse + 3 and then were enriched separately using a biotinylated target repeat oligonucleotide on the streptavidin coated magnetic beads. The eluted fragments were re-amplified in the presence of [ $\alpha^{32}$ P]-dATP using the same set of selective primer combination and separated on a DNA sequencing gel. The bands were cut-off from the sequencing gel, reamplified and purified. The relatively long fragments containing both the MseI or EcoRI restriction sites were directly sequenced using either site selective primer.

The genomic DNA isolation of a durum wheat cultivar, 'Selçuklu-97', was performed according to Saghai-Marooof *et al.* (1984) with some modifications. The reaction steps, components, the adapters, and the primers of AFLP marker generation were essentially the same as Vos *et al.* (1995) with some optimizations below. Pre-selective amplification was performed using Eco + A and Mse + A primers (20 cycles of three steps at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min). Selective amplifications were performed in the absence of [ $\alpha^{32}$ P]-dATP using AFLP primer combinations in Fig. 1 (11 cycles of denaturation at 94 °C for 30 s, annealing at 65 °C (–0.7 °C/each cycle) for 30 s, extension at 72 °C for 1 min and additional 24 cycles of denaturation, annealing and extension at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min, respectively). Each selectively amplified polymerase chain reaction (PCR) product was enriched according Fisher & Bachmann (1998) with the following changes: streptavidin coated magnetic beads (Streptavidin S-2415; Sigma Corp., MO) of 300  $\mu$ g suspension was equilibrated in 20  $\mu$ L of 6 $\times$  SSC. The hybridization complex was formed by incubating 30 pmol of 5'-end biotinylated (GA)<sub>9</sub> oligonucleotide with 20  $\mu$ L of selectively amplified PCR products in a final volume of 50  $\mu$ L (in 6 $\times$  SSC) for 5 min at 94 °C. The samples were loaded on beads, allowing streptavidin and biotin association for 15 min at 25 °C. The immobilized DNA hetero-duplexes were washed twice in 500  $\mu$ L 2 $\times$  SSC, 0.1% SDS, after incubation for 5 min at 25 °C, continued washings were twice in 1 $\times$  SSC at 25 °C and twice in 1 $\times$  SSC at 47 °C for 2 min. The single stranded 'GA' enriched AFLP fragments were eluted in 10  $\mu$ L PCR water after 15 min incubation at 25 °C, in two steps. They were reamplified separately using the same AFLP primer combinations used prior to enrichment, in the presence of 0.1  $\mu$ L [ $\alpha^{32}$ P]-dATP (3000 mCi/mmol) in a final volume of 20  $\mu$ L, containing 10  $\mu$ L eluted DNA, 50 ng of each primers, 3 mM Mg<sup>2+</sup>, 0.2 mM of each dNTP, 75 mM Tris-HCl, pH 9.0, 20 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.01% Tween-20 and 1 U Taq DNA polymerase



**Fig. 1** The scheme of isolation of microsatellite markers. The autoradiograph of AFLP fragments generated after 'GA' repeat enrichment using the set of primers indicated on each lane is on the left. The bands that are labelled and marked with dots (57 bands) were randomly selected for reamplification. Agarose gel picture shows some of the reamplified products from DNA sequence gel cut-off bands. Each agarose gel isolated band (30 bands, approximately 300 bp, and some short ones) was checked for the presence of both of the sites; 21 bands, marked with '+', out of 30 had both *EcoRI* and *MseI* site adaptors. Among 21 purified fragments, seven size representing fragments, marked with '\*', were sequenced. The longer ones (3A, 6B, 9A) had repeat sequences long enough for microsatellite primer design (Table 1).

(MBI-Fermentas, Lithuania). The fragments were separated on a DNA sequencing gel (Fig. 1). The gel was exposed to Kodak Bio-Max/MR film for several days. The bands were cut-off together with the attached filter paper behind or scratched from the paper using a sterile blade and suspended in 50 µL PCR grade water. The DNA fragments were reamplified as above except, in 50 µL reaction volume with 25 ng Eco + ANN and 75 ng of Mse + ANN selective primers, the same set generated the cut-off AFLP band (30 cycles of denaturation, annealing and extension at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min, respectively). The ends of the fragments were checked for the presence of both of the *MseI* and *EcoRI* site adaptors (Fig. 1). The DNA fragments were extracted from agarose gel pieces using QIAquick gel extraction kit (Qiagen, CA). The sequencing reactions were performed using Big-dye fluorescent labelling sequencing reaction mixture. The sequences were read on ABI Prism-310 Genetic Analyser.

Many well-separated bands were observed on the AFLP gel autoradiograph after selective amplification PCR of the 'GA' enriched AFLP fragments, of which 57 bands were reamplified and purified (Fig. 1). All the reamplified bands were observed as pure fragments on the agarose gels. The products approximately 300 bp in length (30 bands) were selected for designation of the ends with both *EcoRI* and *MseI* site adaptors. The microsatellites (3A, 6B and 9B) were found to contain composite repeat types (Table 1). Under the conditions applied for enrichment, 9D with three repeating units was also selected. In order to find loci with the tandem repeats of 10 or more, the stringency can be increased during enrichment, because the larger the repeat number, the higher the observed polymorphism levels.

We believe this approach for finding and sequencing of microsatellite loci is a viable alternative to current practice and may prove to be advantageous and productive for most laboratories.

