Polymorphic DNA microsatellites identified in the yellow dung fly (*Scathophaga stercoraria*)

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Sperm competition in yellow dung flies (*Scathophaga stercoraria*) has been extensively investigated since Parker's (1970a) seminal work (e.g. Parker & Simmons 1991; Ward 1993; Hosken & Ward 2000; reviewed in Hosken 1999). These flies serve as a model system for understanding the mechanisms and outcomes of sperm competition in internal fertilizers. Invariably however, these investigations have been laboratory based, and typically involved competition between only two males. How the results of such studies relates to free-living flies is unknown, but it is unlikely that the experimental conditions employed exist in nature, and therefore outcomes may not reflect true female sperm utilization patterns (Eady & Tubman 1996). This is exemplified by a study of sperm competition in pseudoscorpions, which showed that second-male mating advantage breaks down when females mate with more than two males (Zeh & Zeh 1994). In addition, Ward (2000) has shown that females are able to subtly alter paternity patterns under conditions that are likely to be common in the field. With this in mind, our aim was to develop appropriate genetic markers to allow paternity to be accurately assigned in clutches laid by free-living female yellow dung flies.

A subgenomic library enriched for CA repeat microsatellites was constructed following standard protocols outlined in Tenzer *et al*. (1999), with slight modifications. Genomic DNA isolated from a single *S. stercoraria* male using standard phenol–chloroform extraction and ethanol precipitation (Sambrook *et al*. 1989) was digested using *Tsp*509I (New England Biolabs). A 500–1000 bp size fraction was isolated from a LM-MP agarose (Boehringer Mannheim) gel by first excising the appropriate size range from the gel. The gel fragment was melted in a 65 °C water bath and volume was increased to 500 μL using double distilled water. An equal volume of equilibrated phenol (pH 8.0) was added, the solution vortexed briefly and then put at – 80 °C for 30 min. The sample was then thawed and extraction was completed following standard phenol–chloroform extraction methods (Sambrook *et al*. 1989). This isolate was used for ligation with TSPADSHORT/TSPADLONG linkers (Tenzer *et al*. 1999) and then amplified via the polymerase chain reaction (PCR), using TSPADSHORT as a primer. PCR was performed using the following conditions: Total reaction volume was 25 μL included 100 ng DNA, 1 U *Taq* DNA polymerase (Quantum-Appligene), 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% TritonX100, 0.2 mg BSA (Quantum-Appligene), 100 μM of each dNTP (Promega), and 1 μM of TSPADSHORT. PCR was performed on a Techne Genius thermocycler (Techne Ltd) using the following thermotreatment: 2 min at 72 °C, followed by 25 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C. A total of 32 PCRs were carried out, pooled, cleaned and concentrated to minimize the likelihood of redundant products being detected during screening for positive clones. PCR products were hybridized to biotinylated $(CA)_{20}$ probes bonded to streptavidin-coated magnetic beads (Dynabeads M-280 Streptavadin, DYNAL, France) and amplified again. These final PCR products were cloned following the Original TA Cloning® Kit (Invitrogen) protocol. White colonies were dot-blotted onto nylon membranes (Hybond™-N^+, Amersham Pharmacia) and screened for CA repeats using the ECL 3′-oligolabelling and detection system (Amersham Pharmacia) and a 40mer CA oligonucleotide. All positive clones were sequenced following the ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit protocol, version 2.0 (PE Biosystems) using M13 forward and reverse primers, and using the ABI 377 automated sequencing system (PE Biosystems). Primers were designed using Primer3 software (Rozen & Skaletsky 1998) and all oligonucleotides were synthesized by Microsynth GmbH (Switzerland). Initial tests for amplification and polymorphism were carried out at 55 °C and electrophoresed on 8%, nondenaturing, 14.5 cm × 17 cm acrylamide gels at 80 V overnight. Those primers that amplified polymorphic products using five test templates were used for all following analyses.

Only field-caught male *S. stercoraria* were used for PCR analysis, as almost every field-caught female is already mated (Parker 1970b), and extraction from fertilized females could therefore result in contamination by sperm DNA. Each sample male was extracted using the QIAamp® DNA mini kit (Qiagen). Twenty males were used to characterize suitable primers, and PCR was carried out using approximately 100 ng of template DNA and the following cycle treatment; initial step of 3 min at 94 °C, followed by 27 cycles of 30 s at 94 °C, 30 s at 58–61 °C (see Table 1), and 30 s at 72 °C, with a final extension step of 2 min at 72 °C. Total reaction volume was 25 μL and contained 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% Triton × 100, 0.2 mg BSA (Quantum-Appligene), 100 μM of each dNTP (Promega), 0.5 μM of both forward and reverse primer, and 0.5 U *Taq* DNA polymerase (Quantum-Appligene). All products were electrophoresed on Spreadex™ EL-300 S-100 gels (Elchrom Scientific AG, Switzerland), using the SEA 2000™ advanced submerged gel electrophoresis apparatus (Elchrom Scientific AG, Switzerland). Gels were run at 100 V for 80–90 min, depending on allele sizes, then scored against the M3 Marker ladder (Elchrom Scientific AG, Switzerland). Expected and observed counts for homozygotes/heterozygotes were determined using GENEPOP version 3x (Raymond & Rousset 1995) and homozygote excess was tested for using Chi-square analysis (null hypothesis rejected at $P < 0.05$).

A minimum of five alleles were detected at each of the loci listed in Table 1. Tests for homozygote excess were only

Table 1 Primer sequence and related information for eight microsatellite loci developed for *Scathophaga stercoraria*. Both repeat motif and size of amplification product are based on that detected in the original sequenced clone (GenBank Accession nos: AF292121–8). *n*, number of individuals tested; T_a, annealing temperature; H_O, observed number of homozygotes; H_E, unbiased average heterozygosity estimate (Nei 1978)

Locus	Primer Sequences (5′–3′)	Repeat motif	T_a (°C)	*n*	No. alleles	Size (bp)	H_O	H_E
SsCA3	CCTCAACCCCCTCACTCAC CATCATCATTTAAGTCAACATTAGAAA	$(AC)_1(A)_2(AC)_{11}$ $(A)_3(C)_2(A)_3$	60	20	11	120	0.35	0.795
SsCA16	GACTTTGGTCCGTTGTAGTCC TTGGCGTCACCATACTCAAC	$(C)_3AT(AC)_{11}AT$ $(AC)_2(C)_3$	60	20	7	101	0.10	0.806
SsCA17	AATAAAAACTCAACCAACCATACAC CCTTACTCGATAAGTTGGTATTTGTG	$(TA)_2GA(CA)_4CG$ $(CA)_5$	60	18	6	108	0.40	0.695
SsCA20	TGTTTGCTGGTGCTACCG TGATCGTTGTTGTTTCATACG	$(CA)_{10}$	60	18	5	120	0.55	0.600
SsCA24	CACACACTCGCAGCTACACC AAACTTTAACTTCGATTTTTGCTG	$(C)_4AT(AC)_9$	60	20	8	120	0.30	0.821
SsCA26	TGCCACTTTTGGTGCTTTC CAGCAAAAACCGGCAAAC	$(CA)_{11}(T)_2(CA)_2CG(CA)_4CG$ $(CA)_4(T)_2(CA)_2(T)_2(GTT)_2$	61	20	8	110	0.25	0.845
SsCA28	GTTTGAAACCCTTAAGATAAAAACTC CCATCTTTCACGGGATTTTG	$(CT)_2(CA)_5AACG$ $(CA)_{10}$	58	20	13	127	0.35	0.890
Ss63T7	AAAGAATTTTACGAATTGTGTCTGG CAACAAATGCAACAAATGACC	$(CA)_6(A)_2(CA)_8$	58	18	8	129	0.20	0.869

significant at one locus, SsCa28, which may suggest one or more null alleles operating at this locus.

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Polymorphic microsatellite loci in vespertilionid bats isolated from the noctule bat *Nyctalus noctula*

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