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Short communication

Development of microsatellite markers using 454 sequencing for the rare socially parasitic hoverfly, *Microdon mutabilis*

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Abstract. To date there have been only limited fine-scale investigations into the molecular ecology of the European hoverfly, *Microdon mutabilis*, due to the paucity of available polymorphic markers. We describe the development of primers amplifying five novel microsatellite loci using next-generation sequencing (454) and three previously undescribed *M. mutabilis* microsatellite loci using enrichments. In hoverflies from a population in Ireland, the number of alleles per locus ranged from 2 to 16, and the observed heterozygosity ranged between 0.26 and 0.97

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The rare European hoverfly, *Microdon mutabilis* (Syrphidae: Microdontinae), is a cryptic myrmecophilous social parasite that displays extreme host specificity to *Formica lemani* ant colonies primarily within the British Isles and Scandinavia (Elmes *et al*. 1999; Schönrogge *et al*. 2006). Although cause and effect are unclear, the flies infest host ant nests that have high levels of genetic diversity (Gardner *et al*. 2007). The extreme local specificity is reflected in bioassays where geographic distance from natal nest of ovipositing females affects the survivorship of her eggs (for details see Elmes *et al*. 1999; Schönrogge *et al*. 2006) and we predict this could promote localised genetic structuring within the flies. To date there have been only limited fine-scale investigations into the molecular ecology of *M. mutabilis* due to a lack of available polymorphic markers. An investigation at a fine scale will not only assist in understanding the measures that need to be implemented in the conservation of this rare fly, but also provide insights to the preservation of genetic diversity of its host ant species *F. lemani*. Here we detail the development of eight microsatellite loci; five of these are novel and three were previously used by Schönrogge *et al*. (2006) but remain undescribed.

Two different methods of microsatellite development were used. Three loci were isolated using enrichments following Gardner *et al*. (1999) (Mmut9) or Hale *et al*. (2001) (CEH49, CEH52). All remaining loci were developed using next-generation sequencing and subsequent bioinformatic screening following Gardner *et al*. (2011). Genomic DNA was extracted using the Gentra Puregene Tissue Kit (Qiagen, Doncaster, Australia) from the legs of 15 individuals of *M. mutabilis* collected from the Burren and from the Isle of Mull, and sequenced on a Titanium GS-FLX (454 Life Sciences/Roche FLX) at the Australian Genomic Research Facility (Brisbane, Australia, www.agrf.org.au) following Gardner *et al*. (2011). A total of 55 312 reads were obtained with a mean read sequence length of 354 bp from ~19.6 Mb of sequence. The program QDD ver 1.0 (Méglecz *et al*. 2010) was used on a Windows system with the graphical user interface to screen the 454 raw sequences for microsatellites (newer versions were not available at the time the study was conducted), to check flanking regions to identify unique loci and to design primers for perfect di-, tri- and tetranucleotides with a minimum of eight tandem repeats. Amplicon length was specified between 90 and 450 bp and all other parameters in QDD were set to default. The installation of QDD ver 1.0 followed Méglecz *et al*. (2010) and also required the installation of ActivePerl ver 5.10 (http://www.activestate.com/activeperl/), BLAST ver 2.2.18 (ftp://ftp.ncbi.nih.gov/blast/executables/), CLUSTALw2 ver 1.83 (Larkin *et al*. 2007) (ftp://ftp.ebi.ac.uk/pub/software/clustalw2/) and Primer3 (Rozen and Skaletsky 2000) (http://primer3.sourceforge.net/). This process yielded 94 putative microsatellite loci for *M. mutabilis*.

Following Gardner *et al*. (2011), and using the primer pairs suggested as the 'best' in QDD, 40 loci were tested for polymorphism on eight samples of *M. mutabilis*: four collected from each of The Burren (53°07′29″N, 9°05′19″W) and the Isle of Mull (56°32′44″N, 6°02′13″W). PCR amplifications for all loci except MiMut24 were performed in 15-µL reaction volumes

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Table 1. Characterisation of *Microdon mutabilis* loci

N, sample size; *N*a, number of alleles; *Ho* and *He*, observed and expected heterozygosity respectively; PIC, polymorphic information content; *, significance after corrections for multiple tests; HWE, Hardy–Weinberg Equilibrium. Superscripts F, N, V, and P indicate that loci were 5′ labelled with the dyes 6-FAM, NED, Vic. and PET respectively. Repeat motif indicates the number of repeats in the sequence used for development

Locus		Primer sequence (5′–3′)	GenBank accession number	Repeat motif	*N*	Allele size range	*N*a	*Ho*	*He*	PIC	Null allele freq	HWE *P*
MiMut12	F:	^{F}AAACTGGATGGCATCACCTC	JQ907481	(TGA)8	31	133–136	3	0.257	0.252	0.224	0.018	0.315
	R:	TCTTGAATTCAGCTTTATCGGA										
MiMut14	F:	^{P}AAATAAGGCCAAGACCGGAT	JQ907482	(GAT)8	29	144–160	7	0.500	0.443	0.409	–0.084	0.035
	R:	TGCCCATTAAACATTGCGTA										
MiMut24	F:	^{N}AAGGAGCTCCATAGCCTTCC	JQ907478	(ATC)8	29	241–247	2	0.387	0.419	0.327	–0.031	0.945
	R:	CAGGACTTGCTAAATAAGGCCA										
MiMut27	F:	^{V}GAGGCTGGGATTAGCCAACT	JQ907480	(GT)14	27	323–356	5	0.517	0.699	0.629	0.135	0.019
	R:	ACGCACGCACAGTACAAGAC										
MiMut28	F:	^{F}CACTATCGAGAGCGATGTGC	JQ907479	(ATG)8	31	278–284	3	0.657	0.606	0.523	0.042	0.169
	R:	CACCAACTCTGAGGGAAAGG										
CEH49	F:	^{V}TCCCAACAACATTCTCGTCA	DQ150106	(CA)17	19	139–141	2	0.800	0.513	0.375	–0.231	0.039
	R:	TCTGATAATCTGCGCTTTGG										
CEH52	F:	^{P}TGTGCATGAACATTAATTTGCTAAC	DQ146463	(TC)16	31	197–249	16	0.971	0.891	0.867	–0.055	0.054
	R:	CCAGAAACGAGAAGAGAAATGG										
Mmut9	F:	^{F}GCGCATCGTTGAACAC	DQ146464	(AC)23	30	302–313	4	0.412	0.378	0.348	–0.052	0.761
	R:	CGTCTTTGGCGTCTGATAA										

containing 1× ImmoBuffer, 0.2 mM each dNTP, 200 nM each forward and reverse primer, 0.3U IMMOLASE DNA Polymerase (BIOLINE) and 20–40 ng template gDNA. The reaction conditions for MiMut24 were the same except 2 mM $MgCI_{2}$, 1× PCR Gold Buffer and 0.3U AmpliTaq Gold DNA Polymerase (Applied Biosystems). The PCR cycling conditions were: initial denaturation step at 94°C (MiMut24) or 95°C (all other loci) for 9 min, followed by 36 cycles at 94°C for 30 s, annealing temperature at 50°C for 30 s, and extension at 45°C for 45 s, followed by a single final extension step at 72°C for 30 min. PCR products were inspected for approximate allele size after separation by electrophoresis in 3% Nu-sieve GTG agarose gel (Cambrex).

We selected eight putative loci that amplified products of the expected size from the 40 trialled, attached florescent labels (FAM, GeneWorks; and NED, PET or Vic., Applied Biosystems) 5′ on each forward primer (Table 1) and amplified the loci using the PCR conditions described above. The PCR products were visually inspected on 6% polyacrylamide gels (Gel-scan 2000, Corbett, Sydney, New South Wales, Australia), which revealed that three loci did not produce clearly interpretable bands and these loci were subsequently removed. The five remaining loci and the three previously isolated (CEH49, Mmut9 and CEH52) (Table 1) were amplified in 189 samples of *M. mutabilis* using the two single-locus PCR reactions mentioned above. The PCR products were combined in two pools (Table1) for genotyping on an ABI 3730 DNA Analyser (Applied Biosystems) with the size standard GS500 (–250) LIZ and scored with GENEMAPPER ver 3.7 (Applied Biosystems).

To assess the suitability of markers for analysis we genotyped individuals from the study site in The Burren, Ireland. Samples with more than three missing microsatellite loci were removed from the dataset. We used MICROCHECKER 2.2.3 (Van Oosterhout *et al*. 2004) to examine each locus for potential scoring errors due to genotyping, stuttering and large allele drop-out and found no evidence for errors due to these factors. For each locus (Table 1) we calculated: the polymorphic information content (PIC), the number of alleles, observed (H_O), expected heterozygosity (H_E), and null allele frequency using Cervus 3.0.3 (Kalinowski *et al*. 2007), deviation from Hardy–Weinberg Equilibrium (HWE) and linkage disequilibrium between all pairs of loci using GENEPOP 4.1 (Raymond and Rousset 1995) (1000 dememorisations, 100 batches, 10 000 iterations per batch). *P*-values from HWE tests were manually adjusted for multiple tests of significance using the sequential Bonferroni method (Hochberg 1988). No loci showed significant deviation from HWE expectation after Bonferroni adjustment. No loci pairs showed evidence for linkage disequilibrium after sequential Bonferroni adjustment. The number of alleles and observed heterozygosity ranged from 2 to 16 and 0.257 to 0.971 respectively. No loci exhibited large null allele frequencies (largest 0.135 for MiMut27). With a combined non-exclusion probability (sib identity) of 0.00893, these loci are likely to be informative and valuable for future population-level genetic studies of *M. mutabilis.*

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