Molecular Ecology Notes (2002), **2**, 316–319 doi:10.1046/j.1471-8278 .2002.00227.x

PRIMER NOTE

Isolation and characterization of highly polymorphic microsatellite loci in the 2-Spot Ladybird, *Adalia bipunctata*

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Abstract

Contrary to theoretical predictions, female 2-spot ladybirds (*Adalia bipunctata*) mate many more times than necessary to maintain high fertilisation success and may gain through the acquisition of material or genetic benefits. In order to investigate this mating system in detail, microsatellite markers have been isolated using a modified enrichment technique. Thirty-nine loci were successfully amplified by polymerase chain reaction (PCR), of which only two were monomorphic. Detailed characterization of ten loci revealed very high levels of polymorphism. These markers are likely to be invaluable tools with which to study population genetics and patterns of paternity in this species.

Keywords: *Adalia bipunctata*, Coccinellidae, microsatellites, mating systems, paternity analysis

Received 22 January 2002; revision received 15 March 2002; accepted 10 April 2002

Female 2-spot ladybirds (*Adalia bipunctata*) are highly promiscuous; mating many more times than necessary to maintain fertility, despite high costs of mating to females (Majerus 1994). Females may gain from this behaviour through the acquisition of either material or genetic benefits (reviewed by Jennions & Petrie 2000). To investigate these benefits, reliable markers for the analysis of paternity and genetic diversity are needed. Previous studies investigating sperm precedence in *A. bipunctata* have used unreliable markers (De Jong *et al*. 1993) and markers of low variability (Ransford 1997). Microsatellite markers have therefore been isolated to allow more detailed analysis of the *A. bipunctata* mating system.

Microsatellite-enriched libraries were constructed according to Edwards *et al*. (1996), except that filters were prehybridized for four days (hybridization buffer changed after two days). Eight libraries were constructed in total, one at Horticulture Research International (in collaboration with Dr Celia James, who kindly supplied all the materials except *A. bipunctata* genomic DNA) and seven at the Department of Genetics, University of Cambridge, with further protocol modifications. Various different combinations of repeat motifs, hybridization temperatures and the number of rounds of enrichment were used.

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Colonies were cultured in LB Freezing Buffer (Zimmer & Gibbins 1997), allowing long-term storage at –80 °C. Colonies were transferred onto Hybond N + nylon membranes (Amersham Pharmacia Biotech) and probed with a mixture of eight oligo repeat sequences, end-labelled with [γ-^{32}P]ATP (hereafter referred to as 'end-labelled 8xSSRs'). Inserts were amplified from positive colonies in a PCR reaction, using microtitre stocks as template and universal M13 primers. PCR products were Southern blotted, probed with end-labelled 8xSSRs and positive inserts re-amplified and purified (Concert Rapid Gel Extraction Kit, Life Technologies). 2 μL of purified PCR products were applied to nylon membrane and probed with end-labelled 8xSSRs. Positive inserts were selected and purified PCR products used in sequencing reactions (Big Dye™ Terminator Ready Reaction Cycle Sequencing Kit, Applied Biosystems) which were run on ABI Prism 377 sequencing gels.

Forty-seven microsatellite-containing sequences were identified and primers designed using PRIMER 3 (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Primers were initially screened for amplification of a single PCR product of approximately expected size. PCR reactions were carried out in a PCR Express Gradient machine (Thermo Hybaid), allowing amplification to be tested at 12 annealing temperatures (range 50–65 °C, interval 1.36 °C on average). Amplifications were carried out in 20 μL, containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 10 ng each primer, 1 unit *Taq* DNA

Table 1 Screening of microsatellite loci in the 2-spot ladybird (*Adalia bipunctata*) against 10 individuals. *T* = primer annealing temperature. No. alleles > 1 when locus polymorphic but exact number of alleles unclear. Ten loci were characterized further (see Table 2)

Locus	Primer sequence (5′–3′)	*T* (°C)	Repeat motif	Cloned allele size (bp)	No. alleles	EMBL accession no
Ab1	F: CCAAATCCGTTAGTGGAACG R: CATTTTCCCAAGAGAAGTTTGG	55	$(GA)_{27}$	270	10	AJ427924
Ab2	F: GTAACAATGACCACACCCGTTACA R: CTTGACCCCCGTCCGATTAATATC	65	$(GT)_{13}$	211	> 1	AJ427925
Ab3	F: TTTCTTGACAGAATGATATCCTGAAC R: GGCAACGATTGTTGATCCTTTC	54	$(GT)_{10}$	152	10	AJ427926
Ab4	F: CCCGTTATTACCAAAACTGATT R: AGGCTCGTCAGTTGCTCCGTATTCA	60	$((CA)_4GA)_2(CA)_7$	113	8	AJ427927
Ab5	F: GTTGCCCGGTCGGATATACGTTTC R: CACACCCCCATACACTCACAGACT	65	$(TG)_{18}$	112	> 1	AJ427928
Ab6	F: TGCGGAAGCGAAAGAATCTGCTG R: GACTTGCCACACCCTATGACGTA	65	$(CA)_{12}$	285	5	AJ427929
Ab7	F: CACGAGTGTCAACTTATTCAAACTG R: GGACTAACAGGATATCACCTCAAGA	55	$(TG)_{32}$	154	3	AJ427930
Ab8	F: CCCGGTCGGATTTACGCTTCTTG R: CTGAACGCTTGGCAGCAGTTATT	65	$(TG)_{13}$	227	10	AJ427931
Ab9	F: AGCCACAATTAATCCCCTATCG R: TGTCCATAATTTTGAGCAGAGC	65	$(TC)_{31}$	248	9	AJ427932
Ab10	F: TATTACTTGCTTGTGTCTGTCAGGA R: ATACTCTGTGATCTGAATCGATGTG	65	$(AG)_{13}$	105	> 1	AJ427933
Ab11	F: AATAAATATTTTTGCAGCCCTGAAG R: TGTTGGTTAACATAAATTTCGATTC	60	$(GA)_{32}$	334	10	AJ427934
Ab12	F: GAATCTGTAACGTAGGCCTAATGAA R: GTCAGGAATACAAAATGGCAGGTTA	65	$(CT)_{12}$	159	> 1	AJ427935
Ab13	F: GAATCAGATTTCTTCGACTTCATTC R: GGCATCGTTTTTATTTTATACTAGAGG	54	$(TG)_{32}$	266	5	AJ427936
Ab14	F: CCCTGTAAAGTTTGTTACGTTTTCG R: CCTACCTTTCAAGAATTCATCTCC	60	$(GA)_{15}$	154	8	AJ427937
Ab15	F: TGGACTAGCATTCATAGCTCTACC R: TTTAACCACAACCCAAGTCATCTAC	55	$(GT)_{20}$	250	9	AJ427938
Ab16	F: GATTGCACCGTAGACAAAACTGTAG R: GGTCGATAGCCCTTTAAAACTTTG	65	$(AG)_{16}$	263	6	AJ427939
Ab17	F: TTCCGATGAAGGAGATATTCAAAG R: CGCTAGAGGCATCGTTTTTATTTTATAC	55	$(TG)_{37}$	180	4	AJ427940
Ab18	F: TTAAACAGCAAGGACGAGACTTAT R: TCTTGAATATCAACTGTGTATGTTGG	55	$(GA)_{29}$	230	10	AJ427941
Ab19	F: GTGTCATTTCCGGGGTCATTT R: AATCGGACTTATGATGGTTTATTTTC	60	$(GT)_{11}$	210	8	AJ427942
Ab20	F: GATTTCTGCGAACTGTTTATTTTCC R: CAATATTGAACTCTTCGTCATCAAG	55	$(CT)_3(GT)_{23}$	222	6	AJ427943
Ab21	F: CTGACTATATAGCTACGCCACTGC R: CTAGCTTGAGTTGTGCTTTCTATC	65	$(CT)_{12}$	257	8	AJ427944
Ab22	F: TGAACAACTCAACATCTTCTCAGTG R: GTGAAATGTTTTCTTCTGTGGAGTC	65	$(CA)_{12}$	161	6	AJ427945
Ab23	F: AGATCCCACTAGAGAACAATTTGC R: CTTAAGGGTGAGTGATTAAGTTTGG	54	$(CA)_{12}$	200	> 1	AJ427946
Ab24	F: TGCTTGTGTCTTTCTGGAGAGC R: GAAACAGCTATGACCATGATTACG	60	$(GA)_{10}$	241	> 1	AJ427947
Ab25	F: GTCCCTTTTCTGACATGCTTTCG R: AACTAAACAACTGAGACAGCATTGG	65	$(TG)_{18}(GT)_6$	201	6	AJ427948
Ab26	F: ATTATGAAATGACGGGCACCTG R: GCTAGCTACTGTGCCTTGAGTAGAC	65	$(AG)_{19}$	202	3	AJ427949
Ab27	F: TAGATGTTTCAGGATGTCCCTTG R: AAAATCGATGAGTCCGCTGTAAC	54	$(CT)_{13}$	300	5	AJ427950

Table 1 *Continued*

Locus	Primer sequence (5′–3′)	*T* (°C)	Repeat motif	Cloned allele size (bp)	No. alleles	EMBL accession no
Ab28	F: GTCAGAGTATCGCACGTTGTTAGC R: GTTACAGGTGACGTTGAGTGACG	65	$(GA)_{12}$	219	> 1	AJ427951
Ab29	F: CAGTTCCTCCGTATTTAATGACTCC R: CACTTCTGCAAATTTGGAACACC	55	$(GT)_{22}$	222	7	AJ427952
Ab30	F: CCAAACTGTTTAATCAGCTACCAG R: GAAGGATTCGATTGCTCATTCTC	55	$(CT)_{32}$	271	> 1	AJ427953
Ab31	F: AAAAGGATAACTGGAGTAGCGGTAG R: TAAATCTGTCAATGCAACATTCATC	60	$(TA)_3(CT)_2$	304	3	AJ427954
Ab32	F: TATCCATATCATTCCTGCTCATTCC R: AAGATCAGACCTTGCTTCTCTTAG	65	$(AC)_{12}$	183	8	AJ427955
Ab33	F: TCGTCATTTCTGGGATTTCTCC R: ACATATTCGGCCAGTAGTTATGTCG	55	$(CCG)_5$	191	1	AJ427956
Ab34	F: TACATATTCGGCCAGTAGTTATGTC R: ATGGAGGAAGTTCAACAAAACC	55	$(GGC)_4$	257	1	AJ427957
Ab35	F: GAAATTGTTAGGTTAGGATCGGAAG R: ATTCACTGTGCGGAGTATTACGTTC	65	$(GA)_{14}$	252	5	AJ427958
Ab36	F: CGATAATACGTTTCCAGAGAGAGC R: GAAACAGCTATGACCATGATTACG	55	$(GT)_2(TG)_2(TA)_2$	214	> 1	AJ427959
Ab37	F: GACGAGAAATTAGAAACCCATCTC R: GAAATACCATCAAGGTTCAGTTAGC	65	$(TA)_2(TAA)_2$	293	2	AJ427960
Ab38	F: GGTAATGCTATCCGTAGATATAGACC R: GAAAATATTCACCTTCCGAAGAACC	60	$(CGG)_5(TGG)_2$	185	2	AJ427961
Ab39	F: GATGACAGTCTACCAGTGTTAATCG R: AAGATGCACGTTCAAAGACTCC	60	$(CAA)_6(CAG)_3$	231	4	AJ427962

Table 2 Characterization of 10 microsatellite loci in the 2-spot ladybird (*Adalia bipunctata*) against *n* individuals. H_O/H_E = observed/expected heterozygosity

Locus	*n*	No. of alleles	Allele size range (bp)	H_O	H_E
Ab1	70	19	234–282	0.36	0.92
Ab3	75	17	142–176	0.49	0.92
Ab7	78	10	144–220	0.21	0.42
Ab9	78	28	184–286	0.53	0.94
Ab11	77	25	286–356	0.75	0.92
Ab19	78	8	210–224	0.50	0.68
Ab31	78	7	304–348	0.18	0.17
Ab32	76	16	170–240	0.38	0.86
Ab35	77	20	218–260	0.79	0.89
Ab38	78	3	182–188	0.28	0.27

polymerase (Life Technologies) and 20 ng *A. bipunctata* genomic DNA. PCR cycling conditions were: 94 °C for 3 min; 35 cycles of 94 °C for 1 min, annealing temperature for 1 min, 72 °C for 1 min; 72 °C for 10 min Thirty-nine primer pairs amplified products of expected size and these were screened against 10 *A. bipunctata* to determine whether the loci were polymorphic. Reactions were as above except forward primers were end-labelled with [γ-^{33}P]ATP and products electrophoresed on 6% denaturing polyacrylamide gels which were dried and exposed to autoradiography film. Fragment sizes were estimated by comparison to 30–330 bp AFLP ladder (Life Technologies).

Table 1 shows the results of the initial primer screening. Ten primer pairs were then selected on the basis of polymorphism and reliable amplification. These were screened against two *A. bipunctata* families consisting of both parents and 10 offspring. All loci segregated as expected. These loci were characterized further by screening against 78 *A. bipunctata*. The results of this screening are shown in Table 2. All loci had at least three alleles, with the majority having 10 or more. Observed heterozygosity levels were lower than expected. This is attributed to inbreeding rather than the presence of null alleles (Haddrill 2001). Preliminary tests also indicate that some loci amplify in other coccinellid species. Three loci (Ab1, Ab6 and Ab8) were tested for amplification in *Harmonia axyridis* and *Exochomus 4-pustulatus*. Successful amplification was observed for Ab8 in *H. axyridis* and for Ab6 and Ab8 in *E. 4-pustulatus*.

These microsatellites are the first molecular genetic markers developed for any coccinellid species. *A. bipunctata*, *H. axyridis* and other coccinelids have been widely used in studies of the biological control of plant pests, the impact of genetically modified organisms, sexual selection and the evolutionary genetics of colour pattern polymorphism. These highly polymorphic microsatellites will therefore be invaluable tools with which to study these beetles further.

Acknowledgements

Many thanks to Celia James at HRI and Keith Edwards for the use of plasmid pJV1. This research was funded by a BBSRC studentship to PRH.

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