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PRIMER NOTE

Polymorphic microsatellite loci in Allodapine bees for investigating the evolution of social behaviour

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Abstract

Allodapine bees provide extraordinary systems for investigating the evolution of cooperation and particularly the division of reproduction in animal societies. We present the first microsatellite primers for two native Australian species (*Exoneura nigrescens* and *E. robusta*; Hymenoptera: Apidae: Xylocopinae) which allow the accurate determination of pedigrees, reproductive skew and relatedness in colonies. Up to 55 different alleles were observed per locus.

Keywords: Allodapine bee, *Exoneura nigresecens*, *Exoneura robusta*, Microsatellites, Reproductive Skew

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Allodapine bees form a tribe in the subfamily Xylocopinae (family Apidae). They range from nonsocial to highly social species, displaying great diversity in levels of sociality (Schwarz *et al.* 1998). This makes them ideal for comparative investigations of the evolution of social behaviour. Several species are facultatively eusocial, and whole colonies can often be transferred between experimental plots differing in a given ecological parameter (e.g. Cronin 2001). These properties give unrivalled opportunities for testing models of the evolution of reproductive skew (Reeve & Keller 2001), provided genetic relationships within nests can be inferred. Codominant multiallelic markers such as microsatellites are the best tools for this task (Gerber *et al.* 2000), and we therefore have developed primers for 13 highly variable microsatellite loci in two Allodapine species, *Exoneura nigrescens* and *E. robusta*.

We extracted DNA from the thorax of *Exoneura nigrescens* and *E. robusta* specimens collected from two sites in Victoria, southeastern Australia (Cronin 2001) using a phenol-chlorophorm protocol (Estoup & Turgeon 1996). DNA was digested with *Alu*I, *Hae*III and *Rsa*I; fragments within the 200–800 bp range were ligated into pUC18 plasmids and used to transform Epicurian Coli XL1-Blue MRF′ supercompetent cells (Stratagene, Amsterdam, the Netherlands). Some 6000 positive clones per species were replated on grid Petri dishes and screened for the microsatellite motifs $(AAT)_{10}$, $(TC)_{10}$, $(TG)_{10}$ and $(GGA)_8$ using a digoxygenin nucleic acid detection kit (Boehringer Mannheim, Basel, Switzerland). Plasmids from 310 positive clones were cycle sequenced using BigDye™ chemistry (Applied Biosystems, Foster City, USA) and analysed on an ABI PRISM 377XL DNA sequencer (Applied Biosystems). Primers were designed using GENEFISHER 1.3 (Giegerich *et al.* 1996), and PCR conditions were optimized for fluorescently labelled primers and electrophoresis on an ABI 377XL sequencer. A total of 63 sequences (24′153 bp; 38 for *Exoneura nigrescens*, 25 for *E. robusta*) containing microsatellite motifs from two to eight basepairs are published on GenBank under the accession numbers AY222377 to AY222439. Primer pairs for 13 of the most variable loci are shown in Table 1. Cross amplifications between *E. nigrescens* and *E. robusta* were possible for all loci.

For routine genotyping, DNA was extracted from whole individuals using a modified salt protocol. In brief, samples were frozen in liquid nitrogen, ground with a pestle and digested overnight at 55 °C in 300 μL extraction buffer (50 mM TRIS HCl, 1 mM EDTA, 0.1 M NaCl, 1% SDS, 8 mg/mL DTT, 0.4 mg/mL Proteinase K). Denatured proteins were precipitated with 150 μL NaCl 4.5 M and extracted using 450 μL chloroform-isoamylalcohol (24 : 1), then DNA was precipitated with 250 μL isopropanol, washed with 500 μL 80% ethanol and resuspended in 100 μL or 50 μL ddH_2O (*E. nigrescens* and *E. robusta*, respectively).

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Table 1 Characteristics of microsatellite markers for *Exoneura* ssp

Locus[1]	Core repeat[2]	Product size[2]	Number of alleles in[3]		Heterozygosity in[4]		Primer sequence (For = forward primer, Rev = reverse primer)[5]	GenBank accession no.
			Exoneura nigrescens	*Exoneura robusta*	*Exoneura nigrescens*	*Exoneura robusta*		
N39	$(TG)_{15}$	151 bp	9 (n = 10)	8 (n = 10)	0.700/0.789 (n = 10)	1.000/0.868 (n = 10)	For: 5′-CGCTTATCATATAAAACACTCGCTG-3′ Rev: 5′-TGTTTAAACGGAACCCATCGAATAC-3′	AY222387
N87	$(CT)_{22}$	176 bp	1 (n = 4)	4 (n = 3)	—[6]	1.000/1.000 (n = 3)	For: 5′-ATATGGCTGCAGTAGAGTATTAAAGACC-3′ Rev: 5′-CTTCAACAAAAAGATAAGCAGAGAGAGAGA-3′	AY222405
N149	$(CT)_{15}$	174 bp	7 (n = 4)	7 (n = 8)	0.750/0.964 (n = 4)	0.500/0.792 (n = 8)	For: 5′-CCTGCGATCCTCGATCGATTCC-3′ Rev: 5′-GGATCGGTGCCGTGCTTCTC-3′	AY222413
R1	$(GT)_{25}(GA)_{10}$	193 bp	5 (n = 3)	5 (n = 3)	1.000/0.933 (n = 3)	1.000/0.933 (n = 3)	For: 5′-TATGCGTGTGCAGTGTATGTATATACG-3′ Rev: 5′-GTGCAGTGTGTTTACACATTCTTTCC-3′	AY222415
R76	$(TC)_{19}$	244 bp	6 (n = 3)	3 (n = 3)	1.000/1.000 (n = 3)	1.000/0.733 (n = 3)	For: 5′-CGGAATTTCATACGAACATCTCACATC-3′ Rev: 5′-TTTGTACACTAAATGGTGCAACTGG-3′	AY222427
R145	$(GA)_{11}$	165 bp	9 (n = 8)	10 (n = 10)	0.375/0.917 (n = 8)	0.500/0.900 (n = 10)	For: 5′-GTGCATCGTCCTTGATTCCTGC-3′ Rev: 5′-GCTGGAGAGCGTATGGTAAATGTTG-3′	AY222439
N22[7]	$(TC)_{19}(TA)_{11}$	141 bp	22 (n = 417)	6 (n = 3)	0.754/0.898 (n = 57)	1.000/1.000 (n = 3)	For: 5′-GTTTCTCGAAGGCGGCTAACTTC-3′ Rev: 5′-TCGCCTGTTAATTTCGCCGTAGG-3′	AY222381
N60[7]	$(TC)_4(TA)_2(TC)_{18}$	187 bp	51 (n = 536)	26 (n = 363)	0.908/0.947 (n = 65)	0.919/0.929 (n = 62)	For: 5′-TTTCACTTTGCTGAAGACTTCGAAC-3′ Rev: 5′-CATAACTTCCAAGAAGATTTTCGGGAC-3′	AY222391
N81[7]	$(CA)_8A(GC)_8(AC)_{10}$	188 bp	26 (n = 535)	30 (n = 329)	0.652/0.777 (n = 66)	0.785/0.843 (n = 65)	For: 5′-CTATTTCCTAACCTTTCCATCACACAC-3′ Rev: 5′-AATTCAATCCGATTGACAATGAGCC-3′	AY222402
N83[7]	$(CG)_4(CA)_{14}(TA)_4$	265 bp	39 (n = 527)	55 (n = 636)	0.846/0.919 (n = 65)	0.517/0.970 (n = 58)	For: 5′-CAAGGGTATATACTATACTTCCGAAGGTT-3′ Rev: 5′-TTCTTGACCTATCCGAATCCAAATTCCT-3′	AY222403
R32[7]	$(TC)_6C(CT)_9C(CT)_{11}$	310 bp	3 (n = 3)	39 (n = 231)	1.000/0.733 (n = 3)	0.746/0.889 (n = 63)	For: 5′-CTCACTTCGACTCCGGGGACA-3′ Rev: 5′-AAACGTCTAACAAGCGGGCTGA-3′	AY222417
R74[7]	$(AT)_6G(TA)_6(TG)_9$	209 bp	40 (n = 528)	53 (n = 363)	0.823/0.966 (n = 62)	0.922/0.953 (n = 64)	For: 5′-AAGGAATAAATGGATGGGCTAAACG-3′ Rev: 5′-GTTAAAGAATCTCGAGTGAACATGG-3′	AY222426
R115[7]	$(CT)_{28}$	173 bp	6 (n = 8)	26 (n = 353)	0.875/0.817 (n = 8)	0.738/0.847 (n = 65)	For: 5′-CACGTAAATCTTCTACTAAATAACAGTCCC-3′ Rev: 5′-GACGCTCTCCCCCGATACACT-3′	AY222434

[1] 'N' loci are isolated from *E. nigrescens*, 'R' from *E. robusta* strains.

[2] Sequenced clone allele.

[3] Number of different alleles found (n = number of sampled individuals that amplified).

[4] Observed (H_O) and expected (H_E) heterozygosity in the format: H_O/H_E (n = number of sampled females). Values are calculated from Genepop version 3.1c (Raymond & Rousset 1995) using one female chosen at random per nest.

[5] Forward primers were fluorescently labelled with either 6-FAM, HEX (Microsynth, Balgach, Switzerland) or NED (Perkin-Elmer, Hünenberg, Switzerland).

[6] Locus N87 amplified poorly in *E. nigrescens*. H_O and H_E can not be calculated since only one allele was found in this species.

[7] The primers for loci N22, N60, N81, N83 and R74 (in *E. nigrescens*), and for loci N60, N81, N83, R32, R74 and R115 (in *E. robusta*) are designed for simultaneous migration of their PCR products in a single lane of an ABI 377XL sequencer. These primer combinations were used for subsequent routine genotyping and hence show larger sample sizes.

Prior to polymerase chain reaction (PCR), this solution was diluted 1:50 for *E. nigrescens* adults and pupae (no dilution for larvae and eggs) and 1:250 for *E. robusta* adults, pupae, larvae and eggs. Amplifications for both species were carried out in a 10 μL reaction volume containing 5 μL of template DNA solution and a final concentration of 0.05 U/μL *Taq* DNA polymerase (Qiagen, Oslo, Norway), 1 × PCR buffer (Qiagen), 0.5 μM of each primer, 300 μM of each dNTP and 2.0 mM $MgCl_2$. All individual loci were amplified with a single touch-down PCR protocol: 4 min of denaturation at 94 °C; 45 cycles of 30 s at 94 °C; 30 s at 64 °C – 0.2 °C/cycle (touchdown) and 2 min at 72 °C; and 5 min of final elongation at 72 °C. In *E. nigrescens*, loci N22 and N81 (Table 1) were multiplexed using the same conditions except 1.5 × PCR buffer (Qiagen), 0.3 μM of each primer and 2.25 mM $MgCl_2$. In this case, cycling conditions were 4 min of denaturation at 94 °C; 45 cycles of 30 s at 94 °C; 30 s at 61 °C – 0.2 °C/cycle (touchdown) and 2 min at 68 °C; and 5 min of final elongation at 68 °C. We used Perkin-Elmer 9700 GeneAmp, MJ Research PTC-100 96 V and Biometra T Gradient thermocycling machines for PCR amplifications.

We selected a subset of the developed microsatellite loci for each species (loci N22, N60, N81, N83 and R74 for *E. nigrescens*; loci N60, N81, N83, R32, R74 and R115 for *E. robusta*) based on their variability and product sizes (Table 1). These loci can be analysed in a single lane on an ABI 377XL sequencer, and showing 22–55 alleles per locus, they allow quick and accurate determination of pedigrees, reproductive skew and relatedness in colonies. The loci in these subsets show no significant genotypic linkage disequilibrium, with the exception of N81 vs. R32 in *E. robusta* ($P = 0.002$ with a probability threshold of 0.003 after Bonferroni corrections; Raymond & Rousset 1995). Observed heterozygosities (H_O) in the subsets were lower than expected heterozygosities (H_E) (Table 1), but significant deviations from Hardy–Weinberg equilibrium occurred only at loci N22 and R74 in *E. nigrescens* and at loci N81, N83 and R32 in *E. robusta* (exact Hardy–Weinberg test after Bonferroni corrections; Raymond & Rousset 1995). These deviations and the observed linkage disequilibrium can be explained by the presence of extensive family groups within the sampled populations (containing sister individuals sired by the same haploid male). The marked difference between H_O and H_E at locus N83 in *E. robusta* suggests that there might be null alleles (Table 1).

The 13 microsatellite primers presented here, as well as the 50 additional microsatellite sequences published on GenBank, provide valuable tools for genetic investigations in Allodapine bees.

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