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PERMANENT GENETIC RESOURCES

Isolation and characterization of 30 polymorphic microsatellite loci from the mycophagous fly *Drosophila innubila*

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Abstract

***Drosophila innubila* is a mushroom-feeding member of the quinaria group, found in the woodlands and forests of the 'sky islands' in Arizona and New Mexico and extending south into central Mexico. Here, we describe and characterize 30 polymorphic microsatellite loci from *D. innubila* collected in the Chiricahua Mountains in Arizona. The number of alleles ranged from three to 21, and observed heterozygosity ranged from 0.0513 to 0.9737. Six loci were putatively X-linked, six departed from Hardy–Weinberg equilibrium, seven had evidence of null alleles, and six showed evidence of linkage disequilibrium. These markers will be useful for examining population structure of *D. innubila* and its association with male-killing *Wolbachia*.**

Keywords: *Drosophila innubila*, microsatellites, mycophagous, polymorphic marker, population genetics

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Drosophila innubila is a mushroom-feeding species from the quinaria group in the subgenus *Drosophila* (Patterson 1943). The known range of *D. innubila* extends from the mid- to high-elevation 'sky islands' in the Arizona and New Mexico to central Mexico (Patterson 1943). *Drosophila innubila* is of special interest due to infection with male-killing *Wolbachia* that causes male offspring of infected mothers to die during development (Jaenike *et al*. 2003). Various aspects of this host–parasite interaction have been investigated, including population structure (Dyer *et al*. 2005; Dyer & Jaenike 2005), infection stability (Dyer & Jaenike 2004), male mate choice (Sullivan & Jaenike 2006), and mushrooms as a potential source of antibiotic curing (Jaenike *et al*. 2006). We developed micro-satellite markers for *D. innubila* and describe those markers here.

Microsatellites were isolated from *D. innubila* following the subtractive hybridization protocol of Hamilton *et al*. (1999), and as previously described for *Drosophila neotestacea* (Dyer 2007). Using the Gentra Puregene kit, genomic DNA was extracted from 30 male flies taken from an isofemale line that was collected in 2000 from the Chiricahua Mountains in Arizona. DNA was stored at 4 °C until used, and then at –20 °C for long-term storage. *Sau*3A was used to cut the genomic DNA into small fragments, and then the hybridization procedure was carried out separately for each of four target repeats, which included $(AC)_{15}$, $(CG)_{15}$, $(AGC)_{10}$ and $(ATC)_{10}$. Using standard insert-flanking plasmid primers (M13for/M13rev), between 35 and 60 randomly chosen inserts from each hybridization were amplified via colony polymerase chain reaction (PCR). Excess primers were removed from amplicons using QIAGEN QIAquick columns, and then each insert was sequenced directly using the T7 plasmid primer. Sequencing reactions were 10-μL in volume, and used 1 μL of BigDye with standard reaction conditions, then run on an ABI 3730 DNA sequencer. From 140 successfully sequenced inserts, we recovered 61 unique sequences, of which 100% contained putative microsatellites that were repeated at least four times.

Two forward and two reverse primers flanking each of 43 microsatellites were designed using the program OLIGO (Molecular Biology Insights). Each primer pair was tested on eight individual flies, and for each locus the pair with

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the most consistent amplification across flies was chosen for further use. PCRs were performed on 10–40 ng of genomic DNA in a 10-μL reaction [1.0 μL 10× PCR buffer, 0.4 μL 50 mM $MgCl_2$, 0.4 μL of each primer, 0.25 μL each dNTP (2 mM), 0.04 μL *Taq* DNA polymerase (Invitrogen)] using a Bio-Rad iCylcer with the following conditions: 2 min at 95 °C, 35 cycles of 30 s at 95 °C, 1 min at 55 °C and 1 min at 72 °C and an extension of 10 min at 72 °C. For each of the 30 loci that amplified reliably, we characterized polymorphism by analysing 32 individuals (16 male and 16 female) collected between 2001 and 2006 in the Chiricahua Mountains of Arizona. PCR was performed using 5′ labelled (6-FAM, PET, VIC or NED) forward primers. Fragments were run on an ABI 3730 DNA sequencer with GeneScan-500 LIZ size standard (Applied Biosystems) and analysed using GENEMAPPER (Applied Biosystems).

Table 1 lists the characteristics of each microsatellite locus developed from *D. innubila*. All loci were polymorphic, with the number of alleles per locus ranging from three to 21 and averaging 12.1. Six of the loci are putatively

Table 1 Primers and characteristics of 30 microsatellite loci of *Drosophila innubila*

Locus	Accession no.	Repeat motif in clone	Primer sequence (5′–3′)	Primer label	Size range (bp)	n	N_A	H_E (H_O)	P value†
Di1103	EU126610	$(AG)_8(AT)(AG)_4$ (GG) $(AG)_2$	F: GAAACCGACGAGAACTGC R: ATGGGCGGATGAAGTACG	VIC	280–304	33	12	0.875 (0.950)	0.015
Di1105	EU126611	$(CT)_3(TTCTAT)$ $(CT)_6$	F: AAGAATCACCTTGCACGACG R: GGCTTGAGGAAGGCACAG	6-FAM	90–135	40	12	0.813 (0.903)	
Di1106	EU126612	$(CT)_3(CG)(CT)_4$ (CG) $(CT)_3$	F: CAATGCTCTGGCAGGATATAC R: CGAACGTTGAACAAATGCTC	6-FAM	107–181	40	8	0.688 (0.671)	< 0.0001
Di1110	EU126613	$(TC)_{13}$	F: ACTGCACGCCTTAAAGTTCAG R: ATTGGGAATGATTGGGTATG	VIC	277–307	40	12	0.934 (0.960)	
Di1116	EU126614	$(TC)_{10}(TG)(TC)_4$	F: AATCAAGCACTTGCAAGCTAC R: TGTGCTGTCGTACGCATC	PET	208–245	37	18	0.934 (0.988)	< 0.0001
Di1121	EU126616	$(GA)_{12}$	F: TTTCACACTGCCACGAGC R: TCGCTGACGTCATAATTGAG	6-FAM	379–397	36	8	1.000 (0.938)	< 0.0001
Di1122	EU126617	$(CT)_9$	F: GTTTTCATGTGCGTGCTTAG R: TTTGCTTGTAATGCAGCTCAG	VIC	240–269	40	14	0.934 (0.966)	
Di2054	EU126618	$(ATG)_7(CTG)_6$	F: ACGAGCGTCTGTTTGAGTG R: CCACAGTCGATATCTCC	VIC	308–327	38	6	0.689 (0.796)	
Di2205	EU126619	$(GT)_{11}(GC)(GT)_3$	F: ATGTCATCTGCAACATGCAC R: TATAAGAAGCGAATGGTAATGG	NED	338–371	35	11	0.875 (0.950)	
Di2222f1	EU126620	$(TGC)_5(TTC)_5$	F: TGCCGGCTACTAAATGATTG R: TGGACTAGGCGTCACTCATAG	6-FAM	136–162	40	10	0.934 (0.944)	
Di2222f2	EU126621	$(CA)_4(CCCAG)$ $(CT)_6$	F: TCTATGAGTGACGCCTAGTCC R: AACTTTCAATGCTGCAATGTC	6-FAM	79–110	40	12	0.813 (0.942)	
Di2228	EU126622	$(CAG)_8$	F: CCAACAGATGATGGAAACAAC R: ACTTCTTGGATGCTTGGATTC	NED	311–317	37	3	0.250 (0.234)	
Di2230	EU126623	$(TGC)_7$	F: TTCTGCCAGCTGTGATAAGC R: ACAGCAGCAACATCAGCTATATC	6-FAM	135–161	40	8	0.934 (0.950)	
Di2231	EU126624	$(AC)_{14}$	F: GTCACATGGAACTCTTAACAGC R: GCTAATCGGCAGCTCATTAAC	NED	398–425	35	11	0.934 (0.950)	
Di2235	EU126625	$(TG)_{16}$	F: AAATTACGTGCGAAATGTTATC R: GTCTCGACTAACCAAACTGACC	6-FAM	91–140	33	20	0.875 (0.942)	< 0.0001
Di3051	EU126626	$(CT)_4(AT)(CT)_6$	F: CTTTGTCATGCCCAATGAG R: CCAAGACTTTTCAGGCACAC	PET	215–248	37	15	0.934 (0.980)	
Di3145	EU126628	$(TCA)_9$	F: ACCGTATGCTCAATACAATG R: GATTCCAAGTCTCCGATGC	VIC	303–318	38	4	0.125 (0.123)	
Di4210	EU126630	$(CA)_4(TACG)(CA)_{11}$	F: CGCATGTTCGAATTGGAG R: CATAAATGCACACCCCAATG	VIC	247–268	34	10	0.934 (0.905)	
Di4212	EU126631	$(AC)_3(GC)(AC)_{12}$	F: GACAATTAATTGGTTTATAAGCC R: CATCTGCAACATGCACAAG	VIC	262–293	31	10	0.934 (0.950)	
Di4214	EU126632	$(AC)_6(AT)(AC)_4$	F: TCAAATGCAGCGAAGTAAAG R: CATCTGAACACAGCGCAC	NED	402–435	39	10	0.875 (0.952)	
Di4222	EU126633	$(GT)_{15}$	F: GCACGAAAGTAGGCAACAG R: CTACAAACGTGGCTACATGTG	PET	221–261	38	17	0.938 (0.905)	< 0.0001
Di4229	EU126635	$(AC)_9$	F: GCATGGATGCACAAGAAAG R: GGCTGAGGGTGTGGTAGTG	PET	152–186	33	13	0.938 (0.964)	
Di4240	EU126638	$(GT)_{16}$	F: GAAATGTTATCGCATACATGTG R: ACCAAACTGACCAACTGTCC	6-FAM	70–119	33	20	0.934 (0.988)	
Di4243	EU126610	$(GT)_5(AT)(GT)_5$ (GA)(GT)	F: GAAAGCCAACAACATTCATATC R: AGTTGCAATTCTAAAGTTAGCC	PET	193–225	40	14	1.000 (0.984)	

Table 1 *Continued*

X-linked locus*	Accession no.	Repeat motif in clone	Primer sequence (5′–3′)	Primer label	Size range (bp)	n	N_A	H_E (H_O)‡
Di1118*	EU126615	$(CT)_9$	F: CCTCTTTCTTCTGCTTCTGC R: GTGACATGCGGCAATAATC	VIC	241–260	39	9	1.000 (0.983)
Di3141*	EU126627	$(GAT)_3(GAA)(GAT)_4$	F: AATAAACCCAAGATAAGCGAC R:TACGGTATTGTGTATATCTGAGC	PET	146–159	38	3	0.375 (0.325)
Di4201*	EU126629	$(AC)_4(GC)(AC)_4$ $(GC)(AC)_5(C)(AC)_3$	F: GGCTATCCCACATGTGTTC R: AGGTTTAGAATTGATGCCAAGAGG	NED	355–397	39	13	1.000 (0.983)
Di4223*	EU126634	$(CA)_6(GA)(CA)_{12}$	F: AGCATAATGCACGCTGAC R: TGGATTTAACGGCAATGAG	6-FAM	86–148	36	21	1.000 (0.983)
Di4235*	EU126636	$(GA)_{10}$	F: GAAATGTAACTGGATATGCCAC R: AAGTTTTCCTTCTGCTGGC	PET	164–214	40	16	1.000 (0.967)
Di4236*	EU126637	$(AC)_{14}$	F: TCATTCATTCAAACGGCAAACG R: CCAGAGTCATAAGCCTTAGCAC	PET	226–251	38	13	1.000 (0.975)

*Putative; †P value if significant departure from Hardy-Weinberg equilibrium with Bonferroni correction; ‡heterozygosities calculated for females only.

X-linked, as they were always homozygous in males. We calculated observed and expected heterozygosities using MSA version 4.0 (Dieringer & Schlotterer 2003) and tested for departure from Hardy–Weinberg equilibrium using GENEPOP version 3.4 on the web (Raymond & Rousset 1995; http://wbiomed.curtin.edu.au/genepop/index.html). For putatively X-linked markers, we excluded males when comparing observed and expected heterozygosities. X-linked and autosomal loci did not differ significantly for heterozygosity (Mann–Whitney $U_{\text{X-linked}} = 47$, $n_{\text{X-linked}} = 6$, $n_{\text{autosomal}} = 24$, $P = 0.2041$). Six of the 30 microsatellite loci showed a significant ($P < 0.05$ with Bonferroni correction) departure from Hardy–Weinberg equilibrium, which may be due to the Wahlund effect (which would cause observed heterozygosity to be lower than expected) (Gillespie 2004), because individuals were collected from various populations and in different years in the Chiricahua Mountains. This is further supported by a global heterozygosity deficit ($P < 0.0001$). Null alleles and chromosomal inversions may cause deviations from expected heterozygosity. We also used GENEPOP to examine linkage disequilibrium among pairs of loci, and found six pairs that showed significant (with Bonferroni correction and $P < 0.05$) association. There were two linkage groups each containing three loci (Di1103-Di1105-Di4229 and Di2222f1-Di2222f2-Di3051). Finally, we used GENEPOP to identify null homozygous individuals, an indication of the presence of null alleles. Seven loci (Di2222f2, Di2235, Di3141, Di3145, Di4210, Di4214 and Di4243) showed evidence of null alleles. Two of these (Di2235 and Di4243) also showed departure from Hardy–Weinberg equilibrium.

We tested each pair of primers on six individuals of *Drosophila falleni*, the closest extant relative to *D. innubila* (Perlman *et al*. 2003). Eight of the 30 loci (Di1116, Di1122, Di2054, Di2222f1, Di2231, Di3051, Di4229 and Di4235) amplified in *D. falleni*, of which seven were polymorphic (data not shown). In order to determine if any loci are conserved across a wider range of taxa, we did a BLAST search against the *Drosophila* genomes with each sequence and found no evidence for sequence conservation across more distantly related species.

These microsatellite markers will allow for studies of *D. innubila* biogeography, inbreeding, and other aspects of their ecology and evolutionary biology.

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