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TECHNICAL NOTE

Isolation and characterization of polymorphic microsatellite markers in the grasshopper *Mioscirtus wagneri* (Orthoptera: Acrididae)

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Abstract Eight polymorphic microsatellite markers were developed for the grasshopper *Mioscirtus wagneri*. Polymorphism at these loci was evaluated in 25 individuals from Central Spain. The number of alleles per locus ranged from 3 to 9 and their observed and expected heterozygosities ranged from 0.28 to 0.80 and 0.25 to 0.84, respectively. All genotypic frequencies conformed to Hardy–Weinberg equilibrium expectations, with no evidence of genotypic linkage disequilibrium between any pair of loci. These loci will be highly useful for the study of the population genetic structure and diversity of this grasshopper species forming highly fragmented populations of great conservation concern.

Keywords Dinucleotide · Genetic diversity · Population fragmentation · Tetranucleotide

Mioscirtus wagneri (Kittary 1859) (Orthoptera: Acrididae) is a grasshopper species that shows a classical Mediterranean–Turanian disjunct distribution. It is a highly specialized organism being restricted to hypersaline low-lying areas with patches of *Suaeda vera*, the halophilic plant on which it depends for food (Cordero et al. 2007). In the Western Mediterranean, inland hypersaline environments often constitute small-sized patches of highly fragmented relict habitat (Ortego et al. 2009). A recent phylogeographic study revealed very low levels of mitochondrial DNA nucleotide diversity in all populations of *M. wagneri* that were considered to be a consequence of the small size of their current populations which are known to frequently go through sharp demographic bottlenecks (Ortego et al. 2009). More detailed analyses of population genetic structure of this species forming highly fragmented populations of great conservation concern requires information from more variable genetic markers if appropriate management measures are to be taken (Cordero et al. 2007; Ortego et al. 2009). Here, we report the development of eight polymorphic microsatellite loci from the grasshopper *M. wagneri* and the results of tests of cross-species amplifications in 5 other orthoptera species.

Partial genomic libraries were constructed from size-selected fragments (250–1,500 bp) of *M. wagneri* DNA (Ciudad Real province, central Spain) that had been digested with *Mbo*I, adapter ligated (adapter sequence; Refseth et al. 1997) and enriched for repeat sequence. Hybridisation capture of repeat sequence was carried out using 3′ biotin-labelled AAG, GAT, AAAG, AAAC, GATA, and GT repeat oligos bound to streptavidin coated beads (Dynabeads M-280, Dynal). Hybridisation capture, recovery of enriched DNA by polymerase chain reaction (PCR), cloning and identification of microsatellite-containing clones by colony PCR were carried out as described in Bloor et al. (2006). Positive clones were sequenced on an ABI 3100 DNA Sequencer (Applied Biosystems). Fifty-two primer pairs were designed for 30 microsatellite-containing sequences using the program Primer3 (Rozen and Skaletsky 2000).

All primer pairs were tested for amplification using eight individuals from central Spain. Primers producing products

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Table 1 Characteristics of eight microsatellite markers developed from *Mioscirtus wagneri*

Locus	Primer sequence (5′–3′)	Repeat motif	T_a (°C)	Allele size range (bp)	N_a	H_O	H_E	GenBank accession no.
MwGTC8	F: AACTGTTTCGGCCACCACT	$(CA)_{31}$	59	148–172	6	0.72	0.71	FJ791049
	R: GTTCCATCTGGAGGTTCGAT							
MwGTD9	F: TAAGAGCAGTCGGCACTGAG	$(CA)_{22}$	64	125–135	5	0.72	0.61	FJ791050
	R: GGAAGAGAGACCGTGTGAGC							
MwGTG12	F: AGTGGCCGAGCGCTCTAT	$(GT)_{14}$	66	421–441	4	0.60	0.60	FJ791051
	R: AATCGTCGTGTGGGAACTAC							
MwGTB10	F: GTCCAAGAGAGGCAGAATGTC	$(CA)_{23}$	68	299–319	7	0.64	0.77	FJ791052
	R: AGACACTAAGGTACGGACTGCAT							
MwGTA6	F: TCCCTAGCCTTTTAAACTACTTAAACT	$(CA)_{25}$	63	230–262	7	0.68	0.72	FJ791053
	R: AACAGTGGAGCTTGGCAGAT							
MwGTC12	F: CAGCGTTTGGCACTCTTAGC	$(GT)_{27}$	65	307–311	3	0.28	0.25	FJ791054
	R: GATGCATGACAATGCTCGAT							
MwGTC11	F: AGGTTCGAGTCCTCGCTCAG	$(GT)_{22}$	65	135–167	8	0.44	0.51	FJ791055
	R: AACGGTGTGTCCCTCTCACT							
MwGATAB11	F: GGAGTAGTGGAGTGGCGGTA	$(GATA)_{13}$	59	358–392	9	0.80	0.84	FJ791056
	R: GGTTTATTGACTCTGGAACATGC							

Annealing temperature (T_a), allele size range in base pairs (bp), number of alleles (N_a), observed heterozygosity (H_O) and expected heterozygosity (H_E) are listed for each locus. Data on polymorphism are based on 25 typed individuals

of expected size were labelled with fluorescent dyes (6-FAM, PET NED or VIC) to allow analysis on an automated DNA sequencer and determination of levels of polymorphism. Twenty-two loci that failed to amplify or were difficult to score were excluded from subsequent analyses. The polymorphism of the remaining eight loci was evaluated in 25 individuals from Ciudad Real province, Central Spain. We used NucleoSpin Tissue (Macherey-Nagel, Düren, Germany) kits to extract and purify genomic DNA from a hind leg of each individual. Amplifications were conducted in 10-µl reaction volumes containing 5 ng of genomic DNA, 1× reaction buffer (67 mM Tris–HCL, pH 8.3, 16 mM $(NH_4)_2SO_4$, 0.01% Tween-20, EcoStart Reaction Buffer, Ecogen), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.15 µM of each primer and 0.1 U of *Taq* DNA EcoStart Polymerase (Ecogen). The PCR programme used was 9 min denaturing at 95°C followed by 35 cycles of 30 s at 94°C, 45 s at the annealing temperature (Table 1) and 45 s at 72°C, ending with a 5 min final elongation stage at 72°C. Amplification products were run on an ABI 310 Genetic Analyzer (Applied Biosystems) and genotypes were scored using GeneMapper 3.7 (Applied Biosystems).

Tests for departure from Hardy–Weinberg equilibrium (HWE) and pairwise linkage equilibrium were performed using GENEPOP 3.4 (Raymond and Rousset 1995). Polymorphism characteristics of these eight microsatellite loci are summarized in Table 1. The number of alleles per locus ranged from 3 to 9 and their observed and expected heterozygosities ranged from 0.28 to 0.80 and 0.25 to 0.84, respectively. After applying sequential Bonferroni corrections to compensate for multiple statistical tests ($\alpha = 0.05$), no locus deviated significantly from HWE and no evidence of genotypic linkage disequilibrium at any pair of loci was found. The eight microsatellites developed for *M. wagneri* were also tested for polymorphism in two individuals from each of five other orthoptera species (*Dericorys carthagonovae*, *Dociostaurus crassiusculus*, *Sphingonotus azurescens*, *Aiolopus strepens*, *Eyprepocnemis plorans*), but all PCRs resulted in multiple bands or gave no amplification. Microsatellite markers recently isolated from *Oedaleus decorus* (Berthier et al. 2008) were also tested for cross-amplification in *M. wagneri* but again all PCRs produced smears or multiple bands of unexpected size.

Overall, these novel polymorphic microsatellites will provide a useful genetic tool to study the genetic diversity and structure of *M. wagneri*, an orthoptera species which offers an interesting model system to study the genetic and evolutionary consequences of small population size and fragmentation.

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