PERMANENT GENETIC RESOURCES

Isolation of eight microsatellite markers from *Moina macrocopa* for assessing cryptic genetic structure in the wild

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Abstract

We isolated eight polymorphic microsatellite loci from the zooplankton *Moina macrocopa* (Straus), which is sensitive to pollutants such as insecticides and heavy metals. The isolated loci were polymorphic, with three to seven alleles among 23 individuals. Expected heterozygosities ranged from 0.167 to 0.787. These loci can be used to examine cryptic genetic structure and to infer the connectivity among metapopulations.

Keywords: genetic structure, littoral, magnetic particles, *Moina macrocopa*, Moinidae

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Moina macrocopa (Straus) is a littoral zooplankton that is distributed in ponds and rice fields in Asia (Chuah *et al*. 2007). Research on the species has mainly centred on ecotoxicological studies for hazard assessment of agrochemicals or heavy metals. Because such hazardous chemicals do not generally show homogeneous distribution in water bodies, some variation in resistance against these chemicals can be expected among sites because of different levels of selection pressure for the resistance.

Widely distributed zooplankton such as *M. macrocopa* are suitable for assessing relationships between the levels of resistance to chemicals and different water environment. For such assessments, however, it is necessary to know the extent to which gene flow among metapopulations constrains the evolution of resistance. Particularly in large ponds and lakes, it is difficult to infer the connectivity of metapopulations and to find genetic discontinuities without a direct approach using genetic markers. Accordingly, we identified polymerase chain reaction (PCR) primers for the amplification of eight loci, which can be used for acquiring the necessary population genetic parameters. These primers may also be useful in other closely related *Moina* species.

We constructed an enriched library based on the modified method of Fischer & Bachmann (1998). Samples of *M. macrocopa* were collected from Lake Kasumigaura and its vicinity, Ibaraki, central Japan. Immediately after the collection, we established isofemale lines from each adult individual and reared them in plastic containers until needed. Ten individuals were randomly chosen from each container and genomic DNA was extracted from whole bodies using the QIAamp DNA Mini Kit (QIAGEN). Five micrograms of genomic DNA were digested with 50 U of *Sau*3AI (TaKaRa), and fragments were ligated to *Sau*3AI cassettes (TaKaRa). PCR amplification was carried out with Cassette Primer C1 (TaKaRa) in a GeneAmp 2720 Thermal Cycler (Applied Biosystems). The PCR products were resolved in binding buffer (10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 7.5) and hybridized to 5′ biotin-labelled oligonucleotide probes $(CA)_{10}$ after denaturation. The hybrids were subsequently isolated by binding them to Streptavidin Magnetic Particles (Roche). After rinsing the particles in washing buffer (10 mM Tris-HCl, 1 mM EDTA, 1 M NaCl, pH 7.5), target DNAs were recovered by resuspending the particles in elution buffer (6 M guanidine-HCl). The obtained fragments were then amplified by PCR and digested with *Sau*3AI to remove the cassettes. The enriched fragments ranging in size from 300 to 1000 bp were ligated into *Bam*HI-cut pUC118 (TaKaRa) and transformed into competent *Escherichia coli* cells (Competent High-DH5α, Toyobo).

A total of 960 recombinant colonies were chosen and suspended independently in 20 μL of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8). Inserts were amplified by PCR using an M13 primer pair for the multicloning site of pUC18.

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Table 1 Characteristics of the eight microsatellites: repeat motif, number of alleles (N_a), size range in PCR product, primer sequences, locus-specific annealing temperature (T_a), observed (H_O) and expected (H_E) heterozygosities and results of the test for Hardy–Weinberg (HW) proportions.

Locus	Repeats	N_a	Size range (bp)	Primer sequences (5′–3′)	T_a (°C)	H_O	H_E	HW *P* values	GenBank Accession no.
MM-01	$(GT)_4$ … $(GGA)_3$	4	200–220	GAATTTCACAGTCCGCCAAT GGCTCCCCTATTTCCATCTC	59	0.174	0.167	1.0000	AB449261
MM-02	$(GCT)_6$	7	230–248	AGACGAGAGAGCCCGAAAA GCATGCGTCACTCGTACACT	60	0.348	0.636	0.0002	AB449262
MM-03	$(GT)_4$	4	158–180	TCAGTGTCAATGGCCTGAAA TTGCTTTGAGGTTCGTTCAA	60	0.261	0.310	0.0370	AB449263
MM-04	$(GTC)_5$	3	195–201	TATACGTCGAGCAGCCAGTG CGTGTTACCATCTCCCGTTA	59	0.565	0.530	1.0000	AB449264
MM-05	$(CA)_5$	7	220–238	CGCCGGTAACTAGGTCAGAA GTTGGCAATTTTTCGTCGAT	60	0.652	0.777	0.0064	AB449265
MM-06	$(GCA)_3$ … $(TA)_3(CA)_3$ $A(CA)_3$ … $(GT)_6$	3	164–170	AGCGGCGAAGAAGAGTAACA CGTTGGCCTAAACACTTTGC	60	0.087	0.414	< 0.0001	AB449266
MM-07	$(TTG)_5$	7	220–240	ACCTCTCGTCTCGTCTCGTC CCTTTACTCGAGGGCCGTAT	60	0.087	0.787	< 0.0001	AB449267
MM-08	$(CT)_5$	5	155–169	CAACGGGCAAGAAAGAACAT AACGAACGAAACAGATGTTGG	60	0.783	0.673	0.1852	AB449268

After PCR, 1 μL of each amplified product was dropped separately on a positively charged nylon membrane (Roche). After drying at room temperature, DNAs on the membrane were blotted by normal alkaline transfer. PCR products containing microsatellite regions were detected using a digoxigenin (DIG) nucleic acid detection kit (Roche) and 5′ DIG-labelled oligonucleotide probes mentioned above. Sixty-seven of 960 clones were positive. For positive clones, 1 μL of the remaining PCR product was checked for its length on 10% polyacrylamide gels in TBE. There were 17 unique inserts (length 300–1000 bp). Inserts of the positive recombinants were amplified independently using the M13 primers and sequenced using BigDye version 3.1 cycle sequencing kit (Applied Biosystems) and an automated sequencer (ABI 3730, Applied Biosystems). There were nine different loci in these positive clones and the nine primer pairs were designed using the online primer design software Primer 3.0 (Rozen & Skaletsky 1998).

To test the effectiveness of the obtained microsatellite loci, we checked amplification and polymorphism of the primer pairs in 23 individuals of *M. macrocopa* collected from a locality of Lake Kasumigaura in Ibaraki Prefecture, central Japan. The PCR amplifications were performed on a Gene-Amp 2720 Thermal Cycler (Applied Biosystems) in 10-μL reaction volumes containing 1 μL (50 ng) of template DNA, 0.5 pmol of each microsatellite primer, 1 μL of 10× reaction buffer [20 mM/L Tris-HCl (pH 8.0), 100 mM/L KCl, 0.1 mM/L EDTA, 0.5% Tween20, 0.5% Triton X-100, 1 mM/L DTT, 50% glycerol], 1.5 mM/L $MgCl_2$, 200 mM/L dNTP, 0.05 U of Gene *Taq* NT (Nippon Gene), and 6.15 μL of double-distilled water. Temperature cycles were as follows: 2 min at 94 °C; 30 cycles of 30 s at 94 °C, 30 s at 50–55 °C, 90 s at 65 °C. After optimization of PCR conditions, forward primers were labelled with *Taq*Man Dye fluorescence (Applied Biosystems). To determine allele sizes, fragment analysis was performed on an automated sequencer (ABI 3730, Applied Biosystems). A LIZ size standard (Applied Biosystems) was run with samples to calculate fragment size. Eight primers successfully amplified the target regions across the 23 individuals. Of these eight loci, all showed polymorphism, with three to seven alleles per locus (Table 1).

We tested for genotype frequency deviations from Hardy–Weinberg equilibrium for each locus among the polymorphic loci using GenePop on the Web (Raymond & Rousset 1996). A significant deviation was observed at five of eight loci ($P < 0.05$, Hardy-Weinberg exact tests with Bonferroni correction for multiple comparisons). This species is parthenogenetic that may explain the deviation from Hardy—Weinberg equilibrium. Null allele frequencies were also estimated by the method of Brookfield (1996). Their significance was tested by probability test using Micro-Checker software (version 2.2.3; van Oosterhout *et al*. 2004). Significant evidence of null alleles ($P < 0.05$, probability test) was found for three of eight loci (MM-02, MM-06 and MM-07). There was no evidence of linkage disequilibrium for all loci ($P > 0.05$, Fisher's exact probability test with Bonferroni correction for multiple comparisons). We conclude that the markers are useful for searching cryptic population structure and for inferring the levels of gene flow between metapopulations of *M. macrocopa.*

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