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PRIMER NOTE

Characterization of polymorphic microsatellite loci in the terrestrial isopod *Armadillidium vulgare*

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Abstract

The common pill bug, *Armadillidium vulgare*, is known to harbour two distinct strains of the feminizing proteobacteria *Wolbachia*. In order to study the effect of the presence of *Wolbachia* on the evolution of *A. vulgare* populations, we developed and characterized a set of nine polymorphic microsatellite loci from two microsatellite-enriched genomic libraries. We screened 48 individuals from three French populations and found high genetic variation. Locus-specific allelic diversity ranged from four to 28 and observed heterozygosity from 0.40 to 1.00, which indicates that these markers can be used to conduct population genetic studies in *A. vulgare*.

Keywords: *Armadillidium vulgare*, Crustacea, Isopoda, microsatellites

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The common pill bug, *Armadillidium vulgare* (Crustacean, Isopod), is known to harbour two distantly related *Wolbachia* strains (Cordaux *et al*. 2004). *Wolbachia* are intracytoplasmic α-proteobacteria that modify the reproduction of their hosts. Both strains induce feminization of genetic males into functional females in *A. vulgare*. Changes in host reproduction caused by *Wolbachia* can enhance the spread of the symbiont in infected populations (Werren & O'Neill 1997) by increasing the frequency of infected cytoplasm at the expense of uninfected cytoplasm and by causing a decrease in mitochondrial DNA (mtDNA) polymorphism. However, the situation in *A. vulgare* is more complex because an unidentified non-Mendelian genetic element, called *f*, acts as a third feminizing sex-ratio distorter (Legrand & Juchault 1984). Moreover, at least one 'resistance' gene inhibits vertical transmission of *Wolbachia* (Rigaud & Juchault 1992) and one 'masculinizing' gene restores male phenotype in the presence of *f* or *Wolbachia* (Rigaud & Juchault 1993). As a consequence, *Wolbachia* infection does not spread. This can explain the presence of increased mtDNA polymorphism in infected *A. vulgare* populations compared to other infected isopod species (Rigaud *et al*. 1999). Although the host–parasite model *A. vulgare*/*Wolbachia* has been studied for a long time, very little is known about the dispersal capabilities of *A. vulgare* and *Wolbachia* in the wild. To provide tools for examining the genetic structure of natural *A. vulgare* populations and the role of the feminizing factors on their genetic polymorphisms, we have isolated and characterized nine microsatellite loci for this species.

Isolation of microsatellite markers was achieved by the method of Hamilton *et al*. (1999). A detailed protocol is available at http://bioserver.georgetown.edu/faculty/hamilton/. Briefly, total genomic DNA was extracted by a phenol–chloroform protocol (Kocher *et al*. 1989) from ovaries of one *A. vulgare* (*Wolbachia* non-infected) from a captive population. Twenty-five micrograms of genomic DNA was digested with restriction endonucleases *Hae*III, *Rsa*I, *Alu*I and *Nhe*I (New England Biolabs) to generate DNA fragments within the 200–1000 bp range. These fragments were dephosphorylated using calf intestinal alkaline phosphatase and ligated to SNX linkers (SNX: 5′-CTAAGGCCTTGCTAGCAGAAGC-3′; SNX reverse: 5′-pGCTTCTGCTAGCAAGGCCTTAGAAAA-3′). Two equal volumes of the linker-ligated genomic DNA solution (10 μL) were then probed respectively with biotin-labelled $(AC)_{15}$ and $(AG)_{15}$ oligonucleotides. Library enrichment was achieved by a magnetic separation protocol employing

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streptavidin-coated polystyrene beads (Dynabeads M-280; Dynal, Inc.). The (AC)-enriched and the (AG)-enriched microsatellite libraries were constructed by cutting the enriched fragments with *Nhe*I, ligating the resulting products into dephosphorylated *Xba*I-digested pBluescript plasmid, and transforming the ligated plasmids into Epicurian Coli XL2-Blue MRF Ultracompetent cells (Stratagene). Plasmid DNA was isolated using the QIAprep Spin Plasmid Kit (QIAGEN). Each library was screened with the same probe used for the bead enrichment protocol using a chemiluminescent detection kit (New England Biolabs). A total of 43 positive clones were sequenced using T3/T7 primers and BigDye termination mix (PerkinElmer Applied Biosystems) and resolved on an ABI 377 automated DNA analyser (Applied Biosystems). Sequences containing microsatellites were aligned using SEQUENCHER software (Gene Codes Corporation) to identify duplicate clones. Polymerase chain reaction (PCR) primer sets for amplification of the 43 cloned microsatellites were designed with PRIMER 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

PCRs were performed in a final volume of 12.5 μL [1× *Taq* polymerase buffer (Promega: 500 mM KCl, 100 mM Tris-HCl pH 9.0, 1% Triton X-100 and 15 mM $MgCl_2$; except 1× TaKaRa buffer II for Av7: 100 mM KCl, 100 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 1mM DTT, 0.5% Tween 20, 0.5% Nonidet P-40, 50% Glycerol), 1.2 mM $MgCl_2$, 60 M of each dNTP, 5 pmol of each primer, 0.25 U *Taq* polymerase (Promega, except LA *Taq* from TaKaRa for Av7) and 10 ng of DNA template] using a Trio-Thermoblock (Biometra GmbH). For each reaction, one primer of each pair was end labelled with one fluorescent phosphoramidite (TET, HEX or 6-FAM). The cycling programme consisted of an initial denaturing step of 5 min at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at specific annealing temperature (Table 1), and 45 s at 72 °C followed by a final step of 10 min at 72 °C. PCR products were separated along with the internal size standard GS500-TAMRA on an ABI PRISM 310 automated sequencer. Product sizes were determined using the GENESCAN software (Applied Biosystems).

Nine polymorphic microsatellites were optimized. Table 1 presents details of allelic diversity and heterozygosity

Table 1 Polymorphic repeat microsatellite loci developed for the terrestrial isopod, *Armadillidium vulgare*

Locus (GenBank Accession no.)	Primer sequences (5′–3′)	Repeat in original clone	Annealing temp. (°C)	No. of alleles	Size range (bp)	Pop.	H_O	H_E
Av1	F(1): TGGAGTCAACTCACATTCTG	$(CA)_{10}$	56	4	108–114	Ens.	0.53	0.68
DQ234304	R: TGTCTGTAAAACTTGTGCTACG					Poi.	0.53	0.45
						Montp.	0.40	0.58
Av2	F(1): TGAAGTTCGGGTGAATTGTG	$(CA)_9(CG)_3(CA)_2$	56	4	159–165	Ens.	0.79	0.69
DQ234305	R: ATACCATGACGTGTCGCAAG					Poi.	0.89	0.65
						Montp.	0.60	0.75
Av3	F(2): TGAGTCTCATTATAGTTTGGATGA	$(CA)_4(CG)_2(CA)_{11}$	48	13	185–233	Ens.	0.69	0.83
DQ234306	R: TCCTCTCTATACCCCATAATTTCA					Poi.	0.65	0.68
						Montp.	0.88	0.89
Av4	F(1): CCGAACCTTTCGAAGGTATT	$(GT)_4AT(GT)_{19}$	56	16	198–242	Ens.	0.45	0.79
DQ234307	R: AAGGCACATAACATTTTCACAAA	$(GA)_4AA(GA)_{11}$				Poi.	0.73	0.82
						Montp.	0.90	0.79
Av5	F(1): CGTGCGAAGTTCAGATTCTTT	$(CA)_{13}$	56	28	286–396	Ens.	1.00	0.94
DQ234308	R: GCGCGCTCGAGGATTTAC					Poi.	0.95	0.92
						Montp.	0.88	0.94
Av6	F(1): GGAATGAGGTCGTCGACTATG	$(GT)_8AT(GT)_2$	56	5	166–178	Ens.	0.74	0.56
DQ234309	R: GTCTTTCAAACGGGCACAAT					Poi.	0.63	0.55
						Montp.	0.44	0.53
Av7	F: TCATTTCCGCTTTCCTCATT	$(GT)_2(GC)_7(GT)_{11}$	50	8	229–251	Ens.	0.50	0.74
DQ234310	R(3): CCAACCCTGATTATGGTCTGAT					Poi.	0.40	0.63
						Montp.	0.60	0.71
Av8	F(2): CAACATCCTAATATTCAGTTCTCA	$(CA)_{32}$	48	15	152–192	Ens.	0.74	0.79
DQ234311	R: AGTTTACAGAATACGGCTGAGG					Poi.	0.68	0.84
						Montp.	0.70	0.84
Av9	F(1): TCTCGAAGAATTGCCTCACA	$(CA)_{16}$	56	14	175–221	Ens.	0.88	0.84
DQ234312	R: CGATGACTGGGACAATCTCA					Poi.	0.84	0.87
						Montp.	1.00	0.89

Label used and labelled primer: (1) 6-FAM. (2) HEX (3) TET.

Pop., population's name; Ens., Ensoulesse; Poi., Poitiers; Montp., Montpellier; H_O, proportion of heterozygotes; H_E, expected heterozygosity (calculated with GENEPOP version 3.3; Raymond & Rousset 1995). Forty-eight individuals were amplified for each locus.

Table 2 Allele sizes obtained by cross-species amplification using the primers developed for *Armadillidium vulgare*

	Species				
Locus	*Armadillidium nasatum*	*Armadillidium maculatum*	*Oniscus asellus*	*Porcellio scaber*	*Porcellionides pruinosus*
Av1	—	—	—	—	—
Av2	—	145	—	—	—
Av3	217	177–179	—	—	—
Av4	—	—	—	—	—
Av5	374–386–410	304–306	—	—	—
Av6	174	164	—	—	—
Av7	—	—	—	—	—
Av8	136–144	122–124–130	—	—	—
Av9	—	—	—	—	—

estimates at these nine loci in three widely separated French populations (Poitiers, Ensoulesse and Montpellier with sample sizes of 19, 19 and 10 respectively). The number of alleles per locus ranged from four to 28 (average = 11.9) and the observed heterozygosity from 0.40 to 1.00 (average = 0.75), indicating a high level of polymorphism in *A. vulgare* populations. No significant genotypic linkage disequilibrium was found using Fisher's exact tests implemented in GENEPOP version 3.3 (Raymond & Rousset 1995) after sequential Bonferroni correction ($\alpha = 0.05$, $k = 36$). All the loci were tested separately in each population for Hardy–Weinberg equilibrium using GENEPOP version 3.3. No significant disequilibrium was detected after sequential Bonferroni correction ($\alpha = 0.05$, $k = 27$). We also tested the utility of the primers in five other species representing three additional genera (Table 2). Two individuals from each species were tested at an annealing temperature of 48 °C. Some loci (Av3, Av5, Av6 and Av8) amplified successfully and exhibited polymorphisms in *Armadillidium* species, but none amplified in other species tested.

The polymorphisms observed at the nine microsatellite loci described herein should facilitate better understanding of genetic differentiation and structure among populations of *A. vulgare* and the evolutionary impact of sex-ratio distorters like *Wolbachia* on this species.

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