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TECHNICAL NOTE

Isolation and characterization of ten polymorphic microsatellite markers for three cryptic *Gammarus fossarum* (Amphipoda) species

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Abstract The ecologically important stream invertebrate *Gammarus fossarum* is a morphospecies that includes at least three genetically differentiated biological species. We developed ten microsatellite markers and tested them in a total of 208 individuals from all three known cryptic species (types A, B and C). All markers were polymorphic and successfully amplified in type A, nine in type B and five in type C. There were up to 11 alleles per marker and species.

Keywords Microsatellite markers · Cryptic species · Amphipods · Population genetics

The amphipod *Gammarus fossarum* is an ecologically important stream macroinvertebrate widespread in Central Europe. Müller (2000) identified three cryptic species within this morphospecies, types A, B and C, which probably split several million years ago and seem reproductively isolated. Their geographical distributions overlap in the Rhine drainage (Müller 2000; Westram et al., submitted manuscript). Distinguishing between the species and knowing their ecological and genetic differences is especially important when *G. fossarum* is used for ecotoxicological analyses or habitat quality assessment.

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Ten polymorphic microsatellite markers for the *G. fossarum* species complex were developed. An enriched library was made by ecogenics GmbH (Zürich, Switzerland) from size selected genomic DNA ligated into SAU-LA/SAULB-linker (Armour et al. 1994) and enriched by magnetic bead selection with biotinlabelled $(CT)_{13}$, $(GT)_{13}$, $(TAC)_{10}$, $(ATC)_{10}$, $(ACC)_{10}$, $(AGG)_{10}$, $(GCT)_9$, $(CGT)_8$, $(ACAG)_7$, $(ACCT)_7$, $(GTAT)_7$ and $(GATA)_7$ oligonucleotide repeats (Gautschi et al. 2000a, b). Of 1472 recombinant colonies screened, 323 gave a positive signal after hybridization. Plasmids from 205 positive clones were sequenced and primers were designed for 29 microsatellite inserts, of which 21 were tested for polymorphism. Ten primer pairs produced a polymorphic, interpretable pattern in at least 14 out of 15 test individuals (10 type A, 4 type B, and 1 type C individuals; species identification by 16S sequencing (Müller 2000)). As library development and testing for polymorphism were predominantly performed with type A individuals, the markers were expected to work best in this species.

For further testing of the markers, we used larger samples from eight populations (types A and B: three populations each; type C: two populations), including different major European drainages (type A: Danube and Rhine drainage; types B and C: Rhine and Rhone drainage). While for type A and B we used a minimum of 27 individuals per population, only eight and 17 individuals were available per type C population.

The forward primers were labelled with four different fluorescent dyes (Table 1). A "pigtail" sequence (GTTTCTT) (Brownstein et al. 1996) was attached to each reverse primer to avoid scoring problems due to plus-A artefacts.

After DNA extraction (Montero-Pau et al. 2008) the fragments were amplified by polymerase chain reaction

Table 1 Primers and amplification conditions for ten microsatellite loci in three cryptic *G. fossarum* species

Name	Primer sequences (5′–3′)	Repeat type	Multiplex	Primer concentration (μM)	Dye
Gamfos 08	F: TTTGCTGGATGCTGTGAGAC R: *TGTGTCAATGTTTGCGACTG	$(AC)_8\cdots(AC)_5TT(AC)_{12}GC(AC)_2$	1	0.6	Yakima yellow
Gamfos 10	F: GGCTGGGCTAGTTGTATTGC R: *AAGACGACTAAGGGGTCTGC	$(CTA)_{10}$	1	0.2	ATTO565
Gamfos 13	F: ATCAGGTTGGCGGGTACTG R: *TCAAGTCAAATGAGCCTGGAG	$(CTG)_{11}$	1	0.05	Fam
Gamfos 18	F: CAAAGAAGGGCGTGGTAGTG R: *AGTGTAAAGCTGCCGACCTC	$(GAG)_4(GAC)_{10}$	1	0.1	Fam
Gamfos 19	F: TTTTAGCTCCACGGCTTACC R: *TCTCAGCTTGATGTTGCATTG	$(GTT)_{12}(GCT)_8(ACT)(GCT)_2(GTT)_3$	2	0.2	ATTO550
Gamfos 21	F: GCTGCTATAACCACCGCTTC R: *CAGCGAAGAAGATTTTGCAC	$(GCA)_{22}$	2	0.2	Fam
Gamfos 22	F: TGTAACAGCATCCAAGTGACG R: *ATCGGGGAAAGGTGTTGAG	$(GCA)_7(GCG)(GTG)_3$	1	0.2	Yakima yellow
Gamfos 24	F: AGGTCAGCAACCAAAGAAGG R: *CAACCTGTCCATCAACAACG	$(TGC)_9(TGT)_2(AGC)(TGC)_4$	2	0.2	Yakima yellow
Gamfos 27	F: CGGCGCTAACCTTCTCATAG R: *CAGACTCCCTCCCCCACA	$(TG)_{15}AG(TG)_5$	1	0.2	ATTO550
Gamfos 28	F: ACCTCTCCATCCCTGATGC R: *CATCGACCCGTCAGTATGTG	$(AC)_{13}$	2	0.2	ATTO565

* Indicates "pigtail" (see text). Primer concentration indicates the concentration of the respective primers in the PCR reaction

(PCR) in two multiplex reactions (Table 1), using QIAGEN Multiplex PCR Kit chemicals. Reaction volumes of 12.5 μl contained 6.25 μl of PCR Master Mix, 1.25 μl Q solution and 1 μl DNA. Primers were used in different concentrations (Table 1). Reaction conditions were as follows: 15 min of denaturation at 95°C, followed by 35 cycles of 94°C (30 s), 60°C (90 s), and 72°C (60 s), followed by a final elongation step of 30 min at 60°C. The PCR-amplified fragments were diluted 1:20, combined with GeneScan 500 LIZ size standard (Applied Biosystems) and analyzed on an Applied Biosystems 3730xl DNA Analyzer. The electropherograms were analyzed and manually edited using SoftGenetics GeneMarker software (v. 1.80).

All loci were polymorphic in type A, while one and three markers were monomorphic in types B and C, respectively. Two loci did not amplify in type C (Table 2).

We tested for the presence of null alleles using the program Micro-Checker (v. 2.2.3) and F_{IS}-values calculated in Fstat (v. 2.9.3.2). Both methods gave similar results. We found evidence for null alleles for several population-locus combinations (Table 2), mostly in type B. The observed imperfect cross-species amplification is probably inevitable when species with such long divergence times are considered.

We tested for linkage disequilibrium between loci and calculated basic genetic diversity indices using Fstat (v. 2.9.3.2) and Arlequin (v. 3.5.1.2) software (Table 2). Within types A and B, we detected no significant linkage disequilibrium between any pair of loci (all $P > 0.01$). There were up to 11 alleles per locus and species. Observed heterozygosity ranged from 0.033 to 0.806 (average: 0.446) in type A, from 0.029 to 0.862 (average: 0.456) in type B and from 0.059 to 0.941 (average: 0.337) in type C. Differentiation at these markers is sufficient to discriminate between types A and B. In the samples we used, the two species do not share alleles at five loci (Gamfos 10, 13, 21, 24 and 27).

These polymorphic markers will be useful for analyses of population genetic structure within the cryptic species. In contrast to previously published *G. fossarum* microsatellite markers (Danancher et al. 2009), they were developed explicitly for all three cryptic species, so that comparisons between them with regard to genetic diversity and postglacial recolonization processes are possible.

Table 2 Basic results for ten microsatellite markers tested in three species of the *G. fossarum* complex (total $N = 208$ individuals)

Locus	*N*	Size range	*A*	H_o	H_e	No amplification	Monomorphic	Null alleles
Type A (3 populations)								
Gamfos 08	91	203–249	8	0.343	0.325	0	0	0
Gamfos 10	93	188–194	3	0.467[a]	0.371[a]	0	1	0
Gamfos 13	93	118–137	4	0.611	0.536	0	0	0
Gamfos 18	93	213–235	8	0.444	0.488	0	0	0
Gamfos 19	93	201–228	9	0.806	0.680	0	0	0
Gamfos 21	89	200–242	10	**0.219**	**0.710**	0	0	1
Gamfos 22	93	165–168	2	0.250	0.222	0	0	0
Gamfos 24	93	165–181	5	0.444	0.505	0	0	0
Gamfos 27	93	202–224	6	0.583	0.710	0	0	0
Gamfos 28	93	217–236	11	**0.278**	**0.444**	0	0	1
Type B (3 populations)								
Gamfos 08	90	224–236	5	0.588	0.644	0	0	0
Gamfos 10	53	195–204	3	**0.172**[a]	**0.327**[a]	0	2	1
Gamfos 13	90	113–122	4	0.029	0.029	0	0	1
Gamfos 18	89	210–232	8	0.647	0.814	0	0	1
Gamfos 19	90	200–213	5	0.706	0.673	0	0	0
Gamfos 21	80	196–213	4	0.529	0.710	0	0	2
Gamfos 22	90	165–171	2	0.382	0.472	0	0	0
Gamfos 24	90	172–190	3	0.176	0.166	0	0	0
Gamfos 27	89	205	1	–	–	0	3	0
Gamfos 28	90	209–236	11	**0.529**	**0.815**	0	0	1
Type C (2 populations)								
Gamfos 08	25	222–243	6	**0.941**	**0.708**	0	0	1
Gamfos 10	25	180–189	3	0.412	0.433	0	0	0
Gamfos 13	25	116	1	–	–	0	2	0
Gamfos 18	0	–	–	–	–	2	0	0
Gamfos 19	25	206–216	3	0.176	0.266	0	0	0
Gamfos 21	0	–	–	–	–	2	0	0
Gamfos 22	25	165	1	–	–	0	2	0
Gamfos 24	25	147–157	3	0.412	0.358	0	0	0
Gamfos 27	25	196–200	3	0.059	0.059	0	0	0
Gamfos 28	25	213	1	–	–	0	2	0

N, number of individuals for which this locus was successfully amplified; *A*, number of alleles. Allele sizes include primers and pigtail sequence. H_o (observed heterozygosity) and H_e (expected heterozygosity) are given only for the largest population sample per species. Bold letters indicate significant deviations from Hardy–Weinberg equilibrium ($P < 0.05$). The last three columns per species indicate the number of populations for which the locus could not be amplified, was monomorphic, or contained null alleles according to the program Micro-Checker

[a] As the largest population was monomorphic for this locus, data from another population are shown

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