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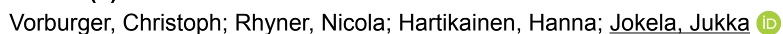

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MICROSATELLITE LETTERS

A set of new and cross-amplifying microsatellite loci for conservation genetics of the endangered stone crayfish (*Austropotamobius torrentium*)

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Abstract Like other indigenous crayfish species in Europe, the stone crayfish (*Austropotamobius torrentium*) is endangered due to habitat degradation, competition with invasive American crayfish and a deadly disease (crayfish plague) vectored by these invaders. To study the genetic diversity and connectivity of the remnant stone crayfish populations, eight new microsatellite loci were developed and supplemented by four cross-amplifying loci developed for related species. These twelve loci were tested for polymorphism in 130 stone crayfish from five streams in Switzerland. Variability was low with 1–7 alleles per locus, but the markers revealed strong genetic differentiation among streams. Use of these microsatellites will provide important information for the conservation and management of this endangered species.

Keywords *Austropotamobius torrentium* · Habitat fragmentation · Microsatellites · Stone crayfish

Microsatellite sequences were isolated by ecogenics GmbH (Switzerland) using the high-throughput genomic sequencing approach detailed in Abdelkrim et al. (2009). Genomic DNA (11 µg) of a single stone crayfish was analyzed on a Roche 454 GS-FLX platform (Roche, Switzerland) using a 1/16th run and the GS FLX Titanium reagents. The 50,529 reads obtained had an average length of 257 bp. Microsatellite inserts suitable for primer design with a tetra- or a trinucleotide motif of at least 6 units or a dinucleotide motif of at least 11 units were present in 137 sequences. Primers were designed for 30 loci and tested in 15 unrelated stone crayfish. Only four primer pairs produced interpretable and polymorphic amplicons of the expected size. The remaining sequences obtained from ecogenics GmbH were therefore subjected to another search using the QDD software (Meglécz et al. 2010) and we designed and tested an additional 23 primer pairs on the same individuals. Of these, again four produced scorable products of variable size. We also tested 13 primer pairs developed for the congeneric species *A. pallipes* and *A. italicus* (Pedraza-Lara et al. 2011; Gouin et al. 2000), of which four yielded interpretable amplification products. The 12 usable microsatellite loci so obtained were then grouped into three multiplex reactions according to annealing temperatures and product sizes to genotype 130 stone crayfish from five streams in northeastern Switzerland (Table 1). Three streams were in close proximity of each other (<1 km) but separated by man-made migration barriers, the other two streams were more distant (20–50 km) and located in different drainages. DNA was extracted from clipped crayfish pleopods using a high salt protocol (Sunnucks and Hales 1996). PCRs were performed in 11 µl final volumes, using the Qiagen multiplex kit and the following thermal profile: 95 °C for 15 min, 35 cycles at 94 °C for 30 s, 1:30 min annealing at 52, 61 or 60 °C (for multiplex I, II or III, respectively) and 1:30 min at 72 °C, and a final extension at 60 °C for 30 min. The forward primer of each pair was labeled with a fluorophore as detailed in Table 1, PCR products were visualized on an ABI 3130 Genetic Analyzer and alleles were sized using GeneMapper® (Applied Biosystems).

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Table 1 Characterization of eight novel microsatellite loci for stone crayfish (*Austropotamobius torrentium*) and four cross-amplifying loci from congeneric species in a sample of 130 individuals from five streams in northeastern Switzerland

Locus	GenBank accession no./reference	Primer sequence (5′–3′) [fluorophore]	Motif	Multiplex	N_A	Allele size range (bp)	H_O	H_E
Newly developed loci								
AT1	KJ150252	F: GAGGTCTAAGGCGACGAGG [ATTO550]	$(AGG)_8\ldots(AGC)_6$	II	3	198–210	0.503	0.506
		R: CAAGTAAGGGCCGGGTGAG						
AT9	KJ150253	F: ACGTGTGTCTGTCTCTATAAATTC [ATTO565]	$(CTGT)_7$	III	2	204–217	0.210	0.213
		R: ATTAATTACGAGGAGGAACAAGAG						
AT22	KJ150254	F: GCACCCCGTTTTCTATGCG [YYE]	$(AAT)_8$	II	2	241–244	0.225	0.226
		R: AGCAAGGTACACCTCTCGG						
AT23	KJ150255	F: CTGTTTCTTGGGTCTCGGC [ATTO565]	$(TGC)_8$	II	3	154–169	0.422	0.423
		R: CTCACCGACACCATACCCG						
AT37	KJ150256	F: ACTATCCGACCGAACGAACC [FAM]	$(TAACC)_{12}$	III	7	287–338	0.489	0.558
		R: ACAGAACCGATTCTTGGCAT						
AT40	KJ150257	F: ATGGGTGTAGACCACGAACG [ATTO550]	$(AT)_{13}$	II	2	113–115	0.273	0.258
		R: CGTGCTAGAATCATTGATCTTACCTT						
AT43	KJ150258	F: TTTCCGAATTTCAATCTGCTT [ATTO565]	$(AAAC)_5\ldots(AC)_{15}$	I	2	119–134	0.381	0.371
		R: CTGCCTTCTCCCTTAACGTG						
AT52	KJ150259	F: AACGAAGGTTTCAGAACGCC [YYE]	$(AG)_9$	I	2	104–108	0.254	0.240
		R: CGCTGTTTCTCGGTTCTTGT						
Cross-amplifying loci								
AP1	Gouin et al. (2000)	F: TCTTGGGGATTGGCTAGTTG [YYE]		I	2	145–147	0.516	0.448
		R: CCTGAACTAAAAGGTGCTTTGG						
AP6	Gouin et al. (2000)	F: GCTGTGTGGGATGGAGGT [FAM]		II	1	342	0.000	0.000
		R: CACTAGCGTATTCAAGCAACT						
Aitali3	Pedraza-Lara et al. (2011)	F: ATATCATGCGCGTCATCTCC [ATTO565]		II	6	249–308	0.579	0.582
		R: CACACGAGCACCAGACATTG						
Aitali4	Pedraza-Lara et al. (2011)	F: CGTTGATGGTAGAGGGGAAG [FAM]		I	1	196	0.000	0.000
		R: CGTCACCGACCATATAAAGTG						

N_A number of different alleles, H_O observed heterozygosity, H_E expected heterozygosity

Polymorphism was relatively low in these samples. Two cross-amplifying loci were not polymorphic, the mean allele number was 2.75 and the mean observed heterozygosity 0.321 (Table 1). No significant deviations from Hardy–Weinberg and linkage equilibria were detected. Genetic differentiation among stone crayfish populations from different drainages was strong and highly significant (F_{ST} values between 0.171 and 0.364). Even the populations from the three closely adjacent but artificially fragmented streams were significantly differentiated (pairwise F_{ST} between 0.054 and 0.101, all $P < 0.005$). These markers will thus be useful to reconstruct historical population structure as well as recent fragmentation effects, despite their limited variability in these remnant populations at the edge of the species' distribution. The microsatellites will also be used to inform management of this endangered crayfish and to identify suitable source populations for reintroduction attempts.

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