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Short communication

Cross-amplification of microsatellite loci in the endangered stone-crayfish *Austropotamobius torrentium* (Crustacea: Decapoda)

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ABSTRACT

Key-words: *polymorphism, cross-species, spatial-scale, conservation, population genetics*

The major aim of this study is to describe the first microsatellite loci for the stone-crayfish (*Austropotamobius torrentium*), by cross-species amplification. *Austropotamobius torrentium* is a priority species in the EU Habitats Directive and it needs effective conservation management efforts throughout Europe. We tested cross-species amplification of 55 decapod microsatellite primer pairs in *A. torrentium* and only ten of these loci, from relatively close related species, yielded PCR products of expected sizes. Five of the ten microsatellites proved to be polymorphic (allele numbers ranging from 4 to 14 in a set of 35 individuals). Three of the loci exhibited departure from Hardy-Weinberg equilibrium which could be explained by the presence of null alleles. A forth locus exhibiting *HWE* deviation, but no null alleles, suggest the possible presence of population substructure of the species in the investigated area. These microsatellite markers are useful for population genetic studies of stone-crayfish.

RÉSUMÉ

Amplification croisée de locus microsatellites chez l'écrevisse des torrents *Austropotamobius torrentium* (Crustacea : Decapoda)

Mots-clés : *polymorphisme, amplification croisée, échelle spatiale, conservation, génétique de population*

Le but principal de cette étude est de décrire les locus microsatellites chez l'écrevisse des torrents (*Austropotamobius torrentium*), par amplification croisée. *Austropotamobius torrentium* est une espèce prioritaire de la Directive Habitats de l'Union Européenne qui nécessite une efficace gestion de conservation à travers l'Europe. Nous avons testé l'amplification croisée de 55 paires de primer microsatellites de décapodes chez *A. Torrentium* et seulement dix de ces locus, d'espèces relativement proches, ont abouti à des résultats PCR. Cinq des dix microsatellites se sont avérés polymorphes (nombres d'allèles de 4 à 14 pour un lot de 35 individus). Trois de ces locus montraient un écart par rapport à l'équilibre de Hardy-Weinberg, qui pourrait s'expliquer par la présence d'allèles nuls. Un quatrième locus présentant une déviation *HWE*, mais pas d'allèles nuls, suggère la présence possible d'une sous-structure de cette espèces dans la zone étudiée. Ces marqueurs microsatellites sont utiles pour les études de génétique des populations de l'écrevisse des torrents.

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INTRODUCTION

The stone-crayfish *Austropotamobius torrentium* (Schrank, 1803) is a crustacean species living mainly in clear and fast-flowing rivers and streams from the mountain and hill areas, being rarely found in lowland rivers or lakes (Băcescu, 1967; Souty-Grosset *et al.*, 2006). This is a native European species, being known from Central and Southeastern Europe. Its range extends from Luxemburg in the West, to Romania and Turkey in the East (Souty-Grosset *et al.*, 2006; Harlioglu and Güner, 2007) and from Saxony (Germany) in the North (Martin *et al.*, 2008) to Northern Greece (Perdikaris *et al.*, 2007) in the South. The species is considered to have originated from the South-west of the Pannonian Basin and has a Danubian biogeographic distribution (Machino and Fureder, 2005; Trontelj *et al.*, 2005). In Romania *A. torrentium* is distributed in running waters from Western Carpathians, from Apuseni Mountains to Cernei and Almaj Mountains and the adjacent Subcarpathian regions (Băcescu, 1967; Pârvulescu, 2010).

Austropotamobius torrentium is listed as a priority species in Annex II of the EU Habitats Directive and needs special conservation measures, while the species IUCN status is "data deficient" at the moment, although the species experiences important declines throughout its range (Füreder *et al.*, 2010). The major threats for this species in Romania are habitat loss and degradation due to domestic, agricultural and industrial pollution and eutrophication (Pârvulescu, 2010). Also, the presence of the spiny-cheek crayfish (*Orconectes limosus*), a non-native crayfish recently discovered in Romania (Pârvulescu *et al.*, 2009), can become an important threat, because this species represents a vector for the crayfish plague (Holdich *et al.*, 2009; Kozubíková *et al.*, 2010).

In order to ensure effective conservation management efforts one needs to evaluate the genetic diversity of the populations of this endangered species. Microsatellite DNA is an ideal molecular marker for studies of genetic diversity in natural populations and can assess population structure and trends (Goldstein and Schlotterer, 1999; Allendorf and Luikart, 2007). However, the development of new species-specific microsatellites by traditional methods entails a substantial effort of time and money. One possible shortcut on the path of describing new microsatellite makers is to cross-amplify markers from other species, based on the fact that the microsatellite flanking regions may be conserved in close related species (Hulák *et al.*, 2010).

In this work we described new microsatellite markers for the species *A. torrentium* by cross-amplifications of microsatellite loci from other decapod species. We also tested the usefulness of the selected microsatellites to assess the genetic variation across a natural population.

MATERIALS AND METHODS

The testing of the cross-species microsatellite amplification was carried out on fifteen individuals of *A. torrentium* from four different Romanian populations (from Nera, Cerna, Topolniţa and Coţuţtea rivers). Total genomic DNA was extracted from a pereiopod segment of these individuals using NucleoSpin® Tissue kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany), according to the producer's specifications. In total, fifty-five primer pairs from six different crustacean species were tested to amplify microsatellite loci in *Austropotamobius torrentium* (Table I). The PCR amplification was performed in 10 μL reaction solution containing 10 ng of template DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5–2.5 mM $MgCl_2$, 0.1 mM dNTP, 0.1 μM from each primer, 0.5 U of JumpStart Taq DNA polymerase (Sigma) and water up to the final volume. The PCR conditions consisted of 3 min initial denaturation at 95 °C, followed by 30 cycles of 95 °C for 30 s, 49–64 °C annealing temperature (Table I) for 30 s, 72 °C for 30 s and a final extension step of 5 min at 72 °C. The PCR fragments were visualized with ethidium bromide on 2% agarose gels.

The primer pairs that exhibited a clear PCR band on the agarose gel were further tested on a LICOR 4300L genotyping system. For this, we performed the PCR reactions, with one of the primers bearing a fluorescent label. Based on this second testing, we selected five microsatellite primer pairs with a clear polymorphic pattern (Table II).

In order to assess basic population genetic parameters we used 35 adult individuals from a population of *A. torrentium* living in Nera river and small tributaries (Romania). The number of alleles per locus (*Na*), effective number of alleles (*Ne*), observed heterozygosity (*Ho*), expected heterozygosity (*He*) and fixation index (F_{IS}) were estimated using GenAlEx 6.4 (Peakall and Smouse, 2006). The exact tests for deviation from the Hardy-Weinberg equilibrium (*HWE*) and the linkage disequilibrium test were carried out using Genepop (ver. 4.0: Rousset, 2008), while the presence of null alleles was tested using Micro-Checker (ver. 2.2.3: Van Oosterhout *et al.*, 2004).

RESULTS AND DISCUSSION

From the 55 tested primer pairs, five primer pairs yielded PCR products that were too long for appropriate scoring, 12 markers yielded multiple bands and 28 markers didn't yield any product at all. Only 10 primer pairs resulted into amplicons of expected sizes and only five of them were variable, with a number of alleles varying from 4 to 10.

Population genetic parameters of the five polymorphic microsatellites in the Nera population are shown in Table II. The total number of alleles per microsatellite ranged from 4 to 14 with an average value of 8.4, and the average effective number of alleles was 3.935. The observed heterozygosity ranged from 0.125 to 0.771 and the expected heterozygosity ranged from 0.613 to 0.834. After non-sequential Bonferroni correction, four of the analyzed loci (AP6, AAS5, AAS6, AAS3040) exhibited significant deviation from the Hardy-Weinberg equilibrium (*HWE*), while no significant linkage disequilibrium was found between our loci, after the same non-sequential Bonferroni correction was applied. Three loci (AP6, AAS5, AAS3040) were identified as possible bearing of null alleles. The deviation from the Hardy-Weinberg equilibrium may be due to several factors like presence of null alleles, inbreeding or population substructure. In our case, the three loci bearing null alleles could also explain the observed deviation from *HWE*. The fourth locus exhibiting deviation from *HWE* was not identified as possibly having null alleles. In this case other factors should be considered to explain the deviation. Among the enumerated causes of *HWE* deviation, we consider inbreeding to be less likely, as the F_{IS} for this locus was small (0.075). The remaining factor, namely population substructure, is more likely to cause the observed *HWE* deviation, as the samples were actually collected not just from the Nera river, but also from small springs tributary to the river. This could indicate that the animals are actually organized in subpopulations among which there is a reduced gene flow, and this could have caused the observed *HWE* deviation. Further studies should be directed toward the identification of population structure/substructure in the area inhabited by the species. While there are concerns that the loci with null alleles might affect the estimation of population differentiation (Paetkau and Strobeck, 1995), there also exist strategies aimed at introducing correction when loci with null alleles are used in population genetics studies (Chapuis and Estoup, 2006).

The microsatellite cross-amplification performed on the stone-crayfish proved successful only with markers from close related species, with 2 loci (out of 8 tested loci, *i.e.* 25%) from *Austropotamobius pallipes* and 3 loci (out of 18 tested loci, *i.e.* 16%) from *Astacus astacus,* species which belong to the same Astacidae family. Other markers from close related families turned out negative results. These results are similar to Hulák *et al.* (2010) on cross-species amplification of microsatellite markers in *Orconectes limosus*. Another aspect concerning the relatedness of the taxa from which the cross-amplified loci yield successful results in a new species is the observation that the null alleles frequencies is increasing as the phylogenetic distance from the focal species is increasing too (Li *et al.*, 2003).

Table I
*Microsatellite loci used for cross-species PCR amplifications in stone-crayfish (*Austropotamobius torrentium*).*

Tableau I
Locus microsatellites utilisés pour les amplifications PCR croisées chez l'écrevisse des torrents (*Austropotamobius torrentium*).

Species	Microsatellite	GenBank accession no.	Annealing temperature (°C)	$MgCl_2$ (mM)	PCR amplification	Reference
Austropotamobius pallipes	Ap1*	AF204815	55	2	Yes	Gouin *et al*. (2000, 2002)
	Ap2	AF204816	55	2.5	Yes – monomorphic	
	Ap3	AF204817	56	1.5	No	
	Ap4	AF204818	51; 52	2.5; 2	Amplification heavy product > 700 bp	
	Ap5	AF204819	56	2.5	Multiband amplification	
	Ap6*	AF204820	57	1.5	Yes	
	Ap7	AF467810	58	2.5	No	
	Ait1	DQ250049	53	1.5; 2.5	Multiband amplification	
Astacus astacus	Aas1	EU692879	56; 57	2.5	Yes – monomorphic	Kõiv *et al*. (2008, 2009)
	Aas2	EU692880	57; 58	2.5; 2	No	
	Aas3	EU692881	56; 57	2.5	Yes – monomorphic	
	Aas4	EU692882	56; 57	2.5; 2	Multiband amplification	
	Aas5*	EU692883	55	2.5	Yes	
	Aas6*	EU692884	59	2	Yes	
	Aas7	EU692885	55	2.5	Amplification heavy product > 700 bp	
	Aas8	EU692886	57	2.5	No	
	Aas9	EU692887	58	2.5	No	
	Aas10	EU692888	59	2.5	No	
	Aas11	EU692889	58	2.5; 2	Yes – monomorphic	
	Aas766	EU352853	59; 60	2.5; 2	Multiband amplification	
	Aas790	EU313798	57	2.5	No	
	Aas1198	EU313799	58	2.5	No	
	Aas2489	EU313797	49	2.5	No	
	Aas3040*	EU313794	58	2.5	Yes	
	Aas3115	EU313795	64	2.5	No	
	Aas3666	EU313796	61; 62	2.5; 2	Multiband amplification	
	Aas3950	EU313800	59	2.5	No	
Orconectes placidus	2.12	AY12993	62	2.5; 1.5	Amplification heavy product > 700 bp	Walker *et al*. (2002)
	3.1	AY112995	60	2.5; 1.5	Multiband amplification	
Procambarus clarkii	Pclg2	AF290920	55	1.5	Multiband amplification	Belfiore and May (2000)
	Pclg8	AF290923	55	1.5	Amplification heavy product > 600 bp	

Table I
Continued.

Tableau I
Suite.

Species	Microsatellite	GenBank accession no.	Annealing temperature (°C)	$MgCl_2$ (mM)	PCR amplification	Reference
Procambarus clarkii	Pclg15	AF290927	60-58	1.5	Yes – monomorphic	Belfiore and May (2000)
	Pclg26	AF290931	55	1.5	No	
	Pclg24	AF290930	58	1.5	No	
	PclG32	AF290935	55	1.5	Multiband amplification	
	PclG37	AF290939	52	1.5	Multiband amplification	
Cherax quadricarinatus	Cqu001	AF156897	56	1.5	No	Baker *et al*. (2000)
	Cqu002	AF156897	59	1.5	No	
	Cqu003	AF156899	59	1.5	No	
	Cqu004	AF156900	57	1.5	No	
	Cqu005	AF156901	56	1.5	No	
	Cqu006	AF156902	60	1.5	No	
	Cqu007	AF156903	60	1.5	Multiband amplification	
Homarus americanus	Ham 9	AF440473	56	1.5	No	Jones *et al.* (2003)
	Ham 10	AF440474	54	1.5	No	
	Ham 15	AF440475	54	1.5	Amplification heavy product > 700 bp	
	Ham 21	AF440476	52	1.5	No	
	Ham 22	AF440477	54	1.5	No	
	Ham 30	AF440478	55	1.5	No	
	Ham 32	AF440479	51	1.5	No	
	Ham 42	AF440480	54	1.5	No	
	Ham 44	AF440481	58	1.5	No	
	Ham 48	AF440482	58	1.5	No	
	Ham 53	AF440483	56	1.5	Multiband amplification	
	Ham 54	AF440484	58	1.5	Multiband amplification	

According to Pandey and Geburek (2009) five microsatellite loci may be useful for the population genetic study of individual populations, even they may not be enough for large spatial-scale studies. In our case, the set of five microsatellites markers exhibited a sufficient level of variation to be used for population genetic studies at a reduced spatial-scale of stone-crayfish populations. Moreover, the preliminary results obtained on the investigated population suggest that further studies are necessary in order to elucidate the possible population substructure of the species in the inhabited area.

Table II

*Characterization and level of variability of five selected microsatellite loci in one population of stone-crayfish from Nera River (Romania). N – number of samples, Na – number of alleles, Ne – effective number of alleles, Ho – observed heterozygosity, He – expected heterozygosity, F_{IS} – fixation index; HWE – Hardy-Weinberg equilibrium test, with non-sequential Bonferroni correction applied: ns – not significant, * – significant deviation.*

Tableau II

Caractérisation et niveau de variabilité de cinq locus microsatellites dans une population d'écrevisses des torrents de la rivière Nera (Roumanie). *N* – nombre d'échantillons, *Na* – nombre d'allèles, *Ne* – nombre effectif d'allèles, *Ho* – hétérozygotie observée, *He* – hétérozygotie attendue, F_{IS} – indice de fixation ; *HWE* – test d'équilibre de Hardy-Weinberg, avec application de la correction de Bonferroni : ns – non significatif, * – déviation significative.

Microsatellite	Primer sequence	Repeat motif	Size range	*N*	*Na*	*Ne*	*Ho*	*He*	F_{IS}	*HWE*
Ap1	F: TCTTGGGGATTGGCTAGTTG R: CCTGAACTAAAAGGTGCTTTGG	$(CA)_{13}(CG)_2(CA)_6$ (Gouin *et al.*, 2000)	161–169	35	4	2.587	0.486	0.613	0.208	ns
Ap6	F: GCTGTGTGGGATGGAGGT R: CACTAGCGTATTCAAGCAACT	$(TG)_7GGGT(TG)_8GG(TG)_{40}TT(TG)_9TT(TG)_7CA(TG)_3$ (Gouin *et al.*, 2000)	330–358	24	8	2.817	0.125	0.645	0.806	*
Aas5	F: CATCAGTCCCATTCCTAATGA R: CGGATTATCTAGGCTGCTGA	$(GA)_{46}$ (Kõiv *et al.*, 2008)	156–210	35	14	4.195	0.629	0.762	0.175	*
Aas6	F: AGACACAAACGCACATGGAA R: GTGTCTGGCAGGCGTATGAT	$(GA)_{26}$ (Kõiv *et al.*, 2008)	159–299	35	10	6.034	0.771	0.834	0.075	*
Aas 3040	F: GTTGTGTGGTAACTCCTGACGA R: CAATCGTATCCCACATGCAG	$(TA)_{20}$ (Kõiv *et al.*, 2009)	230–254	33	6	4.041	0.273	0.753	0.638	*
			Mean	32.4	8.4	3.935	0.457	0.721	0.38	
			SD	2.135	1.72	0.615	0.117	0.041	0.144	

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