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Development of the first polymorphic microsatellite markers for the Roman snail *Helix pomatia* L., 1758 (Helicidae) and cross-species amplification within the genus *Helix*

A.M. Krapal, O.P. Popa, E.I. Iorgu, L.B. Cojocaru, A.F. Popa and L.O. Popa

"Grigore Antipa" National Museum of Natural History, Bucharest, Romania

Corresponding author: L.O. Popa
E-mail: popaluis@antipa.ro

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ABSTRACT. The terrestrial snail *Helix pomatia* (Gastropoda: Stylommatophora: Helicidae) is one of the largest gastropod species in Europe. This species is strictly protected in some European Union countries; however, at the same time, it is also farmed and commercialized for human consumption. Here, we describe 11 microsatellite markers that are very useful in population genetic studies for assessing the status of both wild and farmed populations of this species of community interest. The microsatellites were isolated using 454 pyrosequencing technologies and 11 primer pairs were selected and used for genotyping an *H. pomatia* population and also checked for cross-species amplification on *H. lucorum* and *H. lutescens* specimens. The number of alleles per locus ranged from 3 to 13 and observed

heterozygosity was between 0.458 and 0.917. Seven of these loci were polymorphic in *H. lucorum*, and four in *H. lutescens*. This set of nuclear markers provides a powerful tool for population genetic studies of this species of community interest, and also for closely related species. The described microsatellite markers should also facilitate the identification of populations of conservation concern.

Key words: Conservation concern; STRs; Population genetics; *Helix pomatia*; Helicidae; Cross-species amplification

INTRODUCTION

Helix pomatia Linnaeus, 1758 (Roman snail) (Gastropoda: Stylommatophora: Helicidae) is one of the largest terrestrial species of European gastropods, with a shell reaching 4.5 cm in diameter (Grossu, 1983). Its native range spreads throughout all of Central and Southern Europe, from Eastern Spain (in the West) to Russia (in the East), and from the southern part of Scandinavia to Bulgaria and Italy (Grossu, 1983; Neubert, 2011). This species is the only one in the *Helix* genus present in the northwestern regions of the Alps, reaching the southern part of Scandinavia (Welter-Schultes, 2012).

Due to its large size, *H. pomatia* is farmed for consumption, together with *H. lucorum* and *H. aspersa*, and is highly appreciated for its taste. *H. pomatia* is commonly consumed in many Mediterranean countries. Due to this high interest from a gastronomical point of view, the species has been extensively analyzed in terms of both its biology and ecology, which are important for successful rearing and reproduction of individuals in snail farms. Genetic studies, however, are relatively new and do not focus exclusively on the analysis of this species, but rather on related species in the genus *Helix* (e.g., Mumladze et al., 2013; Korábek et al., 2014) or on the entire Helicidae family (e.g., Korábek et al., 2015). No microsatellite markers have been described to date for *H. pomatia*, as the previously mentioned studies used other types of molecular markers. One study has tested microsatellite markers in *Helicella itala* and *H. pomatia* (Wirth, 2000); however, of the six tested loci, only three have yielded one or two alleles when tested in two *H. pomatia* individuals.

Microsatellite markers are a great tool for assessing genetic diversity, dynamics, and structure of populations. Population genetic studies will be useful in assessing populations of this particular species, and in identifying potentially threatened ones. This will be especially important in those countries where the species is of conservation concern, but can also be useful for determining the genetic characterization of farmed populations.

In this paper, we describe 11 polymorphic microsatellite loci in the species *H. pomatia*. These loci have also been tested on two other closely related species in the *Helix* genus: *H. lucorum* and *H. lutescens*.

MATERIAL AND METHODS

A representative sample consisting of 24 specimens of *H. pomatia* was collected from Bucharest, Romania (44°23'7.92"N, 26°6'3.13"E). Five individuals each of *H. lucorum* and *H. lutescens* were collected from Târgu Jiu, Gorj County, Romania (45°2'18.42"N, 23°16'31.19"E) and Popas Mujdeni, Oraşu Nou, Satu Mare County, Romania (47°51'31.42"N,

23°14'39.18"E), respectively. All collected individuals were preserved in 96% ethanol. Total genomic DNA was isolated from foot muscle tissue using the ISOLATE II Genomic DNA Kit (Bioline, London, UK), according to producer specifications.

Microsatellite-enriched genomic libraries were developed using eight probes (TG, TC, AAC, AAG, AGG, ACG, ACAT, and ACTC). This highly enriched library was sequenced using 454 GS-FLX Titanium (Roche Diagnostics, Risch-Rotkreuz, Switzerland) pyrosequencing technologies, following the protocol described by Malausa et al. (2011). The QDD software was used to validate the best primer pairs for each microsatellite DNA region (Meglécz et al., 2010).

Software-validated primer pairs were tested for successful PCR amplification on a sample of four individuals of *H. pomatia*. Loci that yielded products of the expected size were further tested for polymorphism. After the optimization of PCR for the polymorphic primer pairs, the sampled *H. pomatia* population was genotyped. The PCR genotyping reaction was performed in a 5-µL total volume containing approximately 10 ng DNA template, 0.5 µL 10X NH_4 Reaction Buffer [670 mM Tris-HCl (pH 8.8 at 25°C), 166 mM $(NH_4)_2SO_4$, 4.5% Triton®-X-100, 2 mg/mL gelatin], 2-2.5 mM $MgCl_2$ (see Table 1 for details regarding each locus), 0.1 mM each dNTP, 0.05 µM each primer (one of the primers was M13 tailed), 0.02 µM IRD700 or IRD800-labeled M13 primer, and 0.5 U Taq DNA polymerase (Rovalab GmbH, Teltow, Germany). The PCR conditions used were as follows: an initial denaturation step at 95°C for 2 min, followed by 30-35 cycles of denaturation at 95°C for 30 s, annealing at a specific temperature for each locus (see Table 1) for 30 s and extension at 72°C for 30 s, followed by a final extension step at 72°C for 5 min. The genotyping process was performed on an LI-COR 4300L genetic analyzer (LI-COR Biosciences, Nebraska, USA) and the Saga^{GT} v3.1 software package was used for scoring alleles.

The loci were also tested on several individuals of *H. lucorum* and *H. lutescens* using the same PCR conditions as described for *H. pomatia*. GenAlEx v6.501 (Peakall and Smouse, 2006; 2012) was used to test for Hardy-Weinberg equilibrium at each locus and to estimate the number of alleles (N_A), along with observed (H_O) and expected heterozygosity (H_E). The presence of null alleles, large allele dropout, and scoring errors due to stuttering was tested using MICRO-CHECKER v2.2.3 (Van Oosterhout et al., 2004). Linkage disequilibrium tests were carried out using GENEPOP v4.2 (Raymond and Rousset, 1995; Rousset, 2008).

RESULTS AND DISCUSSION

From the enriched library, a total of 233 sequences containing microsatellite motifs were obtained. We tested 123 primer pairs in PCR and selected 40 primer pairs that provided constant amplification with a PCR product of expected size. A total of 11 polymorphic loci were successfully genotyped in all samples of *H. pomatia*.

The microsatellite loci were polymorphic, with a number of alleles per locus ranging from 3 to 13 (Table 1). The observed and expected heterozygosity values ranged from 0.458 to 0.917 for H_O and between 0.379 and 0.884 for H_E, with a mean of 0.712 ± 0.043 (SE) and 0.667 ± 0.043 (SE), respectively. No departure from the Hardy-Weinberg equilibrium or linkage disequilibrium was observed following Bonferroni correction. The results of the MICRO-CHECKER testing showed no evidence for large allele dropout, null alleles, or scoring errors due to stuttering.

Table 1. Ten polymorphic microsatellites for *Helix pomatia* and their genetic diversity, estimated in a native population from Bucharest (Romania).

Locus	Primer sequence (5'-3')	GenBank accession No.	Repeat motif	Annealing temp. (°C)	$MgCl_2$ (mM)	Bucharest (N = 24 individuals)			
						N_A	Ho	H_E	pHWE
HP 50	F: CATCTTGGCCCGTCAGTAAG R: GATAGACGCTAATCGCAGGC	KU198449	$(GTT)_8$	53	2	8	0.708	0.763	0.450
HP 97	F: TATAAGTGCAGCACCTTGCG R: GGGTGCCTATTACCCGATTT	KU198450	$(ACTG)_{10}$	49	2	6	0.500	0.521	0.144
HP 186	F: TGCCTGTTGGAGCAGAAATA R: ATGCATTATAAAGGCGTGGG	KU198451	$(TC)_{14}$	50	2	13	0.833	0.884	0.586
HP 207	F: TGACAGTGACAAAGACACAGAGA R: TCAAATAGGGACCAGGATCG	KU198452	$(CAGA)_{10}$	50	2	7	0.833	0.815	0.318
HP 228	F: GTACGTCACAGGAGGGCAAT R: GGTGTTCTAGCCAGCCACTC	KU198453	$(TGT)_6$	57	2	3	0.708	0.551	0.075
HP 458	F: TGTGCTGCACCGTCAACTA R: TTCCCTACCCAAGCTCAGAA	KU198454	$(CA)_7$	51	2.5	10	0.833	0.749	0.162
HP 500	F: GGATGGAGAGAATACGCGAG R: AAGCGTGGCAAACACTCAG	KU198455	$(GT)_7$	55	2	5	0.625	0.694	0.548
HP 503	F: TGCTGCACGTGACATAACAA R: CAGCATGGGCTAGAAGAAGG	KU198456	$(TCC)_5$	52	2.5	4	0.917	0.709	0.910
HP 535	F: GGGAGCTTGTGTCTAATGGG R: GAGATATCCGAGGGCAATGA	KU198457	$(AGT)_5$	56	2	3	0.458	0.379	0.546
HP 558	F: TATTCGCTGACACAGGAACG R: CCTGGTTGTCTAATCGGCTC	KU198458	$(GACT)_5$	52	2.5	5	0.667	0.622	0.668
HP 612	F: CACCAACTCACTCCCCACTT R: TGAATGATTAAATAGACAGAATGAAAG	KU198459	$(TTCA)_6$	55	2	5	0.750	0.653	0.215

N - number of individuals, N_A - number of alleles, H_O and H_E - observed and expected heterozygosities, pHWE - P values for Hardy-Weinberg equilibrium test after Bonferroni correction for multiple tests (k = 11).

Given the fact that microsatellite markers are a very useful tool in population genetics studies, we also tested the cross-amplification of these newly developed microsatellite loci in two other helicid species, *H. lucorum* and *H. lutescens* (Table 2).

Table 2. Size ranges (bp) for alleles of the isolated loci in *Helix pomatia* (N = 24), *H. lucorum* (N = 5), and *H. lutescens* (N = 5).

Locus	*H. pomatia*	*H. lucorum*	*H. lutescens*
HP 50	134-170	140-158 (3)	173
HP 97	117-181	133	133
HP 186	142-182	164-196 (4)	174-194 (3)
HP 207	197-229	-	205-219 (3)
HP 228	168-174	165-174 (4)	165
HP 458	130-152	132-134 (2)	146-166 (4)
HP 500	114-126	116	112
HP 503	121-130	112-115 (2)	-
HP 535	175-181	187-205 (2)	184
HP 558	143-159	143-147 (2)	155
HP 612	151-167	167	171-175 (2)

The total number of alleles for the cross-amplifications is indicated in parentheses. A dash indicates no PCR product.

The small sample sizes of *H. lucorum* and *H. lutescens* used in the cross-amplification tests could be the reason for the limited number of observed alleles. Further studies on larger numbers of individuals in these two species should be undertaken to assess polymorphism levels and the usefulness of these microsatellite loci.

H. pomatia is an intensively commercialized species, and is consumed mostly in the Mediterranean region. Although there are farms that grow these snails for consumption, they are also collected from the wild in some European regions; this may eventually threaten the existence of wild populations of this species. Currently, although wild populations are

abundant and stable in most of the snail's native area, there are also some European Union countries in which the species is protected (e.g., the United Kingdom, Denmark, the Czech Republic, and Germany).

Population genetic studies can serve as a baseline for the protection of threatened populations and subpopulations present in regions where this species is rare. The microsatellite markers described in this study should facilitate the identification of populations of conservation concern, thus allowing informed decision-making when preparing conservation plans.

Conflicts of interest

The authors declare no conflict of interest.

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