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MICROSATELLITE LETTERS

Development and characterization of novel microsatellite markers for *Arion* slug species

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Abstract Seventeen polymorphic microsatellite markers were isolated and characterized in *Arion vulgaris/lusitanicus*, which belongs to the worst European slug pests with serious economic and ecological impact. These markers were tested on 23 individuals collected in a population in Switzerland. Numbers of alleles ranged from 2 to 14 per locus, observed and expected heterozygosities ranged from 0.174 to 0.87, and from 0.162 to 0.903, respectively. These loci were also successfully amplified and were polymorphic in the closely related species *A. rufus* and *A. ater*. These loci represent the first highly polymorphic nuclear markers described for *A. vulgaris* and pave the way for population genetics and molecular ecology research of the important *Arion* pest slugs.

Keywords *Arion vulgaris* · *Arion lusitanicus* · *Arion rufus* · *Arion ater* · 454 Pyrosequencing · Invasive species · Mollusks

One of the major threats to global biodiversity is the invasion by alien species. The slug *Arion vulgaris* (syn. *Arion lusitanicus*) belongs to the hundred most invasive species in Europe (DAISIE 2009). It causes significant economic losses in agriculture and horticulture, and it might lead to a decline of native related species, probably by competition or hybridization (Proschwitz 1997). Here, we report on the development of 17 microsatellite markers for *A. vulgaris* and related slugs, that can be applied e.g. in population genetics analyses of the invasion history or tests of hybridization with related species.

Genomic DNA was extracted from one individual of *A. vulgaris* sampled in Gruyères, Switzerland (N 46°34′, W 7°2′) in 2012 using the standard phenol–chloroform extraction protocol. We sheared seven hundred nanograms of DNA by nebulization and used DNA fragments in the length range between 600 and 900 bp for Next-Generation Sequencing. Library preparation and sequencing were done following the manuals for the GS Junior pyrosequencing system FLX Titanium (454 Life Sciences Corp). After initial filtering of sequence reads that failed the Key Pass Filter of the system because they did not start with the sequence expected from library preparation, we retained 110,975 raw sequencing reads of 388 bp average length. These reads were searched for di-, tri-, and tetranucleotide microsatellite motifs of at least 10 repeat units length using MSATCOMMANDER version 0.8.2 for Windows (Faircloth 2008). PCR primers were designed using PRIMER3 release 1.1.1 (Rozen and Skaletsky 2000), implemented in MSATCOMMANDER, using the default parameters without any tagging options. We tested 103 primers on polyacrylamid gels and assessed their polymorphism and amplification strength. We selected 17 primer pairs that successfully amplified products of the expected size based on the length of the sequence from which the primers were designed, and that exhibited polymorphism. These were tested on 23 individuals collected at a single locality near Blumenstein, Switzerland (N 46°44′, W 7°31′). We used PCR reaction volumes of 10 µl, including 5 µl of the

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Table 1 Microsatellite loci for *A. vulgaris* tested in 23 individuals from a Swiss population

Locus	GenBank accession number	Repeat motif	Primer sequences (5′–3′)	Size range (bp)	N_A	H_O	H_E
ALU_02_3	KP247477	ATC	F: CGAGTTCCATTTCTCCCGTG	301–343	9	0.682	0.797
			R: TGGATGGCCTGACTTCGTG				
ALU_06_4	KP247478	AATC	F: ATAACGCGGTCCGAACTGG	280–356	6	0.391	0.729*
			R: CAACTCATTGGCCGACCTC				
ALU_11_2	KP247479	AG	F: GCCTTAAACTCCAGAGGCGT	103–117	8	0.714	0.823
			R: GTCAAGATAACAAGGCACAAACT				
ALU_12_2	KP247480	AG	F: ACAGAAGTGAGATTCAGGAGGT	109–120	5	0.826	0.880
			R: CGTACTTTGTTGCCTGATTGT				
ALU_13_2	KP247481	AG	F: ATACCAGAGGGCAAACGGG	237–251	7	0.696	0.799
			R: ACAGGCACAAACTTAGAAGTCG				
ALU_30_2	KP247482	AG	F: TGGGAAACATCAATGGGAAACG	187–227	4	0.652	0.903*
			R: GCAGCCAGCATAAGACTACC				
ALU_34_2	KP247483	AG	F: TATTGGCTCCAGTGCTCCC	305–321	5	0.478	0.662
			R: ACTGATGGTTCCTGTAGCTCG				
ALU_37_2	KP247484	AG	F: TGCTGTGTATTTGTTACTGCCC	230–259	4	0.652	0.805*
			R: CGTGTCTGCAAGGTTTCCC				
ALU_60_2	KP247485	AT	F: GCATCTATTTCATTTGGTGTTCCC	211–223	7	0.609	0.673
			R: GTATCATGGGAGATCTAAATATCAAGG				
ALU_76_2	KP247486	AT	F: GATCTGCCTGCTCATTCCAC	140–150	6	0.696	0.715
			R: ATGCAGGAGAACGTGCTTG				
ALU_79_2	KP247487	AT	F: TGGTATCTTAGTCCATTTCTTGATG	226–244	6	0.870	0.763
			R: TCAGTGCTGTTCAATATGTCTTC				
ALU_86_2	KP247488	AG	F: TGGGTGTTCTTGATCCCAC	277–280	2	0.565	0.689
			R: ACGCCAGCTCTGGACATC				
ALU_88_2	KP247489	AC	F: ACCGCGAAGGTGATGACAG	298–309	8	0.652	0.809
			R: ACTGCTGGACGTGATGAAAC				
ALU_92_2	KP247490	AG	F: GCTACCCAAAGTACCAAGTGC	332–346	8	0.609	0.620
			R: CGGACATGGAATGGGCTTC				
ALU_94_2	KP247491	AT	F: TGACAATCGCGAAACGGTG	339–357	6	0.609	0.689
			R: AGAACTTAAGATAATCGGCACG				
ALU_96_2	KP247492	AT	F: CATGGCACCGAGCTACAAG	367–381	14	0.478	0.768*
			R: GATGACGCTCAACAACCCG				
ALU_102_2	KP247493	AT	F: GGCCCTATGTAACGTACACC	439–443	6	0.174	0.162
			R: CCAGTGAGTTTGCCTCAGC				

N_A number of alleles, H_O observed heterozygosity, H_E expected heterozygosity: asterisks indicate significant ($P < 0.05$) deviations from HWE

Qiagen multiplex kit, 2 µl of H_2O, 1 µl of the primer mix, and 2 µl of genomic DNA. The temperature profile was as follows: 15 min initial denaturation at 96 °C, followed by 32 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 1 min 30 s, extension at 72 °C for 1 min 30 s, and the final extension step of 30 min at 60 °C. The PCR product was diluted with 20 µl dH_2O and 1.2 µl of the diluted product were mixed with 12 µl of the internal size standard (GeneScan™-500LIZ™, Applied Biosystems) for determining the length of the PCR products. Finally, the amplified fragments were separated on an ABI Prism 3130 Genetic Analyzer and scored with GeneMapper version 3.7 (Applied Biosystems).

The number of alleles per locus (N_A), the observed and expected heterozygosity (H_O and H_E) and tests for deviations from Hardy–Weinberg equilibrium (HWE) were calculated using Arlequin 3.5.1.2 (Excoffier and Lischer 2010). The number of alleles ranged from 2 to 14 per locus, observed and expected heterozygosities ranged from 0.174 to 0.87, and from 0.162 to 0.903, respectively (Table 1).

Significant departures from HWE were detected for four loci, potentially due to the presence of null alleles. We successfully cross-amplified these loci in the closely related species *A. rufus* (3 populations, 72 individuals) and *A. ater* (3 populations, 48 individuals). All loci were polymorphic also within these species, with an average number of alleles per locus of 4 for *A. rufus* and 3 for *A. ater*. These markers will be useful for assessing genetic diversity in native and invasive populations of *Arion* sp., their population structure and parentage analysis in potential hybrids.

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