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ADDITIONAL POLYMORPHIC MICROSATELLITE LOCI FOR DETAILED POPULATION GENETIC STUDIES OF *LETHRUS APTERUS* (COLEOPTERA: GEOTRUPIDAE)

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In this study we attempted to isolate 13 additional microsatellites from the beetle *Lethrus apterus* and then to use them to screen for polymorphisms in 35 specimens collected from the northern Hungarian mountains. The newly developed markers presented in this paper are polymorphic, including between two and seven detected alleles per locus. The observed and expected heterozygosity per locus ranged between 0.057 and 0.686 and between 0.056 and 0.756, respectively, while two loci showed significant deviations from Hardy-Weinberg equilibrium. These deviations can be explained by the presence of null alleles. All loci cross-amplified in at least two out of four congeneric *Lethrus* species (*L. bituberculatus*, *L. scoparius*, *L. strymonensis* and *L. perun*). The polymorphic microsatellite markers we present for *L. apterus* will be valuable for future population genetic studies.

Keywords: dinucleotide repeats, trinucleotide repeats, fragmentation, species conservation, parentage.

INTRODUCTION

Microsatellites are considered to be one of the most powerful genetic markers because of their high variability and co-dominant inheritance (Jarne & Lagoda 1996, Avise 2004, Pemberton 2009). As such, these markers are widely used in population and conservation genetic studies as well as for the reconstruction of pedigrees and the estimation of relationships between individuals (Harris *et al.* 1991, Goldstein & Schlötterer 1999).

A detailed population genetic survey has become necessary for the geotrupid beetle *Lethrus apterus* Laxmann, 1770. Although this species was once

common in Hungary causing severe damage especially to vineyards (Emich 1884, Biró 1886, Horváth 1897), its populations have declined significantly over the last 100 years presumably due to changes in agricultural practices. In particular, the use of broad-spectrum insecticides and deep ploughing are likely to significantly influence population sizes of *L. apterus* overwintering underground. Agricultural intensification has caused this species to retreat to fragmented habitats that are able to maintain only reduced numbers of individuals (Merkl & Vig 2009). Thus, as a result of this astonishing decrease in population size, the species was finally protected by law in Hungary in 2012.

Lethrus apterus has low dispersal abilities as it is flightless, and therefore gene flow among populations that live in isolated habitat patches is strongly limited. As a result, separated populations can differ stochastically as genetic differences accumulate. All these effects may influence the pattern of genetic variation, which, in turn, can strongly affect species conservation. Therefore, genetic surveys on *L. apterus* populations are essential to assess possible effects of fragmentation on genetic diversity and hence the conservation status of this species. Such studies will greatly contribute to the successful protection of *L. apterus* and will enable the more effective planning of further nature conservation activities.

In addition, *L. apterus* exhibits highly developed biparental care where parents perform different tasks (Clutton-Brock 1991). This leads to conflict between male and female parents in terms of their investment in offspring, and extra-pair copulations could play an important role in the game between the two sexes (Houston *et al.* 2005). In order to determine the complex behaviour of this species, in particular the results of the conflict between parents, we need detailed parentage analyses for which microsatellites are also applicable.

Until now, 15 published microsatellite markers were available for *L. apterus* (Rácz *et al.* 2015); however, that marker set showed a moderate level of variability, which can reduce its efficiency in investigation of population genetic structure or pedigree reconstruction. In this study, we attempted to isolate and characterize further microsatellites in order to increase the power of the existing marker set. Here we present 13 new microsatellite loci developed for *L. apterus*.

MATERIAL AND METHODS

Genomic DNA was isolated by homogenizing thorax muscle following the protocol described in Rácz *et al.* (2015). The genomic DNA libraries of two individually tagged specimens were then used for high throughput sequencing using an Illumina HiScan-SQ platform via a commercial service provider (UD-GenoMed Medical Genomic Technologies Ltd., Debrecen, Hungary). Sequencing and assembling were carried out as described in Rácz *et al.* (2015). Subsequent to assembly, we searched for repeating motifs in the assembled contigs using BLASTN (Altschul *et al.* 1997) that fulfilled two conditions: (i)

Table 1. Characteristics of 13 microsatellite loci of *Lethrus apterus*. Values are based on the analyses of 35 individuals.

Locus	Primer sequence (5′–3′)	Repeat motif	Fluo-rescent label	Mul-tiplex set	T_a (C°)	N	Size range (bp)	N_a	H_o	H_e	HWE p-value	GenBank Accession No.
Lapterus1	F:CGTTGCGAAAGATATTGACC R:CACTTCCTGTGGAATAAATCG	$(TG)_{20}$	VIC	3	60	35	126–174	6	0.371	0.551	0.052	KU707884
Lapterus 2	F:GCTTTGAGCGTTAACCATAATCC R:GGATTCCTAGAGTTGTACTTTCAC	$(TGA)_{27}$	NED	2	62	35	202–214	3	0.600	0.509	0.346	KU707885
Lapterus 3	F:CGATCCTCTTGTACCCTATGG R:AACAGTTCAGTAAAGGCTATGC	$(TC)_{17}$	FAM	2	60	35	136–144	3	0.429	0.428	1.000	KU707886
Lapterus 4	F:TACTAATAACTTGCCCCAAGAC R:GATCTCTACACGTATTTATAAACG	$(TA)_{17}$	PET	2	60	35	99–113	3	0.114	0.134	0.058	KU707887
Lapterus 5	F:TAAGAACGCGTCGATCTGTCC R:CCAATCAGGCTTTGTATCAGC	$(GTT)_2GCA(GTT)_{19}$	PET	3	60	35	237–261	7	0.686	0.756	0.406	KU707888
Lapterus 6	F:GGCTGATTGCTCTAGAATAAAAGAC R:GACAAATTGCTACAATTATGAATGC	$(TC)_2TT(TC)_{19}$	VIC	1	60	35	232–244	6	0.657	0.605	0.494	KU707889
Lapterus 7	F:CAACCTGGAAATTTGGACAG R:TTTTCCTGTTTCCTTCGC	$(AG)_{19}A(AG)$	NED	2	62	32	160–170	4	0.156	0.346	0.0004*	KU707890
Lapterus 8	F:GATTGATTTAGAGAAGTTACAATGC R:CACATTCTTCCTGATAGCC	$(AG)_{16}A(AG)$	PET	3	58	35	135–139	3	0.114	0.109	1.000	KU707891
Lapterus 9	F:CCATCAGCAAAATGGACGTAGTC R:GCCTACAGTCAGATTCTTCGTG	$(TG)_{16}$	NED	3	60	35	95–109	3	0.600	0.664	0.013*	KU707892
Lapterus 10	F:CGTGGCGGACAGTTTCAG R:TCTCGATAAGCGAAAGCTTGAG	$(TG)_{16}G(TG)$	FAM	1	62	30	169–175	4	0.167	0.365	0.0005*	KU707893
Lapterus 11	F:ACTCGCTAGTGCTTACTAACTC R:CAATTATCTTTAACTTACGACAGTG	$(TAG)_{16}$	VIC	1	56	32	84–105	3	0.375	0.398	0.181	KU707894
Lapterus 12	F:CATAAAACACACATAAATGGTG R:GACTTTACACATTAGTATAACTC	$(CA)_{16}$	PET	1	60	35	184–186	2	0.057	0.056	1.000	KU707895
Lapterus 13	F:TACCCAAAGCCTTACATTCGG R:TCAAACCATGACTCCTTTGC	$(AAG)_{16}$	VIC	2	62	35	106–139	5	0.600	0.554	0.082	KU707896

T_a optimal annealing temperature (°C), N number of individuals from the 35 in which the locus amplified, N_a number of alleles per locus, H_o observed heterozygosity, H_e expected heterozygosity, *HWE p-value* the exact p-value for Hardy-Weinberg equilibrium test. * Loci that significantly deviated from Hardy-Weinberg equilibrium after Bonferroni correction ($p < 0.00385$)

Table 2. Cross-amplification of *L. apterus* microsatellite loci in four species of the genus *Lethrus*.

Species	*N*	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13
L. perun Král et Hillert, 2013	5	5	5	5	5	5	5	5	5	4	-	5	5	5
L. bituberculatus Ballion, 1870	5	3	1	-	3	4	2	-	5	5	2	4	-	-
L. strymonensis Král et Hillert, 2013	5	5	5	5	5	5	5	4	3	5	1	5	4	5
L. scoparius Fischer von Waldheim, 1822	4	1	4	-	4	4	-	-	4	4	-	3	-	-

N number of individuals tested, L1–L13 abbreviations of loci's names (Lapterus1–Lapterus13 respectively); numbers represent the number of individuals in which the locus amplified (dash means that the locus did not amplify)

The number of repeats was at least 15, and; (ii) A clear size difference is present in repeat lengths between the sequences of the two individuals. This process resulted in 26 potential loci. Primers were designed by manually inspecting potential priming regions, and the potential primers were tested and further modified to meet optimal priming criteria using the Primer Stats program in the Sequence Manipulation Suite v.2 (Stothard 2000).

Microsatellite polymorphism was tested on 35 specimens collected at Dorogháza, Hungary (47°59'29"N, 19°53'36"E) in the Mátra Mountains in 2013. Cross-amplification of markers developed for *L. apterus* was also tested for four congeneric species (Table 2). The amplification procedure was carried out in 10 μl final reaction volumes containing 1 μl of DNA extracts, 10 × PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.05 units/μl of Taq DNA polymerase (Dream Taq Green, Fermentas), and 0.5 μM of each fluorescent dye-labeled primer (Table 1). The cycling conditions on an ABI Veriti Thermal Cycler comprised initial denaturation two min at 95°C, 40 cycles of 15 s at 95°C, 30 s at the locus specific annealing temperature, one min at 72°C, and a final elongation of 14 min at 72°C. The success of PCRs was checked by running of 2 μl of product on 1% agarose gels stained with GelRed Nucleic Acid Stain (Biotium Inc.).

Initial tests showed that 13 primer pairs were not able to amplify target sequences consistently; these were therefore excluded from further investigations. Subsequent to amplification, microsatellite products were pooled in three multiplex sets (Table 1) and fragment sizes were determined using an ABI 3130 Genetic Analyzer in the Molecular Taxonomy Laboratory of the Hungarian Natural History Museum (Budapest, Hungary). Allele sizes were scored with Peak Scanner software version 1.0 (Applied Biosystems). Parameters of polymorphism, including the number of alleles per locus (N_a), observed (H_o) and expected heterozygosity (H_e), were determined using GENALEX 6.4 (Peakall & Smouse 2006). Checking for the presence of null alleles was performed using Micro-Checker 2.2.3 (van Oosterhout *et al.* 2004) using Monte Carlo simulation of expected homozygote frequencies and heterozygote allele size differences. Tests for Hardy-Weinberg equilibrium and linkage disequilibrium were conducted using GENEPOP 4.2 (Raymond & Rousset 1995, Rousset 2008).

RESULTS AND DISCUSSION

Results show that the 13 consistently amplifying markers were polymorphic, with two-to-seven alleles detected per locus (Table 1). Observed (H_o) and expected heterozygosity (H_e) ranged from 0.057 to 0.686 and from 0.056 to 0.756, respectively (Table 1). We found evidence for null alleles for loci Lapterus1, Lapterus7 and Lapterus10. Three loci (Lapterus7, Lapterus9 and Lapterus10) out of 13 were not in Hardy-Weinberg equilibrium, and 6.4% of all pairwise tests for linkage disequilibrium among loci were significant. Following Bonferroni correction (Rice 1989) locus Lapterus7 and Lapterus10 still showed significant deviations from Hardy-Weinberg equilibrium ($p < 0.00385$), although none of the pairs of loci exhibited significant evidence for linkage disequilibrium ($p < 0.00064$). Deviations from Hardy-Weinberg equilibrium were caused by heterozygote deficit, and can be explained by the presence of null alleles as Micro-Checker suggested.

Cross-amplification tests of primer pairs were carried out for four congeneric species, *L. bituberculatus* Ballion, 1870, *L. scoparius* Fischer von Waldheim, 1822, *L. strymonensis* Král et Hillert, 2013, and *L. perun* Král et Hillert, 2013. All loci showed cross-amplification in at least two of the four *Lethrus* species based on agarose gel electrophoresis (Table 2), and the highest number of successful amplifications occurred in *L. perun* and *L. strymonensis* (Table 2).

The polymorphic microsatellite markers presented here for *L. apterus* will be extremely valuable for future detailed population genetic studies. Firstly, they will allow us to reveal the factors that shape the current genetic structure of the species. This kind of knowledge is fundamental to successful species conservation. Secondly, this marker set will contribute to research on the evolution of cooperation between parents via parentage analyses in *L. apterus* families.

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