

1 Isolation and characterisation of 15 microsatellite loci from *Lethrus apterus* (Coleoptera:
2 Geotrupidae)

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24
25 Short title: Microsatellites in *Lethrus apterus*

26 Abstract

27

28 Fifteen new microsatellite markers for the beetle *Lethrus apterus* were developed and tested
29 in 45 specimens from North Hungarian Mountains. Fourteen of the developed markers were
30 polymorphic, and the number of alleles per locus ranged from two to nine. The observed and
31 expected heterozygosity of the polymorphic markers ranged from 0.178 to 0.578 and 0.201 to
32 0.698, respectively. One locus showed significant deviation from Hardy-Weinberg
33 equilibrium, probably due to null alleles. The primers were tested on four other *Lethrus*
34 species (*L. bituberculatus*, *L. scoparius*, *L. strymonensis* and *L. perun*) and six other
35 Coleopteran species (*Copris hispanus*, *Geotrupes stercorarius*, *Melolontha melolontha*,
36 *Onthophagus taurus*, *Oryctes nasicornis* and *Protaetia affinis*). Thirteen loci showed cross-
37 amplification in *Lethrus* species and only three loci could be amplified in some of the six
38 other Coleopteran species. These markers will be valuable to investigate the population
39 genetic structure, behaviour and reproductive biology of *L. apterus*.

40

41 **Keywords:** dinucleotide repeats, trinucleotide repeats, cross-amplification, parentage

42 1. Introduction

43

44 The subfamily Lethrinae within the scarabaeoid family Geotrupidae is represented by a single
45 genus, *Lethrus* Scopoli, 1777, which comprises about 120 species (Hillert 2004, Král and
46 Nikolajev 2006). The genus is considered to be monophyletic based on morphological
47 characters (Nikolajev 2003, Scholz and Grebenikov 2005) with a wide distribution area in the
48 Palearctic, however, most of the species are known from Central Asia (Nikolajev 2003, Král
49 and Nikolajev 2006, Král and Hillert 2013). The beetle *Lethrus apterus* Laxmann, 1770 has
50 Eastern European and Anatolian distribution, and the western edge of its distribution is in
51 Hungary (Merkl and Vig 2009) where the species is protected. The species is well-known for
52 its highly developed parental care (Wilson 1971).

53

54 *Lethrus apterus* is a biparental species, the sexes are dimorphic and according to the literature
55 there is a division of parental roles between the sexes (Emich 1884, Schreiner 1906, von
56 Lengerken 1939, Wilson 1971, Clutton-Brock 1991): males are responsible for leaf collecting
57 and defend the nest burrow from intruders while females prepare food balls for the offspring.
58 Recent observations suggest a change in division of labour between the parents in Northern
59 Hungary: the leaf collecting activity is highly female biased (Kosztolányi *et al.* 2014). One of
60 the several possible explanations for this shift is that the area of this species was fragmented
61 recently, and because of this fragmentation the density of breeding individuals may have
62 increased locally. High male density may increase the frequency of extra-pair mating leading
63 to a reduced incentive to care by males (Kokko and Jennions 2008). The observed change in
64 parental duties may provide a unique opportunity to shed light on the evolutionary origin of
65 biparental care and on how social environment influences this cooperative behaviour.

66

67 Microsatellites are considered as hypervariable and codominant DNA markers, thus they are
68 suited for investigation of genetic structure and reconstruction of pedigrees and estimation of
69 parentage (Harris *et al.* 1991). Until now, there were no published microsatellite markers
70 available for any of the approximately 120 species in the genus *Lethrus*. Here we present 15
71 microsatellite loci developed for *L. apterus*.

72

73

74 2. Materials and methods

75

76 Genomic DNA was isolated by homogenizing the muscle of thorax in 800 µl extraction buffer
77 proposed by Gilbert *et al.* (2007). The samples were incubated for 24 h at 56°C with gentle
78 agitation and then centrifuged at 14000 rpm for 1 min. The supernatant was washed with an
79 equal volume of chloroform-isoamyl alcohol (24:1) to remove proteins. The DNA was
80 precipitated using 80 µl ammonium acetate (7.5 M) and an equal volume of ice-cold
81 isopropanol stored at -20°C for 4 h. The DNA is pelleted by centrifugation at 14000 rpm for
82 10 minutes at 4°C. After centrifugation, the supernatant was discarded and the DNA pellet
83 was washed twice with 70% ice-cold ethanol. The pellet was air dried for 1 h and dissolved in
84 50 µl elution buffer (10 mM Tris HCl, pH 8.0 and 0.5 mM EDTA, pH 9.0).

85

86 High throughput sequencing was performed on Illumina HiScan-SQ platform by a
87 commercial service provider (UD-GenoMed Medical Genomic Technologies Ltd., Debrecen,
88 Hungary). Genomic DNA libraries of two individually tagged specimens were prepared
89 according to Illumina DNA library preparation method, TruSeq DNA Sample Preparation Kit
90 was used (Illumina, San Diego, CA) and paired-end 100 bp sequencing was carried out. The
91 paired-end sequenced reads were de-multiplexed by individuals and assembled using ngopt-
92 a5pipeline v20130326 de novo assembler software (ngopt, NextGenOptimizer,
93 <http://sourceforge.net/projects/ngopt/>), with default settings. A total of 202 .1K and 214 .8K
94 contigs (total length of the contigs: 240.6 and 257.7 Mb, N50 values: 2311 and 2837,
95 respectively) were obtained from 57.7M and 86.9M aligned reads of the two individuals.
96 After assembling we searched in assembled contigs for the motifs (AAT)_n, (GT)_n, (CT)_n
97 fulfilling three conditions: (i) $n \geq 5$; (ii) the length of flanking regions had to be at least 100 bp
98 on both ends; (iii) there had to be a size difference in repeat length between sequences of the
99 two individuals. This process resulted in 18 potential loci. Primers were designed by manually
100 inspecting potential priming regions, and the potential primers were tested and further
101 modified to meet optimal priming criteria using the Primer Stats program of the Sequence
102 Manipulation Suite v.2 (Stothard 2000).

103

104 Microsatellite polymorphism was tested on 45 specimens from four populations of North
105 Hungarian Mountains. DNA was extracted by homogenizing the middle leg in 800 µl

106 extraction buffer proposed by Gilbert *et al.* (2007) and using the protocol described above.
107 DNA aliquots were stored at 4°C. DNA amplification from 1 µl of DNA extracts was carried
108 out in 10 µl final reaction volumes containing 10x PCR buffer, 2 mM MgCl₂, 0.2 mM dNTPs,
109 0.05 units/µl of Taq DNA polymerase (Dream Taq Green, Fermentas) and 0.5 µM of each
110 fluorescent dye-labeled primer (Table 1). The following cycling conditions were used on ABI
111 Verity Thermal Cycler: initial denaturation 2 min at 95°C; 40 cycles of 15 s at 95°C, 30 s at
112 the locus specific annealing temperature of 60°C, 1 min at 72°C; final elongation of 14 min at
113 72°C. PCR amplicons were run on 2% agarose gels. Three primer pairs did not amplify the
114 target sequences consistently, therefore these were excluded from further investigations. After
115 amplification, microsatellite products were multiplexed and fragment analysis was carried out
116 on an ABI 3130 Genetic Analyzer in the Molecular Taxonomy Laboratory of the Hungarian
117 Natural History Museum (Budapest, Hungary). Allele sizes were estimated using Peak
118 Scanner software (Applied Biosystems). All allele sizes were double checked to assure
119 reproducibility and correct readings. Micro-Checker 2.2.3 (van Oosterhout *et al.* 2004) was
120 used for calculating null allele frequency by Monte Carlo simulation of expected homozygote
121 frequencies and heterozygote allele size differences. Parameters of polymorphism, including
122 the number of alleles per locus (N_a), observed heterozygosity (H_o) and expected
123 heterozygosity (H_e) were calculated by GENALEX 6.4 (Peakall and Smouse 2006). Linkage
124 disequilibrium test and deviation from Hardy-Weinberg equilibrium at each locus were
125 performed by GENEPOP 4.2 (Raymond and Rousset 1995, Rousset 2008).

126 127 3. Results and discussion

128
129 Fourteen of the developed markers were polymorphic. The number of alleles per locus ranged
130 from two to nine (Table 1). Observed (H_o) and expected heterozygosity (H_e) ranged from
131 0.178 to 0.578 and from 0.201 to 0.698, respectively (Table 1). Two loci (Lethrus11 and
132 Lethrus13) showed significant deviation from Hardy-Weinberg equilibrium and significant
133 linkage disequilibrium was observed in 16.2% of all possible comparisons. The Micro-
134 Checker analysis detected evidence for null alleles at Lethrus11 locus by general excess of
135 homozygotes for most allele size classes. After Bonferroni correction (Rice 1989) only the
136 Lethrus11 locus displayed deviation from Hardy-Weinberg equilibrium (at $p < 0.0033$)

137 probably due to null alleles and only one significant linkage disequilibrium was observed (at
138 $p < 0.00048$), affecting loci Lethrus01 and Lethrus05.

139

140 The primers were also tested on DNA of 2-5 individuals of four closely related species:
141 *Lethrus bituberculatus*, *L. scoparius*, *L. strymonensis* and *L. perun* and on DNA of six other
142 Coleopteran species: *Copris hispanus*, *Geotrupes stercorarius*, *Melolontha melolontha*,
143 *Onthophagus taurus*, *Oryctes nasicornis* and *Protaetia affinis* in order to investigate the
144 primer pairs' effectiveness in other taxa. Out of the 15 loci 13 showed cross-amplification,
145 and amplifications were successful predominantly in *Lethrus* species (Table 2). The results
146 showed that most of our markers are specific for *Lethrus* species, two of them for *L. apterus*
147 expressly.

148

149 This newly developed microsatellite marker set will allow us to examine the relationship of
150 environmental factors, behaviour, and reproductive biology of *Lethrus apterus* with its
151 genetic structure in a new aspect.

152

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- 218

Table 1 Characteristics of 15 microsatellite loci in *Lethrus apterus*. Values based on the analyses of 45 individuals.

Locus	Primer sequence (5'-3')	Repeat motif	Dye	Mix	T _a	N	Size (bp)	N _a	H _o	H _e	HWE	GenBank
Lethrus01	F: GCACAAAGACGTTATTACGAG R: ATTTTCGTCCATTGTTTGCG	(GT) ₈	FAM	2	60	45	148-150	2	0.289	0.401	0.060	KJ934622
Lethrus02	F: GTAACGTTTGATTTTCCACACG R: GTRGTGATGGATAAGAACAGAGC	(AAT) ₅	VIC	2	60	44	98-101	2	0.386	0.407	0.739	KJ934623
Lethrus03	F: TTCAAATGGGTCATTGATGAAA R: ATGTATAATGGACACACTTATCTG	(AAT) ₅	PET	1	60	45	150-153	2	0.489	0.500	0.884	KJ934624
Lethrus04	F: CGTTTTGACAATAAACCTGC R: GATTGTGTTGCTATCCATGA	(CT) ₉	NED	2	60	45	155-171	5	0.578	0.698	0.004	KJ934625
Lethrus05	F: CGCACAAAGACGTTATTACG R: TTTTCGTCCATTGTTTGCG	(GT) ₈	VIC	1	60	45	149-151	2	0.289	0.401	0.060	KJ934626
Lethrus06	F: TGACCGTATCACCTCCAA R: ACTTGCTGTTTCTAAGTAGCG	(GT) ₈	FAM	1	60	45	189-195	2	0.444	0.480	0.619	KJ934627
Lethrus07	F: GGTTAAATATGGACGAACG R: CCGTAAATCATAACAAGCG	(GT) ₈	NED	1	60	45	165-169	3	0.289	0.363	0.469	KJ934628
Lethrus10	F: GTTTATTAACAATACGCAAACC R: GTTCCTGTTTCCTTATAGTTGG	(CT) ₁₇	FAM	2	60	44	185-197	4	0.455	0.504	0.966	KJ934629
Lethrus11	F: TCCCGTTGTTACTACTTTTCG R: ATGAGGCTGGGAATGGTC	(CT) ₁₀ -TT- (CT) ₁₀	NED	1	60	45	230-238	4	0.511	0.663	0.000 *	KJ934630
Lethrus13	F: AAGATCGCAAATCAATGTCG R: AGGTTTGCGACTTCTTGG	(AAT) ₈	NED	2	60	42	258-261	3	0.262	0.368	0.083	KJ934631
Lethrus14	F: CGAGATGACAAAAATTGTTCC R: TACAAACCAAGAGCCAATCC	(GT) ₇	FAM	1	60	45	366	1	monomorphic			KJ934632
Lethrus15	F: AGTTGAATGTACCGATGACG	(GT) ₁₁ -A-	FAM	1	60	45	259-265	3	0.178	0.201	0.665	KJ934633

	R: GTAACATATGTGTGTTGCAAGC	(GT) ₂ -CA-GT																
Lethrus16	F: GTTCTCATTATTCTAGTGAGC	(GT) ₂ -TT-	PET	1	60	45	324-352	9	0.422	0.446	0.429							KJ934634
	R: TACACGCACAAATCACACG	(GT) ₁₈																
Lethrus17	F: CGTGTAATGACGTGAGC	(GT) ₈	VIC	2	60	45	187-191	2	0.511	0.475	0.613							KJ934635
	R: CCGACTTCCTTATAGACAGG																	
Lethrus19	F: GATTATGTACTAAGGTCAGC	AAT-A-	PET	2	60	41	343-346	2	0.293	0.369	0.186							KJ934636
	R: GCATAGTTCGTTTAGATACG	(AAT) ₇																

221 Dye -- fluorescent dye label, Mix -- the serial number of multiplexed microsatellite sets, T_a -- optimal annealing temperature (°C), N -- number of
 222 individuals from the 45 in which the locus amplified, N_a -- number of alleles per locus, H_o -- observed heterozygosity, H_e -- expected
 223 heterozygosity, HWE -- exact p-value for Hardy-Weinberg equilibrium test (asterisk indicate a significant deviation from Hardy-Weinberg
 224 equilibrium, p<0.0033 after Bonferroni correction).

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Table 2 Cross-amplification of *Lethrus apterus* microsatellite loci in four species of the genus *Lethrus* and six other Coleopteran species.

Species	N	L1	L2	L3	L4	L5	L6	L7	L10	L11	L13	L14	L15	L16	L17	L19
<i>Copris hispanus</i> (Linnaeus, 1764)	4	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-
<i>Geotrupes stercorarius</i> (Linnaeus, 1758)	2	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-
<i>Oryctes nasicornis</i> (Linnaeus, 1758)	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Protaetia affinis</i> (Andersch, 1797)	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Melolontha melolontha</i> (Linnaeus, 1758)	5	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-
<i>Onthophagus taurus</i> (Schreber, 1759)	5	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-
<i>Lethrus scoparius</i> Fischer von Waldheim, 1822	4	1	-	4	-	3	-	-	-	-	4	-	4	-	-	-
<i>Lethrus strymonensis</i> Král & Hillert, 2013	5	5	4	5	5	5	5	-	5	2	5	3	5	-	4	3
<i>Lethrus bituberculatus</i> Ballion, 1870	5	3	-	3	-	3	-	-	-	1	3	-	5	-	4	-
<i>Lethrus perun</i> Král & Hillert, 2013	5	5	5	5	5	5	5	-	4	5	5	5	5	-	5	4

228 N -- number of individuals tested, L1-L19 -- abbreviations of loci's names (*Lethrus*01-*Lethrus*19 respectively) and numbers represent the number
 229 of individuals in which the locus amplified (dash means that the locus did not amplified).