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

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Short title: Microsatellites in *Lethrus apterus*

Abstract

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

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

1. Introduction

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

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

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

2. Materials and methods

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

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

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

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

3. Results and discussion

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

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

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

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

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219 Table 1 Characteristics of 15 microsatellite loci in *Lethrus apterus*. Values based on the analyses of 45 individuals.
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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	$(GT)_8$	FAM	2	60	45	148-150	2	0.289	0.401	0.060	KJ934622
	R: ATTTTCGTCCATTGTTTGCG											
Lethrus02	F: GTAACGTTTGATTTTCCACACG	$(AAT)_5$	VIC	2	60	44	98-101	2	0.386	0.407	0.739	KJ934623
	R: GTRGTGATGGATAAGAACAGAGC											
Lethrus03	F: TTCAAATGGGTCATTGATGAAA	$(AAT)_5$	PET	1	60	45	150-153	2	0.489	0.500	0.884	KJ934624
	R: ATGTATAATGGACACACTTATCTG											
Lethrus04	F: CGTTTTGACAATAAAACCTGC	$(CT)_9$	NED	2	60	45	155-171	5	0.578	0.698	0.004	KJ934625
	R: GATTGTGTTGCTATCCATGA											
Lethrus05	F: CGCACAAAGACGTTATTACG	$(GT)_8$	VIC	1	60	45	149-151	2	0.289	0.401	0.060	KJ934626
	R: TTTTCGTCCATTGTTTGCG											
Lethrus06	F: TGACCGTATCACCTCCAA	$(GT)_8$	FAM	1	60	45	189-195	2	0.444	0.480	0.619	KJ934627
	R: ACTTGCTGTTTCTAAGTAGCG											
Lethrus07	F: GGTTAAATATGGACGAACG	$(GT)_8$	NED	1	60	45	165-169	3	0.289	0.363	0.469	KJ934628
	R: CCGTAAATCATAACAAGCG											
Lethrus10	F: GTTTATTAACAATACGCAAACC	$(CT)_{17}$	FAM	2	60	44	185-197	4	0.455	0.504	0.966	KJ934629
	R: GTTCCTGTTCCTTATAGTTGG											
Lethrus11	F: TCCCGTTGTTACTACTTTCG	$(CT)_{10}$-TT-	NED	1	60	45	230-238	4	0.511	0.663	0.000 *	KJ934630
	R: ATGAGGCTGGGAATGGTC	$(CT)_{10}$										
Lethrus13	F: AAGATCGCAAATCAATGTCG	$(AAT)_8$	NED	2	60	42	258-261	3	0.262	0.368	0.083	KJ934631
	R: AGGTTTGCGACTTCTTGG											
Lethrus14	F: CGAGATGACAAAAATTGTTCC	$(GT)_7$	FAM	1	60	45	366	1	monomorphic			KJ934632
	R: TACAAACCAAGAGCCAATCC											
Lethrus15	F: AGTTGAATGTACCGATGACG	$(GT)_{11}$-A-	FAM	1	60	45	259-265	3	0.178	0.201	0.665	KJ934633

	R: GTAACTATGTGTGTTGCAAGC	$(GT)_2$-CA-GT										
Lethrus16	F: GTTCTCATTTATTCTAGTGAGC	$(GT)_2$-TT-	PET	1	60	45	324-352	9	0.422	0.446	0.429	KJ934634
	R: TACACGCACAAATCACACG	$(GT)_{18}$										
Lethrus17	F: CGTGTAAATGACGTGAGC	$(GT)_8$	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	$(AAT)_7$										

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

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
Copris hispanus (Linnaeus, 1764)	4	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-
Geotrupes stercorarius (Linnaeus, 1758)	2	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-
Oryctes nasicornis (Linnaeus, 1758)	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Protaetia affinis (Andersch, 1797)	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Melolontha melolontha (Linnaeus, 1758)	5	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-
Onthophagus taurus (Schreber, 1759)	5	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-
Lethrus scoparius Fischer von Waldheim, 1822	4	1	-	4	-	3	-	-	-	-	4	-	4	-	-	-
Lethrus strymonensis Král & Hillert, 2013	5	5	4	5	5	5	5	-	5	2	5	3	5	-	4	3
Lethrus bituberculatus Ballion, 1870	5	3	-	3	-	3	-	-	-	1	3	-	5	-	4	-
Lethrus perun Král & Hillert, 2013	5	5	5	5	5	5	5	-	4	5	5	5	5	-	5	4

N -- number of individuals tested, L1-L19 -- abbreviations of loci's names (Lethrus01-Lethrus19 respectively) and numbers represent the number of individuals in which the locus amplified (dash means that the locus did not amplified).