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TECHNICAL NOTE

Development of microsatellite markers for the wetland grasshopper *Stethophyma grossum*

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Abstract *Stethophyma grossum* is a threatened Eurosiberian grasshopper species. Since it is bound to wetlands, *S. grossum* is often used as indicator for extensive wet meadows. To study its movement capability and dispersal habitat in landscape genetic analyses, we developed ten polymorphic microsatellite markers, making use of next generation sequencing. Markers were tested on 75 individuals collected in five populations from Switzerland. We found four to 18 alleles per locus. Observed and expected heterozygosities varied between 0.215–0.893 and 0.397–0.831, respectively. One marker seems to be sex-chromosome X-linked and one showed high null allele frequencies, a phenomenon generally detected in microsatellite studies on grasshoppers.

Keywords Conservation · Next generation sequencing · Orthoptera · SSRs · *Stethophyma grossum*

The large marsh grasshopper *Stethophyma grossum* (Linnaeus, 1758; Acrididae) is distributed throughout Europe and Siberia and is strictly bound to wetlands (Baur et al. 2006). Therefore, *S. grossum* is often used as an indicator species for extensively managed wet meadows. Due to the decline and fragmentation of wet meadows during the last decades, many populations of *S. grossum* have become spatially isolated or extinct. In Switzerland, the species is currently red listed as vulnerable (Monnerat et al. 2007). To plan effective conservation management strategies for this threatened grasshopper, knowledge on its movement capabilities and dispersal habitat is required. Landscape genetic approaches can help investigating these issues (Segelbacher et al. 2010). Microsatellites have widely been used in these analyses, but no markers had been available for species of the *Stethophyma* genus. Here, we describe the development of ten polymorphic microsatellite markers for *S. grossum*, making use of next generation sequencing (Csencsics et al. 2010).

DNA of one individual (tibia and tarsus) was extracted and shotgun sequenced (1/16th run) at Microsynth AG (Balgach, Switzerland) using a Roche 454 Genome Sequencer FLX with the Titanium sequencing kit XLR 70. Details can be found in Margulies et al. (2005). We obtained a total of 52,693 reads with an average read length of 355 bp. MSATCOMMANDER 0.8.2 (Faircloth 2008) was used to search for di-, tri- and tetra-nucleotides in unassembled sequences. We limited the search to dinucleotides of at least eight, trinucleotides of at least ten and tetranucleotides of at least six repeats. A large number of repeats (1,059) matched with these criteria, and primers were designed for 121 repeats using PRIMER3 (Rozen and Skaletsky 2000). CLC SEQUENCE VIEWER 6.0.2 (CLC bio Anhus, Denmark) was used to check the 121 sequences for possible duplicate microsatellite loci. Three sequence pairs were matching and we thus removed all duplicates, resulting in 118 repeats (84 di-, 21 tri-, 22 tetra-nucleotides). Finally, fifty sequences were chosen for further testing. DNA was extracted from grasshopper legs (tibia and tarsus), which were stored in 100% ethanol at −20°C in the dark after collecting. Samples were first lyophilised, ground with an MM300 mixer mill for 4 min (Retsch) and then extracted using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol including the changes recommended for insects. As tibiae of mid legs were only small, half of the recommended quantities of

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extraction chemicals were taken, and elution was performed twice with each 75 µl elution buffer. Seven samples, all collected in different populations in central Switzerland, were used for preliminary testing of all 50 microsatellite markers. Microsatellite amplification was performed with the M13 method (Schuelke 2000). PCR reaction volumes (10 µl) contained approximately 0.7 ng of diluted genomic DNA, 5 µl 2× Multiplex Mix (Qiagen), ddH_2O, 0.01 µM of forward primer and 0.15 µM of each reverse and universal FAM-labelled M13 primers (Schuelke 2000). Amplifications were run on ABI Verity thermocyclers (Applied Biosystems) with polymerase activation at 94°C for 15 min, followed by 30 cycles of 94°C for 30 s, 60°C for 90 s and 72°C for 60 s, and an additional eight cycles of 94°C for 30 s, 53°C for 90 s and 72°C for 60 s, followed by a final elongation step at 72°C for 30 min. Fragments were analysed on an ABI 3130 sequencer (Applied Biosystems) and electropherograms were scored with GENEMAPPER 3.7 (Applied Biosystems). Only 10 out of the 50 markers tested were polymorphic and showed clear and reproducible patterns. We tested these ten markers on five populations with 15 individuals each, collected in central Switzerland in an area of about 50 km^2. Calculations of number of alleles per locus, observed and expected heterozygosities and a test for linkage disequilibrium were performed in FSTAT 2.9.3.2. (Goudet 1995). Departure from Hardy–Weinberg equilibrium was tested in GENEPOP 4.0.10. (Raymond and Rousset 1995) using Fisher's exact test. Frequencies of null alleles were estimated for each marker with FREENA (Chapuis and Estoup 2007).

For the five populations tested, we found four to eighteen alleles per locus (Table 1). Observed and expected heterozygosities varied between 0.215–0.893 and 0.397–0.831, respectively. Linkage disequilibrium was detected for three primer combinations ($P \leq 0.05$), but they were no longer significant after Bonferroni correction ($P \geq 0.001$). Deviations from Hardy–Weinberg equilibrium were found for locus Sgr07 and Sgr29. Locus Sgr29 seemed to be sex-chromosome X-linked. At this locus, males were all homozygous while approximately 50% of females were heterozygous. Females of *S. grossum* are XX and males X0 (Perry and Jones 1974). For locus Sgr07, however, deviations from Hardy–Weinberg equilibrium were probably caused by the presence of null alleles (at a frequency ≥0.2; Table 1). High frequencies of null alleles are often found in orthopteran species (e.g. Ustinova et al. 2006; Chapuis et al.

Table 1 Characteristics of ten microsatellite loci developed for *Stethophyma grossum* and tested for a total of 75 individuals collected from five locations in Switzerland

Locus	Primer sequences (5′–3′)	Repeat motif	N_A	Size range (bp)	H_o	H_e	HWE test	Null allele frequency	GenBank accession number
Sgr07	F: TATGCACAGGGATGGGAGC	$(ATT)_{12}$	10	289–319	0.307	0.736	<0.05	0.232	JQ026312
	R: TTGTCCTCGTCACATGCAG								
Sgr10	F: CTTTCCCGAAGCCCACAAG	$(AAT)_{15}$	7	158–203	0.667	0.676	0.431	0.029	JQ026313
	R: TGCAACAAGTCTGCTTACCG								
Sgr13	F: TGATGGCTGAACATCCCGC	$(AAT)_{12}$	11	298–342	0.710	0.726	0.332	0.029	JQ026314
	R: CCAAATCCGCTTCACAACG								
Sgr14	F: TTCCACAGAAAGGTGGGTC	$(AC)_{12}$	16	264–344	0.597	0.673	0.087	0.047	JQ026315
	R: AGTTTGCATATCACCCGTTTG								
Sgr15	F: CCACCGTGTGATACTTGGC	$(AG)_8$	12	163–203	0.607	0.656	0.479	0.034	JQ026316
	R: CGGCGTTTCCCGTCTATTTAC								
Sgr19	F: GATAGCTTTCAGGTTAGTTGGG	$(ATT)_{11}$	8	229–259	0.779	0.761	0.485	0.014	JQ026317
	R: TCCTCCGACCTTCGAACTG								
Sgr29	F: TCAAGTCCCTATCAAGCACG	$(AC)_8$	7	312–324	0.215	0.538	<0.05*	–	JQ026318
	R: ACGCAGTTCGGTGTTATCG								
Sgr38	F: GGCCAATGTATGACGAGGG	$(GT)_8$	18	186–290	0.893	0.831	0.971	0.002	JQ026319
	R: CTGATGACCTCGCTGTTTGG								
Sgr40	F: TGTGACTTTATTTATCTGGTGGCTC	$(AAAT)_6$	4	304–316	0.283	0.397	0.075	0.080	JQ026320
	R: TATATCCGACGTGGCTCCC								
Sgr45	F: CTAGCCTGGTCATCAGTCCC	$(AC)_8$	7	282–300	0.573	0.654	0.464	0.052	JQ026321
	R: AGAGAGACACGCTATGTGC								

* X-linked marker primer sequence (F: forward, R: reverse), number of alleles (N_A), observed heterozygosity (H_o), expected heterozygosity (H_e) and *P* value of test for Hardy–Weinberg equilibrium (HWE)

2008; Blanchet et al. 2010). Thus, it is important to carefully check loci for the presence of null alleles in each study and to exclude loci with high frequencies of null alleles.

Of the presented microsatellites, eight (excluding Sgr07 and Sgr29 for previously mentioned reasons) will serve as markers for a landscape genetic study focussing on the dispersal of *S. grossum* in a fragmented landscape. Results will help to develop guidelines for the conservation management of this grasshopper species.

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