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PERMANENT GENETIC RESOURCES

Eleven polymorphic microsatellite markers for *Oedaleus decorus* (Orthoptera, Acrididae), an endangered grasshopper in Central Europe

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Abstract

We isolated and characterized 11 microsatellite loci in the grasshopper *Oedaleus decorus* (Orthoptera: Acrididae), an endangered species in Central Europe. Polymorphism was studied from two populations, one out of two populations known from Switzerland (n = 20 individuals) and one site from south of France (n = 20). The number of alleles and the expected heterozygosity ranged from five to 12 and from 0.559 to 0.898, respectively, in the Swiss population, and from 14 to 23 and from 0.895 to 0.974, respectively, in the French population. These microsatellite markers are suitable for further conservation genetic studies of *O. decorus*.

Keywords: microsatellites, multiplex PCR, *Oedaleus decorus*, Orthoptera

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Oedaleus decorus is an Orthopteran of the Acrididae family. This grasshopper has a primarily circum-Mediterranean distribution, and occurs in dry, very open steppe-like habitat with surfaces of bare ground. As other Mediterranean Orthopteran species, *O. decorus* has probably used sandy and gravely steppe-like habitats for expanding its range northwards after Quaternary glaciation recessions (Central Asia, Sergeev 1998). In some northern parts of its distribution, populations of *O. decorus* have dramatically declined over the last century. In Switzerland, populations of *O. decorus* were considered as extinct since the 1950s before being rediscovered in the early 1990s in the Canton of Valais (Switzerland; R. Arlettaz, unpublished), which is the driest region in the Central European Alps. The species is now considered as highly endangered in Switzerland (Nadig & Thorens 1994). A likely reason for this decline may be habitat modification by human activities. Extended areas of natural steppes on the south-exposed slopes have been transformed into vineyards, while grazing by domestic ungulates has ceased in most remaining patches (Arlettaz 1990). From a conservation perspective, the application of population genetic approaches might be useful to, first, determine if the remaining Swiss populations of *O. decorus* are still interconnected — through migration — among each other and with the more abundant populations in southern Europe; second, where are the genetically most suitable populations for possible reinforcements of the threatened Swiss populations through translocations. We present here the development and characterization in populations of 11 polymorphic microsatellite loci for *O. decorus*.

Microsatellite loci were developed for *O. decorus* using an enriched library protocol, slightly modified (Loiseau *et al*. 2008). Genomic DNA was extracted from muscles of a leg of one individual using a standard phenol–chloroform method (Sambrook *et al*. 1989). Total genomic DNA was digested using the *Rsa*1 restriction enzyme. A 600- to 1000-bp fragment of the digested DNA was selected, purified and ligated to *Mlu*I oligo adapters *Rsa*21 and 5′-phosphorylated *Rsa*25 (Edwards *et al*. 1996). Biotinylated oligo probes $(TC)_{10}$ and $(TG)_{10}$ were hybridized to the ligated DNA and captured using streptavidin magnetic particles (Promega). Oligo adapters *Rsa*21 was used as primer to perform polymerase chain reactions (PCR) on the microsatellite-enriched eluates (initial denaturation at 95 C, 1 min, 25 cycles with denaturation at 94 C, 40 s, annealing at 60 C, 1 min,

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Table 1 Characteristics of the 11 microsatellite loci developed for *Oedaleus decorus*

	Locus	Genbank Accession no.	Primer sequences	Repeat motif	Primer (μM)	Dye	No. of alleles		Size range (bp)		H_O		H_E		HW test		$\hat{r}_D$	
							Ga	Cr	Ga	Cr	Ga	Cr	Ga	Cr	Ga	Cr	Ga	Cr
PCR 1	OD2	EU604818	F: GAAACACGCCACGGATTAGT	$(TG)_{11}$-$(AG)_6$	0.4	ATTO550	11	22	216–265	214–264	0.72	0.69	0.90	0.97	0.106	**< 0.001**	0.07	0.13
			R: CTCACCCTAACCAGCCAAAC		0.4													
	OD4	EU604819	F: GTAATCTGCCGGATGGATTC	$(AG)_{16}$	0.2	Yakima yellow	10	23	126–246	132–196	0.83	0.88	0.89	0.97	0.867	0.062	0.00	0.03
			R: GTAGAAACGCCCCAATCACT		0.2													
	OD6	EU604820	F: CTTGCACAATGAATCTCGTGA	$(TG)_{18}$-$(AG)_7$	0.2	Yakima yellow	8	14	286–326	288–344	0.19	0.44	0.84	0.89	**< 0.001**	**< 0.001**	0.34	0.22
			R: GCACACCGTTCGCTACACTA		0.2													
	OD7	EU604821	F: CCATTGAACCTGCTTACCGT	$(CA)_{26}$	0.4	ATTO565	10	23	161–208	169–228	0.89	0.72	0.85	0.96	0.281	**0.005**	0.00	0.11
			R: CGATTGGTGGGACACAGTTT		0.4													
	OD9	EU604822	F: TTCGCAGACAGCACTTCG	$(GA)_{21}$-$(GT)_{27}$	0.2	ATTO565	10	22	298–368	248–359	0.36	0.50	0.88	0.97	**< 0.001**	**< 0.001**	0.27	0.23
			R: CGATGGTTTCCTGGCAGTAT		0.2													
	OD12	EU604823	F: CGAACTAACTGCTTGGCAAC	$(CT)_{10}$-CCC-$(CT)_9$	0.2	FAM	8	16	251–295	251–296	0.94	0.78	0.83	0.94	0.431	**0.020**	0.00	0.07
			R: GCTGCAACGAGGTTAGAATC		0.2													
PCR 2	OD3	EU604824	F: AGCCACTATCCTCTTCCTGTT	$(CT)_{12}$-AGTC-$(CA)_{19}$	0.2	ATTO550	5	18	277–299	263–321	0.33	0.50	0.56	0.96	**0.018**	**< 0.001**	0.14	0.22
			R: GAGGCGGCAAGAGAGAAT		0.2													
	OD5	EU604825	F: TATTGCGTGAGCGCGTGT	$(GA)_{25}$-GG-$(GA)_4$	0.2	Yakima yellow	8	19	224–252	202–278	0.50	0.67	0.81	0.96	**< 0.001**	**< 0.001**	0.16	0.14
			R: CTGTATGTCACTGGAAGTGCCTC		0.2													
	OD8	EU604826	F: TCCATCTTTTCGGCACACAG	$(TG)_5$-TC-$(TG)_{14}$	0.2	ATTO565	8	23	253–275	245–333	0.89	0.89	0.79	0.97	0.884	0.102	0.00	0.03
			R: GGAGCTTCCGGATTATGTTG		0.2													
	OD18	EU604827	F: TTCCTGCCTTGACTCCTGAT	$(TC)_{20}$	0.2	FAM	10	20	136–184	126–186	0.94	0.89	0.90	0.95	0.931	0.486	0.00	0.00
			R: CTCCTCAGATACGACTTGACGA		0.2													
	OD31	EU604828	F: GCATGCAAAGCACATACCAC	$(TC)_{24}$	0.2	FAM	12	21	219–297	211–287	0.83	0.89	0.87	0.97	0.800	0.131	0.00	0.01
			R: GATTCCTACCTGATGTCTCAACA		0.2													

Number of alleles, size range, observed heterozygosities (H_O), unbiased estimates of heterozygosities (H_E) were estimated for each locus from 20 individuals of both the Swiss (Ga for Gampel, Valais) and French (Cr for Crau, Var) populations. HW test indicates the probability associated with the rejection of the Hardy–Weinberg equilibrium; significant probabilities, after false discovery rate correction (Benjamini & Hochberg 1995) are indicated in bold. The mean null allele frequency ($\hat{r}_D$) was computed, for each locus, using the program FreeNA.

extension at 72 C, 2 min). The amplification products were purified (QIAquick PCR Purification Kit, QIAGEN) and ligated into a plasmid vector (pGEM-T, Promega), transformed into JM109 competent cells (Promega) and plated onto Luria-Bertani agar medium containing ampicillin (50 mg/L) for selection and X-Gal/IPTG for identification of recombinant plasmids by means of blue-white screening. Positive transformed cells were boiled and amplified using the plasmid's primers T7 and SP6 and $(TC)_{10}$ or $(TG)_{10}$ primer.

We screened 644 positive transformed cells and we obtained 371 positive transformants (57.6%). Among them, 212 were sequenced. We used the program MicroFamily version 1.0 (Meglécz 2006) to investigate the similarity between the flanking regions of microsatellite-containing sequences. We identified 111 unique sequences. We selected 15 sequences for primer design using the program Primer version 2.0 (Rozen & Skaletsky 1996).

Primers were first tested using a single PCR per locus. Eleven from the 15 new isolated markers were polymorphic and provided good quality amplification products. For each of these 11 loci, the forward primer was 5′-end labelled with a fluorescent dye (i.e. ATTO550, Yakima Yellow, ATTO565 or FAM) from the company Microsynth (Table 1). The 11 loci were amplified in two different multiplex PCRs conducted in 10- L reaction volume containing QIAGEN Multiplex PCR Master Mix (1×) (including *Taq*, dNTPs and 3 mM of $MgCl_2$ as final concentration), 0.2 M or 0.4 M of each primer, 20 ng of genomic DNA and nucleic acid-free water. The two multiplex PCRs were performed on a GeneAmp thermocycler (Applied Biosystems) as following: initial activation step at 94 C, 15 min, 30 cycles with denaturation at 94 C, 30 s, annealing at 60 C, 90 s, extension at 72 C, 60 s and final extension step at 60 C, 30 min. The PCR products were diluted threefold, and then 3 L of the diluted PCR were mixed with 0.25 L of GeneScan 500 LIZ standard and 9.75 L of Hidi Formamide (Applied Biosystems). The samples were separated and detected on an ABI 3130 automated sequencer and analysed using GeneMapper version 3.7 (Applied Biosystems).

As a preliminary assessment of the polymorphism at the 11 loci, we genotyped 20 individual hindlegs collected at Gampel (Valais, Switzerland) and 20 individual hindlegs collected on the plain of Crau (south of France). Computation of observed and expected heterozygosities, tests for genotypic linkage disequilibrium between all pairs of loci, conformation to Hardy–Weinberg equilibrium for each locus, and tests for genotypic differentiation over all loci were performed using GenePop 3.4 (Raymond & Rousset 1995). The numbers of alleles per locus and the expected heterozygosities ranged from five to 12 and from 0.559 to 0.898, respectively, in the Swiss population and from 14 to 23 and from 0.895 to 0.974, respectively, in the French population (Table 1). There was no significant linkage disequilibrium for all pairs of loci. We found significant departure from Hardy–Weinberg equilibrium in the Swiss and French populations for four and seven loci, respectively (Table 1). All loci in Hardy–Weinberg disequilibrium in the Swiss and French populations showed an excess of homozygotes evenly distributed across most allele size classes, suggesting the presence of null alleles at these loci (Micro-Checker; van Oosterhout *et al*. 2004). Estimated frequencies of null alleles per locus were moderate, ranging from 0.000 to 0.344 (mean over loci = 0.090) in the Swiss population and from 0.004 to 0.229 (mean over loci = 0.109) in the French population (Table 1; FreeNA; Chapuis & Estoup 2007). High prevalence of null alleles at microsatellite loci have been observed in Orthopteran species, especially in the Oedipodinae subfamily to which belongs *O. decorus* (Chapuis *et al*. 2005). New methods are now available to analyse microsatellite data in the presence of null alleles. The program FreeNA allows computing unbiased F_{ST} estimates corrected for the presence of null alleles [$F_{ST}^{(ENA)}$, Chapuis & Estoup 2007]. In the present study, the Swiss and French populations were significantly differentiated ($P < 0.001$) and the corrected $F_{ST}^{(ENA)}$ value was 0.064, $CI_{95\%}$ (0.036–0.094).

These 11 newly characterized microsatellite loci will be useful to conduct population genetic studies on *O. decorus*. This will enable to investigate whether the lower genetic diversity in the Swiss populations is due to historic founder effects during northward range expansion or to more recent demographic events such as population size reduction and fragmentation following habitat alterations in Valais.

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