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TECHNICAL NOTE

Characterization of ten polymorphic microsatellite markers for the protected Spanish moon moth *Graellsia isabelae* (Lepidoptera: Saturniidae)

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Abstract Ten polymorphic microsatellite loci were developed for *Graellsia isabelae*. Polymorphism was assessed for 20 individuals from a Spanish population (Els-Ports-de-Beseit, Catalonia) and 39 more individuals from one population in the French Alps and six other Spanish localities. Overall, the number of alleles per locus ranged from 5 to 24. Els-Ports-de-Beseit showed an average number of alleles per locus of 9.80 (SD = 4.32), observed heterozygosity was 0.71 (SD = 0.226), and expected heterozygosity was 0.788 (SD = 0.146). Genotypic frequencies conformed to Hardy–Weinberg equilibrium at the Catalonian population, and no evidence for linkage disequilibrium was observed. Multilocus genotypes resulting from this set of markers will be useful to determine genetic diversity and differentiation within and among populations of this highly protected moth. Several loci amplified and resulted polymorphic in two related species: two loci in *Actias neidhoeferi*, and three loci in *A. luna*.

Keywords *Graellsia isabelae* · *Actias* · Microsatellites · Conservation genetics · Lepidoptera · Saturniidae

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The Spanish moon moth *Graellsia isabelae* (Graells, 1849) (Lepidoptera: Saturniidae) is one of the few European insects protected at both national and international level. It is considered as data deficient by the International Unit for Conservation of Nature (IUCN 2008). There are several conservation issues that require a set of appropriate DNA markers to be addressed. For instance, whether *G. isabelae galliaegloria* (occurring in France) derives from a human introduction of Iberian specimens during the 19th or 20th century or it is a French native species (revised by Fernández Vidal 1992). Another interesting question is if both demographic history and genetic diversity of the different populations justify their inclusion into the French and Spanish red lists.

We aimed at identifying an appropriate set of genetic markers to investigate the genetic diversity and structure showed by this species all throughout its distribution area (i.e. Spain, France, and Switzerland). A high level of variability of these markers would allow other investigations, such as a survey on the genetic diversity of the ex-situ colony kept at INRA-Orléans or parentage analyses. Here we described the isolation and characterization of ten polymorphic microsatellite markers in *G. isabelae* and indicated their effectiveness in identifying patterns of genetic diversity. For this, we genotyped 20 individuals collected in one Spanish population (Els-Ports-de-Beseit, Tarragona, Catalonia). Additionally, we genotyped 39 specimens of *G. isabelae* from one population in the French Alps and six other Spanish localities (Table 1). Tissue was non-lethally collected (Vila et al. 2009) in May 2007 and 2008.

Genomic DNA was extracted from wing and leg samples of *G. isabelae* using the Qiagen's DNeasy® Tissue Kit. An enriched library was made by Ecogenics GmbH (Zurich, Switzerland) from size selected genomic DNA ligated

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Table 1 Details of the sampling sites and specimens collected

Locality	Region, country	Latitude	Longitude	*n*	Sex	*A*	H_E	F_{IS}
Els-Ports-de-Beseit	Catalonia, S	40°47′30″N	0°18′46″E	20	♂	9.8 ± 4.32	0.788 ± 0.146	0.102
L'Ange Gardien	Hautes-Alpes, F	44°44′14″N	6°46′11″E	10	♂	1.7 ± 0.95	0.183 ± 0.232	0.073[a]
INRA-Orléans	Orléans, F	47°49′43″N	1°54′50″E	15	♀	1.9 ± 0.99	0.169 ± 0.208	−0.152[b]
Cazorla	Andalusia, S	37°54′07″N	2°56′18″W	7	♀	4.6 ± 2.27	0.616 ± 0.291	0.103[c]
Albanyà	Catalonia, S	42°18′33″N	2°42′15″E	1	♀	–	–	–
Guillimona	Andalusia, S	38°03′16″N	2°33′12″W	1	♀	–	–	–
Segura	Andalusia, S	38°14′17″N	2°36′47″W	1	♀	–	–	–
Mundo	Castilla-la-Mancha, S	38°27′21″N	2°26′18″W	1	♀	–	–	–
María	Andalusia, S	37°42′32″N	2°03′54″W	1	♀	–	–	–

Sample sizes (*n*), mean number of alleles per locus per population (*A*), expected heterozygosity (H_E) (±SD) and inbreeding coefficient (F_{IS}). Individuals from INRA-Orléans (ex situ conservation programme) emerged as adults in 2007. (S) Spain, (F) France. No population sample ($n \geq 7$) deviated from HWE

[a] Loci GRAISA6, GRAISA15, GRAISA17, GRAISA18, and GRAISA23 resulted monomorphic

[b] Loci GRAISA6, GRAISA15, GRAISA18, and GRAISA23 resulted monomorphic

[c] Locus GRAISA26 monomorphic

into SAULA/SAULB-linker (Armour et al. 1994) and enriched by magnetic bead selection with biotin-labelled $(GTAT)_7$, $(GATA)_7$, $(GT)_{13}$ and $(CT)_{13}$ oligonucleotide repeats (Gautschi et al. 2000a, b). Of 739 recombinant colonies screened, 336 gave a positive signal after hybridization. Plasmids from 214 positive clones were sequenced and primers were designed for 29 microsatellite inserts, of which 23 were tested for polymorphism. Ten microsatellite loci were reliably scored and polymorphic.

Markers were amplified through polymerase chain reaction (PCR). PCR conditions were designed for multiplexing of up to four loci per reaction. Each of the three multiplex PCR amplifications was optimised to be performed in a 10 µl reaction volume. Forward primers were fluorescent-labelled with NED, HEX or 6-FAM dyes (Applied Biosystems, ABI) (Table 2).

Polymerase chain reaction protocol Multiplex 1: 10 ng of DNA, 0.5 U HotStar Taq DNA polymerase, 1× PCR buffer with 1.5 mM $MgCl_2$, 200 µM of each dNTP (Qiagen HotStar Taq Mastermix), 0.3 µM of forward and reverse primers each. Thermotreatment was as follows: 35 cycles with 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Before the first cycle, a prolonged denaturation step (95°C for 15 min) was included and the last cycle was followed by a 30 min extension at 72°C. PCR protocol for Multiplex 2 only differed from Multiplex 1 in the annealing temperature: 56°C. PCR protocol for Multiplex 3 differed from Multiplex 1 in the denaturing, annealing, and extension temperatures and times: 94°C for 30 s, 56°C for 90 s, and 72°C for 60 s. Multiplex 3 ended with a final extension of 30 min at 60°C.

Amplified PCR products (1.2 µl) were mixed with 16 µl formamide containing GENESCAN-500 (ROX) Size Standard (ABI), and the genotype was determined on an ABI Prism 3100 Genetic Analyzer using GeneScan Analysis® Software 3.7 and Genotyper® 3.7 Software (ABI). Dilutions of the labelled and unlabelled primers prior to the PCR step were optimised, so that the PCR product may be loaded on the sequencer with no need for further dilution steps. Details about primer dilutions are available from the authors upon request.

DNA sequences of these ten polymorphic microsatellite markers were confirmed to be unique using BLASTN 2.219 (Zhang et al. 2000). A total of 59 individuals of *G. isabelae* were genotyped for each of the ten loci. Numbers of alleles per locus, expected and observed heterozygosities were estimated using MICROSATELLITE TOOLKIT (Park 2001). Analyses of Hardy–Weinberg (HW) segregations and tests for linkage disequilibrium were computed with FSTAT 2.9.3.2 (Goudet 2001). The frequency of null alleles was calculated with MICRO-CHECKER (Van Oosterhout et al. 2004). We estimated the paternity exclusion probability of this set of markers as implemented in IDENTITY4 (Wagner and Sefc 1999).

All loci were successfully amplified in the 59 *G. isabelae* individuals tested, with the exception of locus GRAISA23 in one specimen (ALB4). The average number of alleles per locus found in population Els-Ports-de-Beseit ($n = 20$) was 9.8 (SD = 4.32). Average observed heterozygosity was 0.71 (SD = 0.226), whereas average expected heterozygosity reached 0.788 (SD = 0.146) (Table 2). The polymorphism information content (PIC) value for that population ranged from 0.392 (locus GRAISA26, 4 alleles) to 0.9 (locus GRAISA11, 15 alleles). Two loci (GRAISA03 and GRAISA18) showed homozygote excess and deviated significantly from HW expectations after correcting for

Table 2 Characteristics of ten polymorphic microsatellite loci developed for *Graellsia isabelae*

Locus	GenBank accession	Repeat motif	Primer sequence (5′–3′)	*Graellsia isabelae*							*Actias neidhoeferi*		*Actias luna*	
				MP	Size	*A*	H_E	H_O	Size′	*A*′	*A*	Size	*A*	Size
GRAISA03	FJ871974	$(AC)_{15}$	F:CGAGGACCCAGAAGATGAAG(6-FAM) R:CATTTTGGCTGCAAATATAACG	1	175–235	16	0.926	0.6[a,b]	175–235	20	1	194	1	198
GRAISA18	FJ871975	$(AC)_{11}$	F:CACACCATACCCCTTATAAATAAAAC(NED) R:CTATTTGTGCGCAATCAAGG	1	115–125	7	0.760	0.2[a,b]	109–133	9	1	140	–	–
GRAISA26	FJ871976	$(GT)_{10}(AT)_5$ $(GT)_3(AT)_2(GT)$	F:TCAGGTTCGCGGGATTAAG(HEX) R:CATATACGCTCAAATGATTGGTTC	1	237–245	4	0.427	0.5	237–247	5	2	243–245	2	243–245
GRAISA06	FJ871977	$(CA)_{17}$	F:TTGTGTTCGCAGTTTACACG(HEX) R:ATGGTTGGAGCGTTGTACG	2	117–161	15	0.906	0.75[b]	117–165	19	1	124	–	–
GRAISA17	FJ871978	$(CA)_{11}$	F:CAGCGTCCATGCAAACTACC(NED) R:GCTAGTGCATTCCTCCTTCG	2	137–151	8	0.863	0.85	137–153	9	–	–	–	–
GRAISA23	FJ871979	$(GT)_{10}(TT)$ $(GT)_3$	F:GATCCGAGAGTTGTACGTGTG(6-FAM) R:TGCCTCTGTATAATGCCTCTATCTC	2	153–173	8	0.792	0.8	153–173	9	–	–	–	–
GRAISA11	FJ871980	$(CA)_5(CC)(CA)_7$ $(GA)(CA)_{14}$	F:TTAAACCACCGGTTAAACACA(HEX) R:CCTCCTGATGAAAGAATGAGC	3	145–207	15	0.931	1	145–207	24	–	–	–	–
GRAISA15	FJ871981	$(TG)_{11}$	F:CGTCGATGACTTCATCTGAATA(6-FAM) R:ATAGCCGTTCCTCGTGAGTC	3	189–199	6	0.772	0.85	189–199	6	1	197	3	187–197
GRAISA21	FJ871982	$(GT)_{10}(GC)(GT)_7$	F:AAAAAGGCATGCAACGAAAC(NED) R:GCTATGCTCATGCATTGTATCC	3	123–175	12	0.758	0.8	123–175	16	2	151–155	1	155
GRAISA25	FJ871983	$(TG)_2(TA)(GT)_{10}$	F:GAACTTAATTGTCGTTACATGCAC(6-FAM) R:ACAAAAGTGCCGTTCGATTC	3	69–91	7	0.747	0.75	69–91	7	1	84	5	71–83
Total						9.8 ± 4.32	0.788 ± 0.146	±0.2262		12.4 ± 6.74				

PCR conditions for each multiplex are specified in the text. Number of alleles per locus (*A*), observed (H_O) and expected (H_E) heterozygosity are reported for population Els-Port-de-Beseit. MP refers to multiplex conditions. *A*′ and Size′ represent number of alleles per locus and size range (bp) of PCR products when analysing 59 individuals from different populations

[a] Significant heterozygote deficiency

[b] Frequency of null alleles >0.05

multiple tests (adjusted *P*-value for 5% nominal level = 0.005) (Table 2). In fact, both of them plus GRAISA06 showed signs of non amplifying alleles at moderate frequencies (0.16, 0.34, and 0.08, respectively) as revealed by MICRO-CHECKER (Van Oosterhout et al. 2004). However, occurrence of null alleles was not detected in other population samples (L'Ange Gardien, $n = 10$; Cazorla, $n = 7$; INRA-Orléans, $n = 15$). Analyses of more populations are needed before drawing further conclusions about the presence of null alleles in those three markers, as loci GRAISA06 and GRAISA18 were monomorphic in L'Ange Gardien and INRA-Orléans (Table 2). We observed no deviations from linkage equilibrium for any locus pair (adjusted *P*-value for 5% nominal level = 0.0011). Total paternity exclusion probability of this set of ten markers was estimated in 0.9999. No locus was found to be sex-linked, as revealed by the presence of heterozygotes in all loci for several of the 29 females analysed.

We tested the usefulness of these markers in two Saturniidae species. For this we genotyped ten individuals of *Actias neidhoeferi* Ong & Yu, 1968 (a Pinaceae-feeding species endemic to Taiwan) and nine specimens of the North American moon moth *Actias luna* (Linnaeus, 1758) collected in Tennessee ($n = 5$) and Wisconsin ($n = 4$). Seven loci amplified in *A. neidhoeferi* and five of them revealed microsatellite product in *A. luna*. Successful annealing temperature for locus GRAISA26 was 60°C, 62°C for loci GRAISA3 and GRAISA18, 56°C for loci GRAISA6 and GRAISA25, and 53°C for loci GRAISA15 and GRAISA21. Supplementary information about sampling localities and PCR conditions for successfully amplified loci is available directly from the authors upon request. The number of polymorphic loci was two for *A. neidhoeferi* and three for *A. luna*. Only GRAISA26 resulted polymorphic in both species (Table 2). The nine individuals of *A. luna* genotyped with GRAISA25 resulted all heterozygotes formed by combinations of five alleles. This high level of observed heterozygosity deserves further investigation as it might be caused by linkage to a gene showing heterozygote advantage.

In summary, these microsatellite markers will be useful for a variety of population genetic studies in *G. isabelae,* including monitoring the genetic effects of habitat fragmentation and parental analysis.

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