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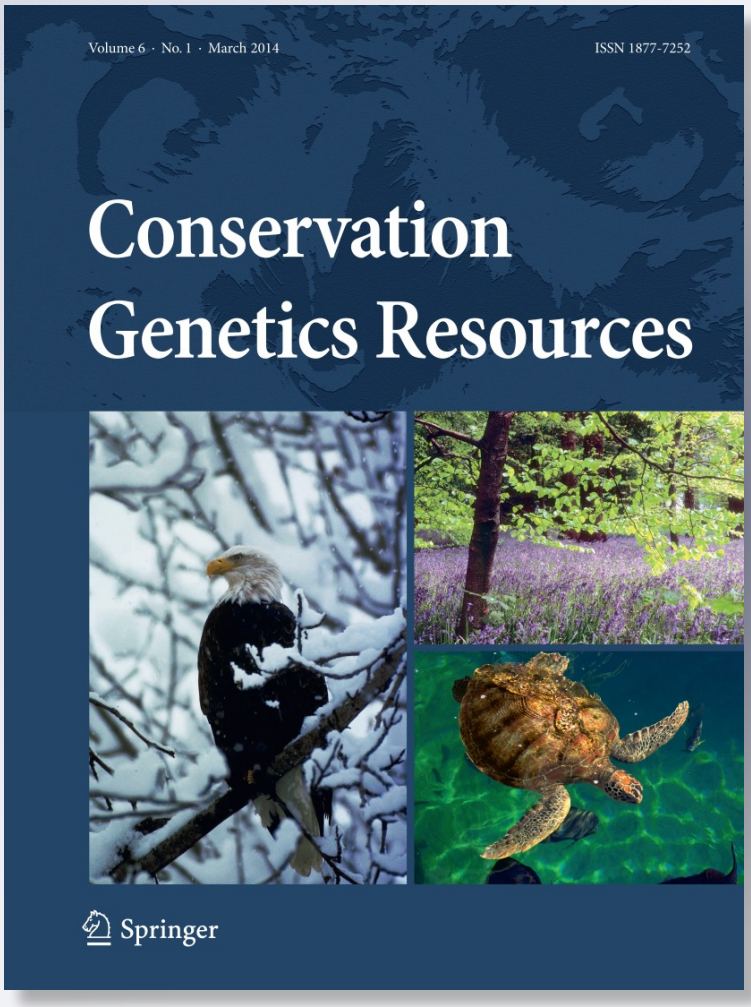

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MICROSATELLITE LETTERS

Isolation and characterization of 15 microsatellite loci in the specialist butterfly *Boloria eunomia*

Delphine Legrand · Audrey Chaput-Bardy · Camille Turlure · Quentin Dubois · Michèle Huet · Nicolas Schtickzelle · Virginie M. Stevens · Michel Baguette

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Abstract *Boloria eunomia* is a boreo-montane butterfly species suffering from habitat loss and isolation in the relictual part of its distribution range. Small populations persist in habitats scattered on plateaux or low mountains in western, central and southern Europe. Quantifying gene flow within and between these remnant populations is thus a crucial point to properly delineate metapopulations, to understand their dynamics and hence to design appropriate conservation plans for this butterfly species. We developed primers for the amplification of 15 microsatellites loci for *B. eunomia*. Thirteen loci were grouped in 2 multiplexes and amplified in 50 individuals from 5 populations to validate their use in population genetics. Gene diversity was on average 0.63 across populations. Null alleles and recurrent Hardy–Weinberg disequilibrium were detected in 7 loci. However, Fst estimates after correction for the presence of null alleles were highly correlated (0.91) to Fst estimates without correction. The loci developed here are thus usable for fine scale population genetic analyses and represent a very useful tool to quantify gene flow within and between metapopulations of *B. eunomia*.

D. Legrand (✉) · M. Huet · V. M. Stevens · M. Baguette
CNRS USR 2936, Station d'Ecologie Expérimentale du CNRS, route du CNRS, 09200 Moulis, France
e-mail: delphine.legrand@ecoex-moulis.cnrs.fr

A. Chaput-Bardy
Groupe Ecologie et Conservation des Vertébrés, Université d'Angers, PRES UNAM, 2 Boulevard Lavoisier, 49045 Angers Cedex 01, France

C. Turlure · Q. Dubois · N. Schtickzelle
Biodiversity Research Centre, Earth and Life Institute, Université catholique de Louvain, Croix du Sud 4 L7.07.04, 1348 Louvain-la-Neuve, Belgium

M. Baguette
Evolution et Biodiversité, UMR 7205, Institut de Systématique, Muséum National d'Histoire Naturelle, 57 rue Cuvier, 75005 Paris Cedex 5, France

Metapopulations consist in spatially structured populations that exchange genes through dispersal of individuals. Individual dispersal allows both the recolonization of locally extinct populations and the rescue of declining local populations, which ensures the long-term persistence of the metapopulation. The study of metapopulation dynamics is central to conservation biology because habitat loss and isolation, major threats to biodiversity, increase the number of species that are forced to function as metapopulations.

The bog fritillary butterfly *Boloria eunomia* is a circumpolar butterfly: in Europe, its distribution is typical of a boreo-montane species with a central, continuous part in Fennoscandia, and marginal parts widely scattered on uplands in Western Europe and mountainous parts of central and southern Europe (Vandewoestijne and Baguette 2004). In these marginal populations in unfertilised wet hay meadows along rivers or in peat bogs, both larvae and adults feed exclusively on *Persicaria bistorta*. These relictual populations are threatened by the destruction of their habitat and *B. eunomia* is protected in France, Belgium, Serbia and Bulgaria. Over the last two decades, Belgian metapopulations of *B. eunomia* have been extensively studied using Capture-Mark-Recapture methods, making the species one of the classical butterfly model species in the study of metapopulation. Detailed studies provided important knowledge on habitat definition, demography, dispersal and movement (Baguette et al. 2011).

An important step forward in our understanding of the effect of habitat fragmentation on *B. eunomia* persistence would be to quantify the amount of gene flow within and

Table 1 Characteristics of the primers developed to amplify microsatellite loci in the butterfly *B. eunomia*

Locus	Primer sequence	Repeat motif	Size range (bp)	NA (n = 15)	Multiplex	NA (n = 50)	Null allele frequency	PIC	Ho/He/Phwe				
									Berismenil	Bievres	Pisserotte	Prés lienne	Langlire
boleun01	F: [NED]CCA TCA CCG AGC AGA AGA TAC	(CA)(TACA)… (CA)(TACA)…(CA)	188–200	5	1	6	0.18	0.59	0.56/0.47/1	0/0.62/ 0.02	0.4/0.48/0.37	0.3/0.65/0.06	0.37/ 0.67/ 0.33
	R: TTG CTA CCA CTG TCC ATT GC												
boleun07	F: [PET]AAC TGC CTA GGG GCA TTT TC	(ACAG)…(AG)G(AG) G(AG)	188–216	6	1	11	0.02	0.81	0.89/0.9/0.48	0.8/ 0.79/ 0.11	0.33/0.58/0.17	0.9/0.83/0.83	0.9/0.8/ 0.67
	R: CGC AGA GGC TAG TCT TGT TTC												
boleun08	F: [NED]CAC ATT GAA AAC GAT CAC GAG	(CT)	152–166	4	2	4	0.22	0.44	0.4/0.51/0.57	**0.12/ 0.6/ 0.007**	0.25/0.57/0.05	0.14/0.36/0.23	**0.1/ 0.43/ 0.01**
	R: CCA CAG GTA TGC CAT GTT TC												
boleun09	F: [VIC]CAT CGA ACA CAC TGT AAG TGA TTG	(GT)	212–216	3	2	4	0.23	0.42	**0/0.66/0.002**	0.14/ 0.47/ 0.02	**Monomorphic**	**Monomorphic**	0.5/ 0.59/1
	R: ATG GAC AGG TCA GCC ATA CC												
boleun10	F: [FAM]CCC CCG GAA GTA GGT TAA AG	(GT)(GC)(GT)	169–186	6	1	7	0.23	0.71	0.44/0.71/0.09	**0.11/ 0.82/ 0.0001**	0.5/0.77/0.05	0/0.44/0.02	0.62/ 0.67/ 0.3
	R: ATC TCG GGG AAG GTG CTG												
boleun11	F: [FAM]AGG GAG AGA CCG GTG AGT G	(TGTA)	114–136	4	2	5	0.2	0.55	0.4/0.44/1	**0.3/ 0.71/ 0.007**	0.5/0.65/0.1	**0.11/0.62/ 0.00**3	0.3/ 0.67/ 0.02
	R: CTA CCC GTG ATA GGC CAG TG												
boleun14	F: GAC TCG AAC CTG GGA CCT C	(CA)	75–94	4	NA	NA	NA	NA	NA	NA	NA	NA	NA
	R: CCA AGT GGG ACA CGG TAA TG												
boleun15	F: [PET]ACT TCG ACC ACA ACC TGT CC	(TG)(TC)(TG)	111–129	5	1	7	0.06	0.77	0.7/0.76/0.33	0.6/ 0.78/ 0.11	0.6/0.77/0.2	**0.55/0.79/ 0.008**	1/0.8/ 0.86
	R: CTA CAT CGC CTC GCA GAT TG												

Table 1 continued

Locus	Primer sequence	Repeat motif	Size range (bp)	NA (n = 15)	Multiplex	NA (n = 50)	Null allele frequency	PIC	Ho/He/Phwe				
									Berismenil	Bievres	Pisserotte	Prés lienne	Langlire
boleun17	F: [NED]CCT CCT ACG AAG TTG GCT ATT C R: CGA CAA CTA GGG GAC ACA GG	(CA)…(CA)	110–162	5	1	9	0	0.66	0.8/0.83/0.053	0.6/0.52/1	0.9/0.72/0.42	0.7/0.56/0.78	0.6/0.54/1
boleun20	F: AAC AAG CAT GGC ACT ACC AC R: CAT GGG ATT GAT GGG ATT TTA G	(TAGA) (GA)	90–111	3	NA	NA	NA	NA	NA	NA	NA	NA	NA
boleun24	F: [FAM]GCA TAC CTA TGC GTG ATC CAG R: GCA CTT ACA CGA CAG GAA AGG	(CA)	206–218	5	2	7	0	0.68	0.7/0.64/0.21	0.9/0.79/0.91	0.78/0.73/1	0.6/0.63/0.66	0.6/0.69/0.045
boleun25	F: [VIC]AAT GTC AGC GTT GAC TGT CG R: GCT TCA TAC TAG CCG GCA TC	(TG)	123–135	3	2	3	0.22	0.52	0.3/0.67/0.11	0.4/0.44/1	**0.1/0.42/0.01**	0.2/0.54/0.02	0.3/0.54/0.08
boleun30	F: [VIC]ATC AAC ACC TGC CTC CAC AC R: TCG GTG ATG ATG TGG ATC TG	(GT)…(GT)…(GT)	177–194	5	1	6	0.06	0.6	0.9/0.68/0.05	0.7/0.73/1	0.5/0.51/1	0.5/0.44/1	0.4/0.5/0.57
boleun33	F: [NED]TTG TTT CCA ACG GTT TTG AAG R: GAG ACA GGG CCG GAT TAA G	(CT)	213–251	5	2	7	0.24	0.77	**Monomorphic**	**0.29/0.67/0.006**	0.83/0.8/0.75	0.29/0.67/0.03	**0.14/0.78/0.0003**
boleun35	F: [PET]TCG AGT CGT TCC ACC ATA AAC(AC)ATGC R: TCA CGA TAT TAG CGC ACG TAA C	(AC)GA(AC) GA(AC)	156–160	3	2	NA	NA	NA	NA	NA	NA	NA	NA

Table 1 continued

Locus	Primer sequence	Repeat motif	Size range (bp)	NA (n = 15)	Multiplex	NA (n = 50)	Null allele frequency	PIC	Ho/He/Phwe				
									Berismenil	Bievres	Pisserotte	Prés lienne	Langlire
Mean				4.25	–	6.5		0.63	0.68	0.66	0.58	0.55	0.64

N_A number of alleles; *bp* base pair; *n* number of individuals with 15 individuals referring to the sampling used to the development of the loci and 50 individuals referring to the sampling used to realize the multiplex procedure and to validate their use in population genetic studies; *PIC* polymorphic information content; *Ho* observed heterozygosity; *He* expected heterozygosity; *pHWE p* value Hardy–Weinberg equilibrium test, bold values refers to significant deviation from HWE after False Discovery Rate correction

between these metapopulations. Allozymes and RAPD have already been used to study the genetic structuring of European populations (Vandewoestijne and Baguette 2004, Nève et al. 2008), but these markers are limited for most population genetic inferences. Allozymes can be under current selection and may reveal to be monomorphic at small spatial scale, while codominance and reproducibility problems occur with RAPD.

We developed 15 microsatellites in *B. eunomia* to provide better genetic markers for population and landscape genetics. Using 15 individuals, Ecogenics GmbH (Zurich, Switzerland) made an enriched library from size selected genomic DNA ligated into SNX forward/SNX reverse linker (Hamilton et al. 1999) and enriched by magnetic bead selection with biotin-labelled (CT)13, (GT)13, (GTAT)7 and (GATA)7 oligonucleotide repeats. Of 528 recombinant colonies screened, 347 gave a positive signal after hybridization. Plasmids from 175 positive clones were sequenced and primers were designed for 32 microsatellite inserts, of which 25 were tested for polymorphism. Among them, only 15 amplified correctly (specific product, simple allelic pattern) and were polymorphic (Table 1). To test for their utility in population genetics, we re-amplified them on a second set of 50 individuals originating from 5 populations (10 individuals per population) from the Ardenne region, Belgium. Individual DNA was extracted from a leg preserved in absolute alcohol with DNeasy Tissue Kits (QIAGEN). We performed Polymerase Chain Reactions (PCR) to test for single amplification in 10 µL reaction volumes using Qiagen Type-it Microsatellite PCR Kits with 5 µL of PCR MasterMix (HotStarTaq *Plus* DNA polymerase, PCR buffer, dNTP mix), 1 µL of template DNA (1–10 ng), 1 µL of primer mix (final concentration: 0.2 µM each) and 3 µL of high purity water. PCR were conducted using a Labcycler (Sensoquest) with initial denaturation of 95 °C for 15 min followed by 30 cycles of 94 °C for 30 s, 59 °C for 1 min 30 s and 72 °C for 30 s, and a final extension at 72 °C for 45 min. Thirteen loci amplified correctly, as revealed by the clear signal of amplicons on agarose 0.8 % gels. We grouped them in 2 multiplexes (Table 1) and re-amplified individuals using forward primers labelled with fluorescent dyes. Fragments were visualized using an ABI PRISM 3730 sequencer (Applied Biosystems). After this multiplexing procedure, one locus did not amplify. The total number of alleles per locus for the 12 others ranged from 3 to 11 (Table 1). We verified that there was no linkage disequilibrium between pairs of loci after False Discovery Rate correction using GENEPOP. Mean Polymorphic Information Content overall loci was 0.63, indicating a reasonable discrimination between individuals. Mean expected heterozygosity overall loci was 0.68. Four loci were repeatedly monomorphic or repeatedly departed

from Hardy–Weinberg Equilibrium, and null alleles were detected in 7 loci using FreeNA. This problem is recurrent when developing microsatellite in butterfly species, even when a combination of biotin-enrichment protocol and 454 GS-FLX Titanium technology is used (Sinama et al. 2011). Nonetheless, estimated Fst between the 5 populations after correction for the presence of null alleles were highly correlated with non-corrected Fst indicating that the total dataset may be usable in population genetics.

The set of loci described here opens new perspectives for the use of *B. eunomia* as a model species for meta-population and dispersal ecology. Among others, it is now possible to build pedigrees and estimate gene flow, which is crucial for the delineation of metapopulations and the management of operational conservation units.

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