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Characterisation of sixteen additional polymorphic microsatellite loci for the spreading but locally rare European butterfly, *Brenthis ino* (Lepidoptera: Nymphalidae)

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Abstract. Whilst the overall geographic range of the lesser marbled fritillary, *Brenthis ino* (Rottemburg, 1775), is currently expanding, this species is patchily distributed at a local spatial scale due to its use of flower rich semi-natural meadows and the aggregated distribution of its host plant. Therefore, understanding the dispersal patterns of this butterfly and the effect of increasing fragmentation of the landscapes in central and Western Europe on its population dynamics is key to determining whether this patchy distribution can lead to metapopulation structuring and dynamics. One way to determine the degree to which local populations are isolated from one another is to use high resolution molecular genetic markers and thence quantify gene flow and genetic drift. Eleven microsatellite loci have previously been developed for this species, but six showed evidence of null alleles, effectively violating key assumptions of the models used to infer gene flow. We therefore developed a set of new primer pairs to amplify a suite of 16 highly polymorphic microsatellite loci (number of alleles ranged from 2–30) of which nine were found to conform to the Hardy-Weinberg's expectations, whilst at the same time not showing any clear signature for the presence of null alleles. We further describe how these primers were optimized for landscape and metapopulation genetics studies in Belgian Ardenne.

INTODUCTION

The lesser marbled fritillary, *Brenthis ino* (Rottemburg, 1775) (Lepidoptera: Nymphalidae), mainly inhabits humid grasslands, and wet areas such as bogs and marshes. Its large geographical distribution ranges from Northern Japan to Western Europe, with populations assessed as either stable (Parmesan et al., 1999) or slightly expanding southward in Europe (Parmesan et al., 1999; Van Swaay & Warren, 1999; Zimmermann et al., 2005). This expansion and the associated increase in population sizes have primarily been associated with the expanding range of the meadowsweet (*Filipendula ulmaria*; family Rosaceae), the main larval host plant of this butterfly species which often grows during the eutrophication of wet meadows (Zimmerman et al., 2005). The local dynamics of *B. ino* is therefore in stark contrast with the decline observed in many other butterfly species inhabiting (semi-) natural wet meadows, such as the bog fritillary (*Boloria eunomia* Esper, 1799). Such contrasted population trends make *B. ino* invaluable for purposes of species comparison (see e.g. Zimmermann et al., 2005; Fric et al., 2010) and for further understanding the variation observed in the species' response to environmental changes and habitat degradation.

Determining whether the patchy distribution of this butterfly leads to the formation of metapopulation structuring (Hanski, 1999) requires quantifying the degree to which local populations are isolated and the frequency of movement among patches. While individuals' emigration propensity can be high and the dispersal distance of *B. ino* can be relatively large (exceeding 2 km, Fric et al., 2010), another study showed that individuals may be very philopatric (Weyer & Schmitt, 2013). These two previous studies were performed at very local scales in populations with very high densities, whereas to better understand the movements and gene flow of this butterfly species requires working at larger scales with populations of varying distribution and densities.

Microsatellites are highly polymorphic genetic markers that are widely used for the detection of genetic differentiations and diversities over a variety of spatio-temporal scales (e.g. Hall & Beissinger, 2014). However, the development and availability of species-specific primers for polymorphic microsatellites is challenging in butterflies due to the widespread presence of null alleles and large allele dropout (Nève & Meglécz, 2000; Meglécz et al., 2007; Sinama et al., 2011). This violates many assumptions of the models used to quantify spatial genetic structure and hence may lead to substantial biases in estimates of butterfly genetic characteristics. Eleven microsatellite markers have previously been described for *B. ino* (Molecular Ecology Resources Primer Development Consortium, 2012), but their suitability may be limited as two of the loci did not conform to Hardy-Weinberg expectations (HWe), whilst null alleles appeared likely in three others. As the six remaining markers had only a moderate number of alleles (range 4–9), we developed an additional set of microsatellite markers for *B. ino* to improve the statistical power of spatial genetic analyses and implement small scale landscape genetic studies.

MATERIAL AND METHODS

Genomic DNA was extracted from the legs of adults captured in five populations in the Belgian Ardenne. Each leg was kept in 90% ethanol in an individual Eppendorf tube and stored in –20°C freezers prior to DNA extraction. DNA was extracted using a phenol-chloroform-isoamyl alcohol method as described in Vandewoestijne & Baguette (2002). The last step of this method was modified such that dried genomic DNA was re-suspended in 20 µL, instead of 50 µL 10 mM Tris-HCl pH 8.0, 1 mM EDTA. The DNA extraction was subsequently treated by RNase (2 µL of 100 µg/mL RNase; incubation: 30 min at 37°C). Microsatellites were isolated by GENOSCREEN (Lille, France) using the

Table 1. Description of 16 polymorphic microsatellite loci developed for *Brenthis ino*. Abbreviations: Access. no. – the Genebank accession number; Range – allele size range; Ta – annealing temperature; Group – Multiplex group.

Primer	Access. no.	Forward sequence	Reverse sequence	Colour	Range	Ta	Group
Bi_111	KP164838	CCGAAATCAGGCAGGATG	ACATGAGACTACTAACATAACCATGC	NED	173–201	55	1
Bi_112	KP164839	ATGTCACAGGATTAAAATAACTACGTG	GATATGCCAGGACCCTCCTT	6-FAM	196–283	53	1
Bi_115	KP164840	GTTGAAGTACATTTATGTTCGTATGC	AACCATTTGCTCTTTGACAGC	NED	192–250	55	3
Bi_116	KP164841	TGTGTTGAAGCTTCTTGTTTAATAGG	GCTTGTGTTAATCCAGGGGT	PET	115–205	55	1
Bi_117	KP164842	AGTACCAAGGAGTTTGGCGA	CGTTACTGTGGCTGCGAGT	NED	129–145	53	1
Bi_119	KP164843	TCCTACGTGTCTGCTCCATTC	ATTGTAGGCTACCAACGCCA	NED	151–187	57	2
Bi_121	KP164844	TCATGATTGAGGACGGTGAA	CGTATACAAACGTATACTGTGACATCT	VIC	127–156	53	1
Bi_125	KP164845	GCGACGCAAAGGAGGATA	ACCGCGGAAAGGTATAAACG	VIC	151–263	53	3
Bi_126	KP164846	GTTCGAGCGATGGGTTGT	CACGTGACGCACCTAACATC	PET	188–218	57	2
Bi_128	KP164847	GTTTGTGTCACATGCGTGC	GGCGTGGTTCGTCAATAAAA	VIC	291–361	53	1
Bi_131	KP164848	GAGCTACGATGCCACAGACA	TTCGACTTAGCTTATCTCTGCAA	VIC	152–186	57	2
Bi_135	KP164849	CCCTAACAGAAAGGTCAAAATGA	CGTCACTCCGTTTTCGTCTT	PET	131–169	57	2
Bi_139	KP164850	TCGGGTGCGTCTCTAGGTAT	AAACAAATAATTTATGAGGATCGGA	VIC	123–207	57	2
Bi_141	KP164851	AGTGCTGCGATCCTCAGGT	TACCTAGGGACGAACCCGAT	PET	145–147	55	3
Bi_143	KP164852	CGAGTTTAGCTTCTGGCGTT	AGTCGCAAAGTCCGTTGAGT	PET	110–128	57	3
Bi_144	KP164853	GTCCTGGGTTCAACTTCGGT	TCACTCTTCAAAATTTTACAGGACC	6-FAM	135–161	57	2

method described in Malausa et al. (2011). Briefly, a pool of the genomic DNA extracted from eight individuals was fragmented using the enzyme RsaI and subsequently enriched using eight probes with the repeat units TG, AAC, AGG, ACAT, TC, AAG, ACG, ACTC. The enriched DNA was amplified and sequenced using a GS-FLX® sequencer (Roche Diagnostics, Basel, Switzerland) to provide a total of 5,831 sequences containing microsatellite repeats. The software QDD (Meglécz et al., 2010) was used to analyze the sequences containing microsatellite repeats and to design primers. In total, 197 primer pairs were available of which 51 pairs were selected having a repeat number >6, and calculated annealing temperatures between 53–63°C (all pairs were di- or tetranucleotide repeats). The amplification of these 51 primer pairs was tested using polymerase chain reaction (PCR) in 10 µL reaction volumes containing: 2 µL of DNA, 0.7 units of DNA polymerase (Roche Diagnostics, Ref. 11 435 094 001), 0.3 mM BSA, 10 mM Tris-HCl, pH 8.0, 50 mM of KCl, 100 µM dNTPs, 1 µM primer, and 1.5 mM of $MgCl_2$ (these conditions were subsequently optimized and the range of primer and $MgCl_2$ ranged from 0.5–1.3 µM and 1.5–2.0 mM, respectively). Following a denaturation step at 95°C for 15 min, DNA was amplified by 32 cycles of denaturation (94°C for 30 s), incubation at the annealing temperature (30 s), followed by an extension step at 72°C (90 s) and an additional elongation step (74°C for 25 min). The amplified fragments were subsequently run on 1% agarose gels. Across the 51 pairs of primers selected, 31 were discarded as they yielded no PCR products (9 primers), produced a large number of bands indicating a lack of specificity (17 primers), or

Table 2. Characteristics of the 16 polymorphic microsatellite loci developed for *Brenthis ino* from five Belgian sampling sites. Descriptions are provided across all sites and within each site. Abbreviations: A – total number of alleles; *Ho* – observed heterozygosity; *He* – expected heterozygosity; $P_{GENEPOP}$ – significance test for deviations of the Hardy-Weinberg's expectations (estimated across all sites using GENEPOP); P_{null} – the frequency of null alleles (estimated using MICROCHECKER). For each study site, the number of genotypes (n) and the *P*-value of the deviation from the Hardy-Weinberg's expectations calculated using Arlequin ($P_{Arlequin}$) are provided.

Primer	Overall					Within sites				
	A	*Ho*	*He*	$P_{GENEPOP}$	P_{null}	Bérismenil (n; $P_{Arlequin}$)	Chapons (n; $P_{Arlequin}$)	Prés de la Lienne (n; $P_{Arlequin}$)	Pont Hierlot (n; $P_{Arlequin}$)	Verleumont (n; $P_{Arlequin}$)
Bi_111	13	0.769	0.807	0.255	–0.096	10; 1.00	15; 0.01	15; 0.92	15; 0.73	14; 0.10
Bi_112	17	0.680	0.816	0.054	–0.038	10; 0.37	15; 0.01	15; 0.43	14; 0.21	15; 0.09
Bi_115	13	0.423	0.757	<0.001	0.136	10; 0.06	14; <0.01	15; 0.23	15; 0.05	13; <0.01
Bi_116	19	0.468	0.866	<0.001	0.243	10; <0.01	15; 0.05	15; 0.03	14; 0.02	15; <0.01
Bi_117	9	0.500	0.886	<0.001	0.164	10; 0.04	14; 0.10	15; <0.01	15; <0.01	14; <0.01
Bi_119	11	0.740	0.842	0.290	0.058	10; 0.45	15; 0.45	15; 0.25	15; 0.05	15; 0.45
Bi_121	12	0.487	0.771	<0.001	0.133	10; 0.10	15; 0.02	15; <0.01	15; 0.03	15; <0.01
Bi_125	30	0.881	0.949	0.099	–0.003	10; 0.72	14; 0.30	14; 0.30	15; 0.09	14; <0.01
Bi_126	11	0.837	0.787	0.542	–0.159	10; 0.62	15; 0.31	14; 0.85	15; 0.49	15; 0.14
Bi_128	15	0.647	0.829	0.001	0.022	10; 0.35	15; 0.05	15; 0.63	15; 0.01	15; <0.01
Bi_131	7	0.264	0.423	0.005	0.281	10; <0.01	14; 0.49	15; 0.07	14; 1.00	15; 0.31
Bi_135	10	0.256	0.805	<0.001	0.284	7; <0.01	14; <0.01	13; <0.01	12; <0.01	8; <0.01
Bi_139	12	0.811	0.854	0.737	0.126	10; 0.19	15; 0.97	14; 0.98	15; 0.82	15; 0.34
Bi_141	2	0.071	0.071	–	–	10; NA	14; 1.00	15; NA	15; NA	15; NA
Bi_143	9	0.770	0.756	0.687	–0.102	10; 1.00	14; 0.88	15; 0.93	15; 0.34	15; 0.07
Bi_144	7	0.673	0.754	0.113	–0.143	10; 0.45	15; 0.08	15; 0.85	15; 0.02	15; 0.16

yielded weak PCR products (5 primers). For the remaining 20 primers, forward primers were labelled using fluorescent dyes NED, 6-FAM, PET, and VIC from Applied Biosystems (Carlsbad, CA, USA; Table 1). Individuals were genotyped using a Prism® 3100 Applied BioSystems genetic analyser, allele sizes being scored using GeneMapper 3.7® (Applied Biosystems). The size of the alleles was estimated by scoring their size against an internal standard (600-LIZ, Applied Biosystems). Multiplex amplifications were performed using QIAGEN Multiplex PCR Kit (Qiagen, Venlo, Netherlands) using the protocol: 95°C for 15 min followed by 25 cycles denaturation (94°C for 30 s), incubation at the annealing temperatures (90 s), denaturation (72°C for 90 s) and with a final denaturation (72°C for 45 min). At this stage, four additional primers were discarded because of interactions with other primers when performing multiplex PCRs. The remaining 16 primers therefore had similar annealing temperatures and allowed multiplex amplifications of the target amplicons (Table 1).

Deviations from the HWe were estimated across all studied populations using GENEPOP 3.3 (Raymond & Rousset, 1995) and within each population using ARLEQUIN v3.1 (Excoffier et al., 2007). We more specifically tested whether some loci were consistently lacking heterozygosity by calculating for each locus the proportion of populations in which significant deviations from the HWe were recorded (α adjusted = 0.003). We further quantified the frequency of null alleles, stutter bands and large allele dropout using the program MICROCHECKER 2.2.1 (van Oosterhout et al., 2004). Linkage disequilibrium was quantified using ARLEQUIN v3.1 (Excoffier et al., 2007). The overall *F*-statistics θ_{ST} and *f*, measuring respectively the genetic structure and inbreeding rate, were quantified using FSTAT 2.93 (Goudet, 1995, 2001).

RESULTS AND DISCUSSION

The total number of alleles per locus ranged from 2–30 (median = 11.5 alleles per locus; Table 2). There was no evidence of linkage disequilibrium between any pair of loci. Significant homozygote excess was found in six loci (Bi_115, Bi_116, Bi_117, Bi_121, Bi_128, and Bi_135) and were very likely in another locus (Bi_131; Table 2). MICROCHECKER found significant null alleles for three loci (Bi_116, Bi_117, and Bi_135) but there was no evidence of stuttering or large allele dropout (Table 2). We therefore estimated the *F*-statistics across the five study populations using all loci complying with HWe (Bi_111, Bi_112, Bi_119, Bi_125, Bi_126, Bi_139, Bi_141, Bi_143, and Bi_144). The overall genetic structure was weak and marginally significant (θ_{ST} = 0.007, 95% CI = –0.001–0.017) and the inbreeding coefficient was significantly higher than zero (*f* = 0.067, 95% CI = 0.020–0.113). These nine highly polymorphic loci are hence hereby optimized for quick and cost effective genotyping. This dataset will be expanded to other surrounding *B. ino* populations (i) to determine the degree to which local populations are isolated and hence whether this system shows metapopulation structuring and dynamics; (ii) determine whether the observed genetic structure is due to barriers to gene flow and/or isolation by distance; and (iii) quantify the degree to which measures of local habitat quality influence the genetic characteristics of this butterfly species.

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