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Characterization of ten polymorphic microsatellite markers for an endangered butterfly *Argynnis niobe* and their cross-species utility in the closely related species *A. adippe* (Lepidoptera: Nymphalidae)

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Abstract. The Niobe Fritillary, *Argynnis niobe*, is a habitat specialist and as a consequence is highly endangered in contemporary Europe. To investigate its genetic diversity and population structure, 10 polymorphic microsatellite loci were developed and characterized, using a recently developed pyrosequencing method. The number of alleles per locus ranged from 2 to 21, and the observed and expected heterozygosities varied from 0.17 to 0.53 and from 0.24 to 0.92, respectively. These loci were also successfully used to study the genetic diversity of a closely related species, the High Brown Fritillary, *Argynnis adippe*, and will be used in future population structure studies of both these species.

INTRODUCTION

Molecular markers are increasingly used in insect conservation biology as they are a cheaper and more reliable way of estimating critical population parameters important for population management, such as intra-population genetic diversity, gene flow, spatial population differentiation and effective population size (Amos & Balmford, 2001; Palsboll et al., 2007). By using these techniques, researchers can consider scales that are much larger than the local scales of pre-molecular population ecology (Sigaard et al., 2008; Brattstrom et al., 2010) and make it possible to compare the genetic makeup over large regions (Finger et al., 2009; Habel et al., 2010), even up to continent-wide comparisons in order to unravel the colonization-extinction history within the current ranges of species (Todisco et al., 2010; Zachos & Hartl, 2011; Runquist et al., 2012). Microsatellite markers are particularly widely used because they are co-dominant, hyper variable, mostly neutral and reproducible (Jarne & Lagoda, 1996). Despite these advantages microsatellite markers have not been widely used in studies on some well studied model groups, notably butterflies and other Lepidoptera, because the sequences flanking microsatellites in this group are very similar for different loci. These similarities are generated by recombination mediated events, such as unequal crossing-over or gene conversion and through transposition of mobile elements (Van't Hof et al., 2007). These problems have now been resolved thanks to next-generation sequencing methodology, which allow the rapid identification of large numbers of bioinformatically variable loci, without the necessity of laborious and costly cloning (Vandewoestijne et al., 2012).

Perhaps due to the difficulties stated above and associated high costs, population genetic studies using microsatellites have so far targeted species with only a subset of potentially diverse lepidopteran population structures. The majority of the species so far studied are sedentary, forming high-density populations in discrete patches of habitat, which are interconnected, to varying extents, by gene flow (e.g., Keyghobadi et al., 1999; Harper et al., 2000; Cassel, 2002; Zeisset et al., 2005; Sarhan, 2006; Habel et al., 2008). As a consequence of habitat loss and population isolation, such species frequently suffer declines of within-population genetic diversity caused by inbreeding and/or genetic drift (Habel et al., 2011), making it important in the context of the conservation of these species to consider their population genetic structure. The population structures of these mainly sedentary species, however, were rather well rendered by pre-molecular approaches, such as mark-recapture, and the molecular genetics results often tended to elaborate what was already expected. There are studies on species at the opposite extreme of possible butterfly population structures, i.e. migratory species that occur as panmictic populations spread over huge geographic distances (Vandewoestijne & Van Dyck, 2010), but such species are seldom of concern to conservationists because they easily replace population losses through immigration.

Species with intermediate dispersal ability fall between these two extremes and have been studied much less, although their fates in modern landscapes should be of great concern to conservationists. More than a decade ago, Thomas (2000) observed that in Western Europe butterflies of intermediate mobility were declining more than their sedentary and mobile counterparts and rapid declines were subsequently reported for several intermediately mobile species (e.g., Konvicka et al., 2008; Kadlec et al., 2010). This is due to the inverse relationship between dispersal and population density (Cowley et al., 2001a, b; Konvicka et al., 2012), which predicts that species that are too mobile to be restricted to patches of a few hectares (cf. Ehrlich & Hanski, 2004), but not as mobile as true migrants (cf. Vandewoestijne et al., 1999), will require relatively large areas of habitat in order to sustain viable populations. As many European species depend on habitats maintained for centuries by preindustrial land use patterns (Settele et al., 2009), conserving such species requires maintaining or mimicking traditional land use over scales that are too large for a single-site approach. A better understanding of the genetic makeup of populations of intermediately mobile species' is a necessary first step in delimiting the boundaries of conservation management units from the point of view of the species concerned. Until now, very few of the species that are declining in abundance and have an intermediate gene flow

have been subjected to genetic analyses, a notable exception being the North American fritillary *Speyeria idalia* (Drury, 1773) (Williams et al., 2003).

We developed microsatellite markers for two intermediately mobile, low-density species of high conservation concern in Europe, the Niobe Fritillary *Argynnis niobe* (Linnaeus, 1758) and High Brown Fritillary *Argynnis adippe* (Denis & Schiffermüller, 1775) (Nymphalidae: Heliconinae). These two closely related species belong to the subgenus *Fabriciana* of the genus *Argynnis* (Simonsen, 2006; Simonsen et al., 2006) and both have broad ranges in the Palaearctic, were historically widespread across Central Europe and inhabit a diverse range of landscapes with grassland, pastures, orchards and woodlots (Tolman & Levington, 1997; Kudrna et al., 2011). As a likely result of land use changes, they became less widespread in many countries and retreated to remote regions that still practice small-scale farming (cf. Spitzer et al., 2009), thus indicating their dependency on large areas consisting of a mosaic of habitats as existed before the intensification of agriculture. Examples of such regions include the offshore islands of Germany (Salz & Fartmann, 2009) and Estonia (Sang et al., 2010) or mountainous regions (Spitzer et al., 2009b; Verovnik et al., 2012). The declines in the abundance of *A. adippe* are less severe and of little concern on a continental basis (Van Swaay et al., 2010), but this species is severely threatened in Britain, for example (Fox et al., 2011). The situation appears much worse for *A. niobe*, which has disappeared from most of Germany (Salz & Fartmann, 2009), and is now near-threatened continentally (Van Swaay et al., 2010). In the Czech Republic, where the material for this study was collected, the extent of the distribution of *A. adippe* was at its lowest a decade ago and is now recovering, whereas the current distribution of *A. niobe* is 40 per-cent of what it was in the 1950s and this species is now critically endangered (Benes et al., 2002).

MATERIAL AND METHODS

Samples were collected during summer 2011 in the Vsetínská Bečva valley, a sub-mountainous area extending for approximately 200 km^2 (centroid coordinates: 49°19′N, 18°9′E) close to the Czech-Slovakian border, which is still traditionally farmed and where both species reach their highest densities within the Czech Republic.

Genomic DNA was extracted from a little piece of wing using the DNeasy Blood&Tissue kit (QIAGEN), which is a way of obtaining tissue samples from butterflies without damaging them (Hamm et al., 2010) and especially important in the case of endangered species. Initially, we reviewed the literature for microsatellites already developed for related species in order to try cross-species amplification. The only existing primers potentially suitable for cross-species amplification were those designed by Williams et al. (2002) for a related fritillary, *Speyeria idalia*. Forward primers were fluorescently labelled and PCR products were analyzed using fragment analysis on an automated sequencer. A variety of PCR conditions were tested, but none of the primer pairs provided microsatellite PCR products.

For the next step, primers were developed de novo using the recent and highly efficient pyrosequencing method (Ronaghi, 2001). DNA from four *Argynnis niobe* individuals was pooled and sent to GenoScreen (France) for the GenoSat service, combining DNA enrichment procedures with the use of multiplexed microsatellite probes and the update Titanium of the 454 GS-FLX technology (Malausa et al., 2011) (see Vandewoestijne et al., 2012 for more details). Obtained primers were analyzed for all primer secondary structures including hairpins, self-dimers and cross-dimers in primer pairs, using the on-line application NetPrimer, available at http://www.premierbiosoft.com/netprimer/.

These secondary structures should be avoided if possible, because they could reduce amplification success. Primers were also checked for the presence of G or C bases within the last five bases from the 3' end of primers (GC clamp), which helps to promote specific binding at the 3' end due to the stronger bonding of G and C bases. In general, we followed the PCR primer design guidelines reviewed at http://www.premierbiosoft.com/tech_notes/PCR_Primer_Design.html.

Initially, we tried PCR amplification with unlabelled primers, followed by 1% agarose gel electrophoresis, on eight individuals (four *A. addipe* and four *A. niobe*), to determine which primer pairs provided suitable PCR products. Subsequently, we ordered fluorescently labelled primers (only forward: 6-FAM, NED, PET, or VIC, Applied Biosystems). We performed monoplex PCR on the same eight individuals for each of the fluorescently labelled primers using Combi PPP-MasterMix (Top-Bio). The total reaction volume was 12 µl, containing 4 µl of MasterMix, 1 µl of each primer (final c = 0.08 nmol), 4 µl of PCR H_2O and 20 ng of DNA. Cycling parameters were: 4 min of initial denaturation at 94°C, followed by 35 cycles of 94°C (30 s), 54°C (60 s), 72°C (60 s), with final elongation at 72°C for 5 min. We also tested different annealing temperatures (50°, 52°, 56°, 58°), but 54°C provided the best results. The PCR products were then analysed by fragment analysis on automated sequencer ABI 3130 and allelic patterns were scored with software GeneMapper (Applied Biosystems) to determine which loci were polymorphic and can be reliably scored.

For the selected loci, we genotyped another 24 individuals of each species and thus had a total of 32 genotyped individuals per species (24 males and 8 females). Basic parameters of loci, such as number of alleles (Na), observed (H_o) and expected (H_e) heterozygosities, were calculated using the software GenAlEx (Peakall & Smouse, 2006). All loci in both species were also tested for Hardy-Weinberg equilibrium (HWE). The occurrence of null alleles was tested using the software FreeNA (Chapuis & Estoup, 2007) and linkage disequilibrium tests were computed in Genepop 4.0 (Rousset, 2008). Because butterflies have heterogametic females and there is always the possibility that genetic markers are sex-linked, we checked for at least one heterozygotic female genotype at every locus.

RESULTS

From the GenoSat service we obtained 67671 sequences, of which 8144 contained microsatellite motifs and the software analysis (provided by GenoScreen) resulted in 388 bioinformatically validated pairs of primers. Based on the NetPrimer analyses, we selected the thirty most suitable primer pairs. Out of these thirty primer pairs, eighteen provided consistent products of between 100 and 300 bp. Of these eighteen loci, analysed with fluorescent labels on the forward primer, two were monomorphic, six exhibited an unclear pattern with unspecific products indistinguishable from real alleles, and ten were polymorphic with reliably scorable alleles.

The basic parameters of the ten loci selected, based on 32 genotyped individuals of each species, are given in Table 1. The mean number of alleles per locus was 8.2 for *A. niobe* and 4.8 for *A. adippe*. The average observed proportion of heterozygotes over all loci (H_o) was 0.33 in *A. niobe* and 0.30 in *A. adippe*, while the level of expected heterozygosity (H_e) reached 0.62 in the former and 0.44 in the latter species. Seven loci in *A. niobe* and four in *A. adippe* were not in HWE. These loci exhibited null alleles with frequencies 0.09–0.34. The exact test for linkage disequilibrium resulted in non-significant P-values, allowing us to consider the analysed loci independent. We found

TABLE 1. Characterization of 10 polymorphic microsatellite loci in *Argynnis niobe* and *A. adippe*. Locus name, forward (F) and reverse (R) primer sequences, repeat motif, size range of alleles (Na), observed (H_o) and expected (H_e) heterozygosities, and statistical significance of the HWE test (ns = not significant, *** P < 0.001) for 32 individuals of each species.

Locus name	Primer sequences (5 → 3′)	Motif	*A. niobe*					*A. adippe*				
			Range	Na	*Ho*	*He*	HWE	Range	Na	*Ho*	*He*	HWE
An_5	F:CGATTGCATATACATCGTGC	CTAT	118–148	11	0.25	0.80	***	118–140	3	0.00	0.23	***
	R:CCTGTTCAAAAGATTCCGTCA											
An_20	F:TAGATCCAGTGGTCGCCTTT	TGTA	186–428	21	0.33	0.92	***	186–206	4	0.50	0.54	ns
	R:ATATGACAGTCGGGAGACGG											
An_21	F:CATCGTGACGAAATCTGCAT	ATAC	213–228	8	0.28	0.68	***	213–229	4	0.10	0.62	***
	R:AGGCTACATTTTGCCCTGTG											
An_22	F:TCCGTTCGCTACCAAACTTC	TA	168–254	14	0.27	0.82	***	176–226	11	0.28	0.64	***
	R:AGTTATCATCGCTTCGCTCG											
An_24	F:GTGCAGGGAAGGAAGAGAAG	AG	74–122	7	0.53	0.66	***	100–106	4	0.19	0.60	***
	R:ATGAATGGAGTTTCGCCAAG											
An_25	F:TTAAAAGAGCTTGCTGCGGT	TG	186–194	4	0.50	0.57	ns	188–200	4	0.40	0.35	ns
	R:TGCATCAAATGTGTTACGTGC											
An_26	F:TGTCGAATCAAGAAATTAGAATGC	ATCT	86–102	5	0.22	0.30	***	90	1	0.00	0.00	–
	R:CAAGAATTGCTCGTTTAAAAGTATT											
An_27	F:ACCAAGTTCCACCCATCTGA	CTT	175–181	2	0.28	0.24	ns	175–190	5	0.44	0.36	ns
	R:CACAGAAGCCACTGCCACTA											
An_28	F:TTACAACATGATTACCATTAGCCA	GT	126–146	6	0.17	0.75	***	134–148	8	0.84	0.83	ns
	R:GGTACGAACCTTCTACCTGGTC											
An_30	F:GCCATTATTGTATCCTCCTTGG	TTG	240–249	4	0.50	0.43	ns	237–246	4	0.25	0.23	ns
	R:CGTCAAAAGAGCAATCAGTGG											

at least one heterozygotic female at each of the loci, indicating that none of the loci were sex-linked (i.e. localised on the W-chromosome).

DISCUSSION AND CONCLUSIONS

The development of microsatellite markers using the pyrosequencing method has greatly facilitated their use in molecular ecology by reducing the cost and the time required to analyze samples (Santana et al., 2009). Now almost anyone can easily access a useful tool for investigating natural populations of non-model organisms. In this report, we describe ten polymorphic microsatellites for two closely related butterfly species of high conservation importance. We see this paper as a pilot study, taking advantage of only a small part of the raw data set, the rest of which can be used to isolate more microsatellite loci in the future. However, the population genetic parameters based on our ten loci are already informative and non-trivial, hinting at the occurrence of interesting evolutionary phenomena within the populations studied.

Primers were designed for *Argynnis niobe*, but the testing of polymorphism was performed for both species simultaneously, which led to relatively high cross-species amplification success, which is uncommon in Lepidoptera. Although some loci exhibited null alleles, the homozygote excess was more likely caused by the strong prevalence of only one type of allele at most loci. We did not detect any locus for which some individuals would fail to amplify at least one allele, which suggests that the homozygote excess was not due to null alleles, but represented genuine homozygosity (Dakin & Avise, 2004). Homozygote excess is usually interpreted as evidence for inbreeding and/or genetic drift (Frankham et al., 2008), but this needs to be confirmed by an analysis of more individuals.

In Table 2, we provide a brief comparison of the microsatellite loci parameters obtained in several other studies on Lepi-

TABLE 2. Comparison of microsatellite loci parameters obtained in other studies on Lepidoptera that used similar sized samples. Mean number of alleles per locus (Na), expected (H_e) and observed (H_o) heterozygosities.

Source study	Species	Sample size	Number of loci	Na	H_e/H_o
Vila et al., 2009	*Erebia palarica*	35	10	14.3	0.82/0.75
Petenian et al., 2005	*Parnassius apollo*	40	6	7.5	0.45/0.33
Petenian et al., 2005	*Euphydryas aurinia*	40	5	14	0.86/0.42
Rousselet et al., 2004	*Thaumetopoea pityocampa*	30	5	6.6	0.70/0.62
This study	*Argynnis adippe*	32	10	4.8	0.44/0.30
This study	*Argynnis niobe*	32	10	8.2	0.62/0.33

doptera that used similar sized samples. Genetic variability, expressed in terms of the number of alleles per locus and observed heterozygosity, was very low in our focal species, which indicates a strong influence of genetic drift and inbreeding on their population genetic structures. Surprisingly, the number of alleles per locus was two times lower in the locally more abundant and less threatened *A. adippe*. The long-term dataset (Benes et al., 2002; and Czech Butterfly Recording) available for this species suggests that this could have been caused by a bottleneck event that happened several decades ago, when there was a more drastic decline in numbers in *A. adippe* than *A. niobe*, but for a more accurate interpretation a more robust dataset is needed.

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