Isolation and characterization of six microsatellite loci in the larch budmoth *Zeiraphera diniana* (Lepidoptera: Tortricidae)

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Abstract. Six microsatellite markers were developed for the larch budmoth *Zeiraphera diniana* Guénée 1845, using two enrichment protocols. The number of alleles ranged from 3 to 15 per locus and observed heterozygosities ranged from 0.09 to 0.98 for the 69 individuals genotyped. Using these markers significant genetic differentiation between one population from Poland and samples from Alpine populations in France and Switzerland (overall $F_{ST} = 0.0298$) was detected. However, the two Alpine samples did not differ significantly. These microsatellite markers are valuable tools for studying the population genetics of *Zeiraphera diniana*.

INTRODUCTION

The larch budmoth, *Zeiraphera diniana* Guénée 1845 (Lepidoptera: Tortricidae), is a Palaearctic species (Bovey & Grison, 1975). In the Alps population densities fluctuate dramatically, with an outbreak every eight to ten years, causing spectacular defoliation of large stands of larch (*Larix decidua*) (Baltensweiler & Rubli, 1999). The outbreak takes the form of a wave that spreads from the South-Western to North-Eastern Alps over a period of five to six years (Johnson et al., 2004). The temporal and spatial characteristics of this insect make it a good model for studies on population dynamics and genetics. So far, genetic studies have focused on the differentiation between the host races on larch and pine, using allozymes and AFLP's (Emelianov et al., 1995, 2003a, b). Here we develop microsatellite markers to study population genetics in space and time, which will allow us to investigate the demographic processes observed.

MATERIAL AND METHODS

This paper reports two different microsatellite screening procedures done in two separate laboratories: Plant Research International, The Netherlands and Forest Zoology Research Unit, INRA, France.

First protocol (Plant Research International, The Netherlands)

Microsatellite loci were isolated from a genomic DNA library using an enrichment procedure after Karagyozov et al. (1993). Genomic DNA was isolated from the head of a caterpillar of *Zeiraphera diniana* using the DNeasy Tissue Kit (Qiagen, Venlo, The Netherlands). Five hundred nanograms of genomic DNA were restricted/ligated with *Alu*I, *Rsa*I or *Hae*III, pooled and amplified as previously described (Esselink et al., 2003; Schroeder et al., 2009). Microsatellite-containing fragments were selected by hybridization with Hybond N⁺ membranes using separately spotted synthetic oligonucleotides: $(TCT)_{10}$, $(TGT)_{10}$, $(GAG)_8$, $(GTG)_8$, $(TGA)_9$, $(AGT)_9$, $(CGT)_8$, $(GCT)_8$, $(GCC)_7$, $(GT)_{12}$. Fragments were eluted from the membranes with increasing stringencies: (1) $0.5 \times$ SSC, 1% SDS for 30 min at 62°C, (2) $0.2 \times$ SSC, 1% SDS for 30 min at 62°C, and (3) 0.1% SDS for 10 min at 100°C. Eluted fragments were precipitated and re-amplified. To obtain microsatellites with a high number of repeats and sufficient flanking sequence, fraction 1 was used for trinucleotide motifs with 30% CG content, fraction 2 for motifs with 60% GC and fraction 3 for motif CGG (100% CG content). For the GT motif fraction 1 was used. Amplified fragments were cloned and for each motif 192 colonies were screened as previously described (van de Wiel et al., 1999). The most abundant trinucleotide repeat was GCT (11% positive clones) followed by GCC (6%), CGT (4%), and TCT (0.5%). For the other motifs no repeats were isolated. In total 13 sequences with a unique repeat were obtained. All sequences were submitted to the EMBL database (FN185943–185953, FN263245). Primer pairs were designed using LASERGENE v8.1 (DNASTAR, Inc., Madison, WI, USA). Reverse primers were PIG-tailed (Brownstein et al., 1996). Using this enrichment, five polymorphic loci (Zd01, Zd02, Zd05, Zd09, and Zd16) with unambiguously scorable alleles were obtained.

Second protocol (INRA, France)

Microsatellite loci were isolated following a biotin-enrichment protocol modified from Kijas & Fowler (1994) and Rousselet et al. (2004). Genomic DNA was extracted from 20 caterpillar heads using the DNeasy Tissue Kit (Qiagen). Total DNA was digested with *Rsa*I and the 300–900 bp-long fragments were isolated on a 1% agarose gel using the Qiaquick Gel Extraction kit (Qiagen). Biotin-labelled oligonucleotides corresponding to microsatellite motifs $(CT)_{10}$, $(GT)_{10}$, $(CG)_{10}$, $(AT)_{10}$, $(AAT)_7$, $(GAA)_7$, $(AGC)_7$, $(AAC)_7$, $(TACA)_5$, and $(TACC)_5$ were hybridized to the modified DNA, and the enrichment was completed using Streptavidine Magnesphere Paramagnetic Particles (Promega, Madison, WI, USA). After re-amplification and cloning, 5623 recombinant clones were screened by PCR using vector primers plus the repeat-specific primer (Rousselet et al., 2004). A total of 269 positive clones were purified using the Wizard Plus SV Miniprep DNA purification System Kit (Promega). Of the 269 clones, 34 different inserts containing microsatellites were found after sequencing. PCR primers were designed for all of them, but only one locus (ZD32; FN185954)

TABLE 1. Characteristics of 6 polymorphic microsatellite loci from *Zeiraphera diniana* tested on 69 individuals (23 from France, 23 from Switzerland, and 23 from Poland).

Locus	Accession No.	Repeat motif[1]	Primer sequences (5′–3′)		Ta^2	Allele size range (bp)	No. of alleles	$H_O{}^3$	H_E	F_{IS}
Zd01	FN185943	$(CAA)_5$	F (FAM*):	TAGTGCCAACCCGCGGAAAATG	55	204–231	5	0.725	0.621	–0.167
			R:	GTTTCGGATATCCCACGGGGACCAG						
Zd02	FN185944	$(CGA)_{34-3}$	F (HEX*):	TGGTCAGGAACCGGGTATCCA	59	116–227	11	0.091	0.456	0.802
			R:	GTTTGAACCCTGGCTGGACCTCTAG						
Zd05	FN185946	$(GCT)_{28-10}$	F (HEX*):	CCTCATGCCCTTCCCTCCAG	55	193–211	3	0.217	0.200	–0.085
			R:	GTTTATTGCTTCGTGTTTTTACCACTTA						
Zd09	FN185945	$(ACC)_{12-3}$	F (NED*):	CCCAAGCGGCGTCTGCT	55	246–261	6	0.580	0.692	0.162
			R:	GTTTGTACTGACTACATCGCACGAAGTA						
Zd16	FN263245	$(GAT)_{64-34}$	F (FAM*):	TAGATTCCCCGGAACTAATGATCC	53	276–345	15	0.986	0.716	–0.377
			R:	GTTTAGATCCTTTGGTCTTATCTTCATT						
ZD32	FN185954	$(CA)_9$	F (FAM*):	ATGCCTACCTACTTGATGCC	49	181–213	13	0.580	0.871	0.334
			R:	AGTTTCCATACATTTGACACCTAC						

[1]minus denotes mismatch; [2]Ta = annealing temperature; * fluorescent dyes used; [3]GENEPOP v3.4 (Raymond & Rousset, 1995) was used to calculate H_O (observed heterozygosity), H_E (expected heterozygosity), and F_{IS} (inbreeding coefficient).

was polymorphic (13 alleles detected). In addition, this locus has an insertion/deletion of an "A" in its microsatellite motif (determined by the sequencing of the different alleles), which was considered as additional information when scoring.

Technical details and accession numbers are given in Table 1. The six loci were amplified in 69 individuals collected in 2006 within the range of the species where outbreaks occur (France, coordinates 45°01′N, 6°33′E, and Switzerland, coordinates 46°25′N, 9°44′E) and outside this outbreak range (Poland, coordinates 50°24′N, 16°96′E). The amplifications were done using a 9800 Fast thermal cycler (Applied Biosystems, Foster City, CA, USA) and the RedTaq package (Sigma-Aldrich, Inc., St Louis, MO, USA). The total reaction volume was 10 µL, containing 10 ng of genomic DNA, 0.4 U of *Taq* DNA polymerase (Sigma), 1X buffer (100 mM Tris HCl, pH 8.3, 500 mM KCl, and 0.1% gelatin), 20 mg/L of Betain, 250 µM of each dNTP, 0.4 µM of each primer, and 1.5 mM MgCl₂. Twenty percent of the forward primer was 5' end-labelled with a fluorescent dye, either 6-FAM (Sigma), HEX (Sigma) or NED (Applied Biosystems). The cycling conditions were 3 min at 95°C followed by 30 cycles of 50 s at 95°C, 1 min at annealing temperature (Table 1), and 30 s at 72°C. The amplified products were detected on an ABI-3100 automatic sequencer and their sizes were estimated using GENESCAN software (Applied Biosystems).

GENEPOP v3.4 (Raymond & Rousset, 1995) was used to calculate observed and expected heterozygosities, to test for Hardy-Weinberg equilibrium (HWE) deviation and linkage disequilibrium. The presence of null alleles was estimated using MICRO-CHECKER (Van Oosterhout et al., 2004).

RESULTS

All individuals were successfully amplified for all loci, except two for locus Zd02. The number of alleles per locus ranged from 3 to 15 and observed heterozygosities ranged from 0.091 to 0.985 (Table 1). Zd16 showed an excess of heterozygotes, while Zd02, Zd09, and Zd32 showed a deficit of heterozygotes ($P < 0.05$). HWE was accepted for Zd01 and Zd05 ($P > 0.05$). No linkage disequilibrium was found between loci ($P > 0.05$). Moreover, only one allele occurred in Poland at the locus Zd02. We estimated the presence of null alleles (MICRO-CHECKER, Van Oosterhout et al., 2004) for locus Zd02 (for the French and Swiss populations), locus Zd09 (for the French population), and locus ZD32 (for the French and Swiss populations).

Overall F_{ST} (Fixation index measuring population differentiation; Weir & Cockerham, 1984) was 0.0298. Pairwise F_{ST} values (averaged across loci) were 0.0535 ($P < 0.05$) and 0.0428 ($P < 0.05$) between France and Poland and between Switzerland and Poland, respectively. There was no differentiation between France and Switzerland ($F_{ST} = –0.0065$, $P > 0.05$).

DISCUSSION

Microsatellites are very suitable markers for studying population genetics, given their high variability, their neutrality, codominance, and reproducibility. Unfortunately, it is often difficult to develop them for species of Lepidoptera (Meglécz & Solignac, 1998; Nève & Meglécz, 2000; Zhang, 2004; Meglécz et al., 2004; Habel et al., 2008; Schroeder et al., 2009). Next to very low microsatellite cloning efficiencies, often multiple copies of microsatellite sequences exist with similar flanking regions and null alleles are also frequent (Meglécz & Solignac, 1998; Nève & Meglécz, 2000; Meglécz et al., 2007; Zhang, 2004; Vila et al., 2009). In this study two different microsatellite screening procedures, processed in two independent laboratories, were used. This resulted in the development of a total of six polymorphic loci. These microsatellite markers will be valuable tools for studies on the population genetics of *Zeiraphera diniana*.

Departures from Hardy-Weinberg equilibrium were found in our data for four of the microsatellite markers, Zd02, Zd09, and ZD32 with a deficit of heterozygotes, and Zd16 with an excess of heterozygotes. Departures from HWE can be due to strong inbreeding, population subdivision, selection, null alleles or frequent or severe bottlenecks (revised by Selkoe & Toonen, 2006). MICRO-CHECKER estimated the presence of null alleles in the three markers in which there was a shortage of heterozygotes. The effect of null alleles could be accompanied by bottleneck effects that may be a consequence of the cyclic outbreak dynamics of *Z. diniana*. No linkage disequilibrium was found, meaning that all loci can be considered as independent.

F_{ST} values indicate that these microsatellite markers are suitable for detecting genetic differentiation throughout the distribution range of *Z. diniana*. Indeed, our results showed that the population in Poland, located outside the range in which outbreaks occur, is significantly differentiated from the population in the Alps, where outbreaks occur ($F_{ST} = 0.0535$ with French Alps; $F_{ST} = 0.0428$ with Swiss Alps). The absence of differentia-

tion between the French and the Swiss Alps is consistent with data obtained using mtDNA, which show a high level of gene flow at the alpine scale (Delamaire et al., in prep).

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