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Identification of 18 polymorphic microsatellite loci in the spruce bark beetle *Ips typographus* (Coleoptera: Scolytidae) using high-throughput sequence data

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Abstract. Novel microsatellite markers for the spruce bark beetle (*Ips typographus* L.) are identified and characterized using next generation sequencing technology. 18 polymorphic loci were obtained by screening 10,684 reads and tested on 35 bark beetle samples from different locations in Bavaria, Germany. Allelic richness ranged from two to 38 alleles, observed heterozygosity from 0.03 to 0.66 and expected heterozygosity from 0.08 to 0.97. Four loci showed significant deviation from the Hardy-Weinberg equilibrium and no linkage between loci was detected. The 18 loci, along with another six loci previously described, provide effective analytical tools for analyzing the fine-scale genetic structure of bark beetle populations. The result of this study demonstrates that next generation sequencing technology is a valuable method for isolating microsatellites of a coleopteran species.

INTRODUCTION

In European forests the bark beetle, *Ips typographus* L., is a serious pest of Norway spruce (*Picea abies*). In order to improve the management strategies for pest species it is important to have a detailed knowledge of their population structure, mode of dispersal and reproductive characteristics. The six microsatellites for *I. typographus* cited in the literature (Sallé et al., 2003; GQ372845) indicate that this species is very good at dispersing (Sallé et al., 2007). The aim of the present study was to identify additional polymorphic loci for *I. typographus* in order to study its fine-scale molecular genetic structure based on the identification of individuals (within infested trees and nests). As several attempts to isolate microsatellite markers for *I. typographus* using traditional methods have failed (Sallé et al., 2003) it was decided to use the output of a next generation sequencing platform to identify new microsatellite markers. This new technology was previously successfully used to identify microsatellite loci for two hymenopteran species (*Sirex noctilio*; *Solenopsis invicta*) (Santana et al., 2009; Ross et al., 2010). To our knowledge no microsatellites for a coleopteran species developed directly from randomly amplified genomic DNA using a high-throughput sequencer have been previously reported.

MATERIAL AND METHODS

Genomic DNA was isolated from ten bark beetles according to the method of Hogan et al. (1994), following standard phenol-chloroform extraction. The DNA was subjected to 454 sequencing at the GS-FLX LAB of Eurofins MWG Operon (Ebersberg, Germany). 1/16 plate generated 10,684 reads (3.1 Mb) with an average length of 228 bp. A single Fasta file containing all reads was screened for di, tri-, tetra and pentanucleotide repeats using MSATCOMMANDER (Faircloth, 2008) with at least four repeats except for dinucleotides (6). 269 loci suitable for primer design with Primer3 software (Rozen & Skaletsky, 2000) were detected. Loci with non-desired properties were ignored as described by Opgenoorth (2009). Subsequently, the Primer sequences flanking the remaining loci were transferred into an Access© database (Microsoft Access 2007) and sorted by similarity in order to avoid the repeated selection of the same loci. In addition, the sequences were aligned by using GENEIOUS v4.7 (Drummond et al., 2009) for the same reason. Out of 135 potential loci 60 primer pairs flanking 25 di-, 25 tri-, nine tetra- and one pentanucleotide from Biomers (Ulm, Germany) were randomly ordered. The fluorescent labelling was done according to Schuelke (2000) using the universal M13 Primer. From the 60 primer pairs tested on three DNA samples of *I. typographus*, amplified products of expected size with low rates of intense stuttering were obtained for 24 of the 60 candidates.

For genotyping, 35 individual bark beetles originating from five regions (populations) in Bavaria (Germany) were used. DNA extraction was done using the method described previously. Polymerase chain reactions (PCRs) were performed in a total volume of 15 µl with the following components: 25 ng of genomic DNA, 0.2 µM of each reverse primer and the M13 universal primer (fluorescently labelled with 6-FAM), 0.05 µM of each forward primer, 0.2 mM of each dNTP (Solis BioDyne), 1.5–3 mM $MgCl_2$ (Table 1), 1× PCR buffer (Solis BioDyne) and 0.5 U *Taq* DNA Polymerase (FIREPol®, Solis BioDyne). For PCR amplification a thermal cycler (Mastercycler Gradient, Eppendorf, Germany) was used with the following PCR profile: initial denaturation at 94°C for 5 min, 30 cycles of 30 s at 94°C, 30 s at the primer specific annealing temperature (Table 1), 45 s at 72°C, followed by eight cycles of 30 s at 94°C, 45 s at 53°C, 45 s at 72°C and a final elongation step at 72°C for 10 min. PCR products were separated on 6% polyacrylamide gels on an ABI Prism 377 automated sequencer (Perkin Elmer) and scored by reference to a ROX standard (79–540 bp) by GENESCAN® 3.1.2 and GENOTYPER® 2.5 software (Applied Biosystems, Foster City, CA, USA). GENEPOP 4.0 (Rousset, 2008) was used to generate allele frequencies, to calculate expected and observed heterozygosities (H_E, H_O), and to test for linkage disequilibrium and deviations from Hardy-Weinberg equilibrium (HWE). MICRO-CHECKER 2.2.3 (Van Oosterhout et al.,

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TABLE 1. Characteristics of 18 microsatellite loci of the spruce bark beetle *I. typographus* L.: locus designation, GenBank Accession no., repeat motif (referring to 454 sequence data), primer sequences, annealing temperature (T_a), $MgCl_2$ concentration, number of observed alleles (N_A), allele size range (including length of M13 primer), level of observed (H_O) and expected (H_E) heterozygosity per locus and number (bold) of populations that deviated from Hardy-Weinberg equilibrium.

Locus	GenBank Accession no.	Repeat motif	Primer sequences (5'–3')	T_a (°C)	$MgCl_2$	N_A	Allelic size range (bp)	H_O	H_E	*HW*
ITY8	GU989603	$(GT)_8$	F: CATGCATAAAACAGTTCGTT R: CGCACTAGCGCTTCTATTTA	60	3.0	2	175–177	0.086	0.083	
ITY10	GU989604	$(GAT)_7$	F: CGTAGGCAAATCAAAAGTAGA R: CCTGAAAATAGTGCGAAATTA	60	3.0	4	256–277	0.171	0.163	
ITY11	GU989605	$(ATC)_5$	F: TCAACCATCAGTTTACTTGT R: CTTATATTTGGGTGCCATGT	60	3.0	2	174–177	0.143	0.373	
ITY12	GU989606	$(ATT)_5$	F: ATTTAGGGAAAAATCGCTGA R: TTTTAAAACAGCCTCTCTGTC	60	3.0	7	203–213	0.486	0.674	**2**
ITY14	GU989607	$(AAT)_5$	F: CAAGTAATGGTCAAGATCAAGTCA R: GTATGGTTAGGGGTGCGATG	60	3.0	2	182–185	0.314	0.269	
ITY16	GU989608	$(AAG)_5$	F: TTGATCCACACGGCAATAAA R: CGGTAAGTTGCTTCCAGAGC	60	1.5	3	202–213	0.143	0.115	
ITY19	GU989609	$(AG)_6$	F: CGAGTTGACAAGTCAAGCAAA R: ATCACACGCAATCGCAAC	60	3.0	4	155–161	0.314	0.595	
ITY22	GU989610	$(ATT)_{22}$	F: GACGTTGCTTTGTGATCGTG R: ATGGCAATTACGGACCTGAA	57	1.5	15	131–194	0.457	0.820	**3**
ITY23	GU989611	$(ATT)_7$	F: CCTACCGGCAAGAATCAAAG R: GCGTACGTCACGACCTACCTA	60	3.0	4	187–196	0.229	0.385	
ITY25	GU989612	$(ATT)_4$	F: GAAAAATTGACTGATTGGAAACG R: AAGCTGTTAACGCAGTGACG	57	3.0	4	220–229	0.457	0.560	
ITY26	GU989613	$(AACCT)_{27}$	F: GCGCGACG TGCATTATTC R: AACCAATCACAAACCATGAACA	60	1.5	38	120–540	0.657	0.969	**2**
ITY28	GU989614	$(AG)_8$	F: GCATATTTACCCCTCGGAAT R: CCCACGGAGGTTTTGGAG	60	3.0	6	182–192	0.571	0.697	**1**
ITY29	GU989615	$(AAG)_4$	F: TAAAGCAAGAGGGAGGCAGA R: TTCCCTTACTCTGAATTGTCACC	52	3.0	3	204–213	0.171	0.161	
ITY30	GU989616	$(ACT)_4$	F: CAACGTTGCCGCATATTTTT R: CCTGGATGGATGACAATTCC	60	3.0	3	324–333	0.029	0.273	
ITY33	GU989617	$(AAT)_7$	F: TGTGTTCCATCAGCACAACA R: CGTTATACCGGTTATTATGCCTTG	54	3.0	2	221–224	0.057	0.414	
ITY41	GU989618	$(ATT)_5$	F: TCTCAATTAGCTAGACGAAACA R: TTTGCTGACTTTGATTTGAA	55	3.0	2	227–236	0.257	0.227	
ITY44	GU989619	$(ATCT)_5$	F: AAAAGTGCAACGTTCTTAGC R: TAATGGGTTATCGAGTGGTG	60	3.0	4	174–220	0.429	0.367	
ITY50	GU989620	$(CTGT)_6$	F: CTGTTATTCTTAAGAGCCCATC R: ACACAGTGAGCAAAATACGA	55	3.0	2	169–173	0.101	0.114	

2004) was used to test the data set for genotyping errors and the frequency of null alleles.

RESULTS AND DISCUSSION

Eighteen loci were polymorphic with 2 to 38 alleles, averaging 5.9 alleles per locus. Their characteristics and amplification conditions are described in Table 1. Observed heterozygosity values ranged from 0.029 to 0.657, and those for expected heterozygosity from 0.083 to 0.969. No linkage was detected between loci pairs across populations in the *I. typographus* samples tested (all adjusted P values > 0.083). The test for HWE for each locus in each population revealed eight deviations in four loci after Bonferroni correction (Table 1). MICRO-CHECKER did not detect any genotyping error (e.g. scoring error) among the data set, although possible null alleles were likely at two loci (ITY12 and ITY22) resulting in the significant deviation from HWE. However, tests for HWE have a low power when sample sizes are small (Selkoe & Toonen, 2006).

This work provided 18 new polymorphic microsatellites for *I. typographus*. The six loci previously published (Sallé et al., 2003; Stoeckle et al., 2010) together with a set of the most informative loci described above provide an effective analytical tool for genetically identifying individuals and their offspring.

The major difficult encountered in developing microsatellites for Coleoptera and Lepidoptera using traditional methods is the repeated isolation of identical motifs (Arthofer et al., 2007; Meglecz et al., 2004). This is not a problem if the next generation sequencing technology in combination with the method of data screening described above is used. This technique can be used to identify microsatellites for species for which the conventional methods may be unsuccessful.

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