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Isolation and characterization of microsatellite loci for acorn weevil *Curculio bimaculatus* Faust (Coleoptera: Curculionidae)

XING-HUA HU[1], LI REN[2] and XIAO-YONG CHEN[1]*

[1]*School of Resources and Environmental Sciences, Tiantong National Field Station for Forest Ecosystems, East China Normal University, Shanghai 200062, People's Republic of China*
[2]*Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, People's Republic of China*

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Introduction

Curculio bimaculatus Faust (Coleoptera: Curculionidae) is a generalist acorn weevil in China, with female beetles laying eggs in seeds of oak trees and larvae feeding on seeds before leaving and overwintering in the soil, where they pupate, and emerge as adults in summer (Sun *et al.* 2004). This weevil infests species of Fagaceae, including stone oaks, chestnuts and oaks. Soon after its first record in Chinese chestnut in Yunnan, southwestern China, in 1987 (Luo *et al.* 1991), it has been found infesting chestnut trees in a vast area, even in northern China (Hou *et al.* 1993), and is considered as one of the most serious pests of Chinese chestnut (Zhao *et al.* 2008).

Despite the economic impact of *C. bimaculatus*, there is no information about its population genetic structure and gene flow patterns. Population genetic information can provide critical insights into range expansion and evolutionary potential to adapt to environmental changes, such as host shift and agricultural management changes. In insects, microsatellites have been proven to be a powerful tool in assessing population structure and gene flow (Voudouris *et al.* 2012), analysing inbreeding and sex ratio selection (Keller *et al.* 2011), and revealing population dynamics and rapid range expansion (Hochkirch and Damerau 2009). Polymorphic microsatellites had been developed for several Curculionidae species (Dhuyvetter *et al.* 2002; Kim and Sappington 2004; Forgie *et al.* 2006; Guzman *et al.* 2010). However, due to the species specificity of microsatellite primers, few microsatellites can be amplified across different genera. Further, cross-amplification of microsatellites can frequently cause null alleles, constraining the usefulness of microsatellites (Selkoe and Toonen 2006).

In the present study, we isolated and characterized 11 microsatellites from *C. bimaculatus*, of which 10 showed high levels of polymorphism. These markers will be a powerful tool for studies of population demographic history, genetic structure and gene flow in this species.

Materials and methods

Genomic DNA was extracted from the thoracic muscles of *C. bimaculatus* using a standard proteinase K/SDS digestion and phenol–chloroform extraction method modified from Sambrook *et al.* (1989). DNA was enriched following the enrichment protocol of Liu *et al.* (2009) and Xu *et al.* (2010). About 250 ng of genomic DNA was digested with the enzyme *Mse*I (New England Biolabs, Beverly, USA) and fragments of 200 to 800 bp were ligated with an *Mse*I site adapter pair. The adapter-ligated fragments were used as templates for PCR using *Mse*I-N primer (5′-GATGAGTCCTGAGTAAN-3′). The PCR products were denatured and hybridized to 5′-biotinylated $(AG)_{15}$ probes. Hybridization products containing microsatellites were selectively captured with streptavidin-coated magnetic beads (Promega, Madison, USA). After stringent washing, the captured DNA fragments were eluted in 50 μL of TE buffer. Using *Mse*I-N as primer, the enriched products were amplified, and then purified with a multifunctional DNA extraction kit (Bioteke Corporation, Beijing, China). The purified products were ligated into pMD19-T vector (Takara, Dalian, China) and used to transform *Escherichia coli* strain TOP10. Six hundred and eighty clones were randomly picked and tested by PCR using $(AG)_{10}$ and $M13^+/M13^-$ as primers, respectively. Of the 680 clones, 253 positive clones were scored and sequenced in an ABI 3730 DNA Sequence Analyzer (Applied Biosystems, Foster City, USA).

*For correspondence. E-mail: xychen@des.ecnu.edu.cn.

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One hundred and forty nine microsatellites were identified, of which 87 were discarded because they contained short flanking sequence, and 62 primer pairs were designed using the program Primer Premier 5.0 (http://www.premierbiosoft.com). Of the 62 primer pairs, 11 produced clear bands of amplification products with expected sizes on 1.2% agarose gel. To look for polymorphism, eight individuals from Gutian and eight from Dongshan of Zhejiang province, were randomly collected for PCR using the 11 primer pairs. Polymorphism was observed at 10 loci using 8% polyacrylamide gel electrophoresis. Subsequently, the forward primer of each polymorphic locus was labelled with one of the fluorescent dyes HEX, 6-FAM or ROX (Sangon, Shanghai, China) for further screening (table 1). The polymorphism was tested in 48 individuals collected from Gutian and Dongshan. Amplifications of microsatellite loci were performed in a final volume of 20 μL containing approximate 50 ng of genomic DNA, 0.2 mM of each dNTPs, 0.2 μM of each primer, 2.0 μL of 1× PCR buffer, 1.5 mM Mg^{2+}, and 0.4 U of *Taq* polymerase (Sangon, Shanghai, China). PCR was carried out on a Mastercycler Pro S (Eppendorf AG, Hamburg, Germany) PCR machine using an initial denaturation step at 95°C for 4 min, followed by 30 cycles of 95°C for 30 s, locus-specific annealing temperature (T_m in table 1) for 30 s, and 72°C for 30 s, and a final 10 min extension at 72°C. The amplification products were combined into four pools (pool I: *Cb107*, *Cb158*, and *Cb168*; pool II: *Cb181*, *Cb280* and *Cb393*; pool III: *Cb447* and *Cb509*; pool IV: *Cb356* and *Cb415*), each pool was diluted 10-fold and scanned by an ABI 3130 automated sequencer (Applied Biosystems, Foster City, USA) using an internal lane standard (GS500 (-250LIZ)). Allele binning and calling were done using GeneMapper 4.0 (Applied Biosystems).

We calculated genetic diversity indices at the population level using TFPGA software v1.3 (Miller 1997). Micro-Checker 2.2.3 (Van Oosterhout *et al.* 2004) was used to check for null alleles. Linkage disequilibrium was checked using FSTAT 2.9.3 (Goudet 2001).

Results and discussion

Characteristics of the microsatellite loci of *C. bimaculatus* are shown in table 1. Ten of the 11 loci were polymorphic. No significant linkage disequilibrium was found between any pair of polymorphic loci. The polymorphic loci had 4 to 26 alleles, with a mean of 13 alleles ($n = 48$ individuals). Observed and expected heterozygosity ranged from 0.167 to 1.000 and 0.451 to 0.958, respectively (table 2). Exact tests for Hardy–Weinberg equilibrium revealed a significant homozygote excess for locus *Cb447* in both Gutian and Dongshan populations. Analysis with Micro-Checker indicated null alleles for locus *Cb447* in Gutian and Dongshan populations, which may be a possible cause of its deviation from Hardy–Weinberg equilibrium in the two populations.

Although one locus in the studied populations was monomorphic, it may be polymorphic in other populations. These microsatellites will provide a powerful tool for future studies of population genetic structure, gene flow and parentage in this species.

Table 1. Characterization of 11 pairs of microsatellite primers developed for *Curculio bimaculatus*.

Locus	Primer sequences (5′-3′)	Motif	Size range (bp)	T_m (°C)	GenBank accession no.
Cb107	F: <HEX>TGCCGCTGGACAGGAAGG R: CACTTCATCTTGATCTTGTCCCGTT	$(TC)_{30}$	120–192	48	JQ083298
Cb158	F: <6-FAM>AGTCCGTTTTAGGGCACA R: TCTTCGTAGGGTATTTCG	$(AG)_{17}$	88–164	51	JQ083299
Cb168	F: <6-FAM>TGGCGAAATGACCAGAAG R: AGACGCAACGGAGCAAGA	$(TC)_{22}CC(TC)_5$	198–246	56	JQ083300
Cb181	F: <HEX>TTTTGTATGGACCTCTATTG R: GAAGGATTGACCACCTACTC	$(TC)_{18}$	162–174	50	JQ083301
Cb280	F: <6-FAM>AGAACAGATCATCTCCGACG R: GTCATTTTGGGTAACTTGTG	$(TC)_{20}$	89–95	51	JQ083302
Cb356	F:< HEX >AAAGGATAATTGCACGAC R:ACGATGATGAAAAGGAGC	$(TC)_{11}AC(TC)_{19}$	163–249	51	JQ619162
Cb393	F: <6-FAM>TAGTCCAGGAGGCAGTGAAGC R: TACGACAGGATAAAGGATAATTGCA	$(AG)_3GG(AG)_{10}$	203–247	52	JQ083303
Cb412	F: TGAGTTGTCCGCAGAATA R: ACCTTTAGCACGCTTTTC	$(AG)_{23}$	147	54	JQ083304
Cb415	F:<6-FAM>CCGATAGGCAATGGACTAAAAC R:CAAGATCACCCGACATCAGAAT	$(CT)_{15}CG(CT)_3$	225–319	50	JQ619163
Cb447	F: <6-FAM>ACACCAATCTCCGTCGTC R: CTTTCGTTTTCCCCTACC	$(GA)_{25}$	228–340	50	JQ083305
Cb509	F: <ROX>TTGGTCTGTCTACTGTGC R: CTACGTTAGCGTTTCTTC	$(GA)_7 \ldots (GA)_5$	205–215	50	JQ083307

T_m, annealing temperature. Polymorphic loci are 5′ fluorescently labelled with HEX, 6-FAM or ROX.

Table 2. Genetic variability of 10 polymorphic microsatellite loci tested in two *Curculio bimaculatus* populations.

Locus	Gutian (29°14′36.3″N, 118°06′29.8″E)				Dongshan (29°12′52.2″N, 118°08′14.0″E)			
	N	N_A	H_O	H_E	*N*	N_A	H_O	H_E
Cb107	24	23	0.833	0.942	24	20	0.958	0.958
Cb158	24	19	0.834	0.926	24	16	0.792	0.929
Cb168	24	17	0.875	0.939	24	18	1.000	0.935
Cb181	24	5	0.625	0.597	24	5	0.625	0.596
Cb280	24	4	0.667	0.567	24	4	0.626	0.490
Cb356	24	7	0.708	0.623	24	5	0.583	0.566
Cb393	24	9	0.666	0.802	24	7	0.668	0.716
Cb415	24	12	0.750	0.870	24	12	0.750	0.822
Cb447	24	9	0.333**	0.651	24	6	0.167**	0.451
Cb509	24	6	0.667	0.582	24	7	0.583	0.645
Mean	24	11	0.696	0.750	24	10	0.675	0.711

N, sample size; N_A, number of alleles per locus; H_O, observed heterozygosity; H_E, expected heterozygosity.
** Significant deviation from Hardy–Weinberg equilibrium in the populations Gutian and Dongshan.

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