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Polymorphic microsatellite loci for the rice stem borer, *Chilo suppressalis* (Walker) (Lepidoptera: Crambidae)

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Abstract

Four microsatellite loci were isolated and characterized from the rice stem borer moth *Chilo suppressalis*, which is an important insect pest of rice plants in Japan. These loci were not in linkage disequilibrium; however, in two of four loci, the observed heterozygosities were significantly less than expected, possibly due to the effect of null alleles. Although caution should be taken in analysis, these loci were sufficiently polymorphic to be used for population genetic analysis of this species.

Key words: *Chilo suppressalis*; mating time; reproductive isolation; microsatellites

INTRODUCTION

The rice stem borer moth, *Chilo suppressalis* (Walker) is an important insect pest of rice plants in Japan (Kiritani, 1988). Two host races are known in the species: one feeds on rice (*Oryza sativa*) and the other on water-oats (*Zizania latifolia*). Many studies have tried to detect the difference between rice feeders and water-oat feeders in various aspects, such as body size and mating time. There is consistent agreement that the body size and mass of adults and immature insects collected from water-oats are larger than those from rice (e.g. Maki and Yamashita, 1956; Takasaki et al., 1969). Konno and Tanaka (1996a) observed a clear difference in the mating time between the two feeders under laboratory conditions: most rice feeder mating was observed during the first half of the scotophase, while that of water-oat feeders was observed during the late scotophase. Konno and Tanaka (1996b) also showed significant differences between the two feeders in insecticide susceptibility and activity of the aliesterase isozyme. These findings suggest that the two feeders may essentially belong to different strains and could be reproductively isolated from each other. To evaluate the magnitude of gene flow between the two feeders with a fine scale, it is desirable to develop a specific microsatellite marker for this species. Here, we reported polymorphic microsatellite loci of *C. suppressalis* developed using two methods, a traditional colony hybridization method (Rassmann et al., 1991) and a dual-suppression PCR technique (Lian et al., 2001).

MATERIALS AND METHODS

Insects. Larvae of *C. suppressalis* were collected from the remaining stub of rice plants after harvesting in Kaizu town, Gifu Prefecture, Japan. The larvae were reared on rice seedlings under laboratory conditions (15L:9D, 25±1°C), and the resulting adult moths were used for the isolation and characterization of microsatellites.

The traditional colony hybridization method. Template DNA was extracted from the heads and metasomata of five adult males and females of *C. suppressalis* and digested to completion with the restriction enzyme *Sau*3A. DNA fragments ranging from 300 to 600 bp were collected from 1.5% agarose gel. The fragments were ligated into plasmid pUC18 previously digested with *Bam*HI. These ligation mixes were used to transform *Escherichia coli* DH5α. Transformed bacterial colonies were screened with oligonucleotides (5′-$(GT)_{10}$-3′) labeled by the ECL 3′-oligolabelling

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system (Amersham). Positive clones were sequenced with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems), and primer pairs were designed for flanking regions surrounding the microsatellites.

Dual-suppression PCR technique (Lian et al., 2001). Template DNA was extracted as described above. Adaptor-ligated, restricted DNA libraries were constructed after Siebert et al. (1995). DNA was separately digested with *Eco*RV, *Ssp*I, *Alu*I, *Sma*I, *Acc*II or *Hae*III blunt-end restriction enzymes. The fragments were then ligated to a specific adaptor (consisting of a 48-mer: 5′-GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT-3′ and an 8-mer with the 3′-end capped by an amino residue: 5′-ACCAGCCC-NH_2 3′) by a DNA Ligation Kit ver. 2 (Takara).

As the first step for isolating microsatellite loci, fragments flanked by the $(AC)_n$ microsatellite at one end were amplified from the constructed *Eco*RV DNA library by the $(AC)_{10}$ primer and adaptor primer (AP2; sequence shown below) designed from the longer strand of the adaptor. The amplified fragments were directly ligated into the TA vector that attached dTTP to plasmid pUC18 previously digested with *Eco*RV. The plasmids were transformed into *E. coli* DH5α. Plasmid DNA extracted from positive clones was sequenced with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Fragments containing $(AC)_n$ microsatellite sequences at one end were chosen for the next step analysis.

The second step was to determine the sequence of the other flanking region of each microsatellite. The first primer (IP1) designed from the sequenced region flanking the microsatellite and, for nested polymerase chain reaction (PCR), another first primer (IP2) based on the sequence between IP1 and the microsatellite, were prepared. As adaptor primers (AP1 and AP2) for nested PCR, AP1 (5′-CCATCGTAATACGACTCACTATAGGGC-3′) and AP2 (5′-CTATAGGGCACGCGTGGT-3′) were also prepared. The primary PCR reaction was conducted with each constructed DNA library using IP1 and AP1 primers. The secondary PCR reaction was conducted with 100-fold dilution of primary PCR products using IP2 and AP2 primers. Single-banded fragments were cloned and sequenced as described above. A second primer from the newly defined flanking sequence was designed for amplification of the region containing a microsatellite.

Characteristics of microsatellite loci. To investigate the characteristics of microsatellite loci amplified by our primer pairs, template DNA was extracted from adult moths (rice feeders collected as described above, $n=30$). The tissue was suspended in 300 μl of extraction buffer (3 μl of 1 M Tris HCl pH 8.0, 6 μl of 0.5 M EDTA, 45 μl of 1 M NaCl, 3 μl of 10% SDS, 243 μl of double-distilled water and 0.09 μl of 20 mg/ml proteinase K) and incubated overnight at 37°C. DNA was extracted by phenol/chloroform (Sambrook and Russell, 2001). Isolated DNA was resuspended in 100 μl of TE and stored at −20°C until the polymerase chain reaction (PCR). The PCR solution contained 10 μl total volume made up of 1 μl genomic DNA (≈1 ng), 2 μl of primer mix (5 pmol/μl), 1 μl of 10×PCR buffer, 0.1 μl of dNTP (2.5 mM each), 5.85 μl of double-distilled water and 0.05 μl Taq DNA polymerase (5 units/μl). PCR was carried out using a thermal cycler (TP-240; Takara). After a denaturing step of 3 min at 94°C, the samples were processed through 27 cycles (Table 1) consisting of 1 min at 94°C, 1 min at 56°C (Table 1) and 1 min at 72°C, followed by a final extension step of 7 min at 72°C. PCR products were run on 8% native polyacrylamide gels (140 mm in height) for 2 h (250 V) and visualized using silver staining (Bassam et al., 1991). Genotyping was conducted using 1D IMAGE ANALYSIS software (Kodak) installed on a Macintosh computer.

RESULTS AND DISCUSSION

In the traditional colony hybridization method, fifteen positive clones from 2,300 colonies were sequenced. Five primer pairs could be designed for the flanking regions surrounding the microsatellites. However, no appropriate primer pairs could be designed for the other 10 sequences because the sequences lacked microsatellites or the flanking region adjacent to either side of the microsatellite. Two of the five primer pairs successfully amplified products showing polymorphisms (Cs1 and Cs5). The other three primer pairs could not successfully amplify the products to the lengths expected from the sequences. The number of isolated microsatellites of the rice stem borer moth was approximately

seven-fold lower than that of the Japanese paper wasp *Polistes chinensis antennalis*, isolated by the same laboratory and method (Tsuchida et al., 2003). Developing microsatellite markers in lepidopteran insects is problematic due to the low frequency of microsatellites in the genome (Bogdanowicz et al., 1997; Meglécz and Solignac, 1998; Nève and Meglécz, 2000; Ji et al., 2003). Therefore, we used the dual-suppression PCR technique to isolate microsatellites because this method is thought to develop efficient microsatellite markers without enrichment and screening procedures (Lian et al., 2001). We designed seventeen first primers (IP1 and IP2) and the resulting 75 clones were sequenced, but only two second primers were successfully designed (Cs11 and Cs20). The sequences, including microsatellites, have been deposited in DDBJ under Accession numbers AB219971–AB219974 for loci Cs1–Cs20.

Although the dual-suppression PCR technique was thought to identify microsatellites efficiently in the first step, we were not able to design second primers efficiently in the second step. In the second step, single-banded fragments amplified by nested PCR using the first primers (IP1 and IP2) and adopter primers (AP1 and AP2) were needed; however, our first primers amplified multiple-banded fragments. It is thought that fragments other than the target sequence were amplified because the sequence of our first primers straggled within the genome. Ji et al. (2003) reported that more than 70% of microsatellite loci cloned from the cotton bollworm *Helicoverpa armigera* have one or both flanking regions formed of repetitive DNA that occur several times in the genome (see also Zhang, 2004). Thus, the association of microsatellites with such repetitive sequences in the flanking regions appears to be a characteristic of lepidopteran microsatellites (Zhang, 2004). Therefore, this specific microsatellite characteristic in lepidopteran insects might hamper the acquisition of single-banded fragments including the target microsatellite sequence in the second step in our study.

Each locus was tested for genotyping linkage disequilibrium using ARLEQUIN version 2.000 software (Schneider et al., 2000). None of the tested loci showed significant linkage disequilibrium ($p>0.05$). The number of alleles detected ranged from 3 to 12 per locus, with an average of 7.0 (Table 1). ARLEQUIN version 2.000 (Schnei-

Table 1. Microsatellite loci for *Chilo suppressalis* (rice feeders, $n=30$). T_a, optimal annealing temperature for PCR amplification; N_a, number of alleles observed; H_E, expected heterozygosity; H_O, observed heterozygosity; N_n, number of non-amplified samples

Locus	Repeat motif	Primer sequence (5′–3′)	Allele size[a] (bp)	T_a (°C)	Cycles	N_a	H_E	H_O	N_n
Cs1	$(AC)_9$	ACATCCAAGCGTATTCACCC ACTCCTACGGTCAGATTGTG	73 [1], 75 [21], 77 [7], 79 [9], 81 [7], 83 [3], 85 [9], 87 [1]	60	29	8	0.806	0.571	1
Cs5	$(GT)_{12}$	GCAAAAAAGGAGCAACG TGAATTACGAGCCAGTCCC	164 [2], 168 [11], 170 [6], 172 [3], 174 [7], 176 [3], 178 [11], 180 [7], 188 [2], 190 [2]	61	27	12	0.880	0.500	3
Cs11	$(CA)_7$	CCTGTGGGTAGACGGAAAAT GAGTTGGGACCGATTTGGGA	137 [30], 139 [9], 141 [5], 143 [12], 145 [2]	55	28	5	0.691	0.345	1
Cs20	$(CA)_{14}$	CTTTTGAGTTTATCCCGG TACATCGTCCAAGCAT	179 [7], 185 [29], 187 [16]	55	29	3	0.620	0.654	4

[a] Numbers in brackets indicate the observed number of alleles.

der et al., 2000) was employed to calculate the expected and observed heterozygosities and Hardy-Weinberg equilibrium for all loci. The expected heterozygosities ranged from 0.620 to 0.880. Two of the four loci (Cs5 and Cs11) significantly deviated from the Hardy-Weinberg equilibrium due to an excess of homozygotes (Table 1). Furthermore, tests for null alleles using MICRO-CHECHER software (van Oosterhout et al., 2004) were significant at Cs5 and Cs11, and the frequencies of null alleles were 0.142 and 0.184, respectively. Additionally, there were one to four non-amplified samples in Cs1–Cs20: these could be null allele homozygotes (Table 1). In previous studies of microsatellite markers in Lepidoptera, excess homozygotes and the presence of null alleles were observed (Bogdanowicz et al., 1997; Meglécz and Solignac, 1998), suggesting that the flanking regions of microsatellites in Lepidoptera are more variable than in other insect orders like Hemiptera (Meglecz et al., 2004). Although caution should be taken in analysis, these microsatellite loci provide a powerful means to examine the genetic structure of *C. suppressalis*.

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