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Identification of Y Chromosomal PCR Marker and Production of a Selected Strain for Molecular Sexing in the Brown Planthopper, *Nilaparvata lugens*

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A laboratory colony was established in order to enable molecular sexing in premature stages in the brown planthopper, *Nilaparvata lugens*. We found four male-specific amplified fragment length polymorphisms (AFLPs) in the planthopper, and sequenced one of the AFLPs along with its 5′ flanking region (1,423 bp in total). PCR primers were designed based on the nucleotide sequence information so that the PCR product was present in male planthoppers and absent in female planthoppers. However, we could not completely distinguish males from females, because the PCR amplification product was absent in some of the males screened. We, therefore, established a laboratory colony, in which all males carried this sequence. We can directly sex pre-adult stages in this colony using our PCR primers, making this strain of considerable value for studies that require sex separation in egg and nymphal stages. Arch. Insect Biochem. Physiol. 65:1–10, 2007.

KEYWORDS: molecular sexing; Y chromosome; sex-specific DNA sequence; AFLP; *Nilaparvata lugens*

INTRODUCTION

In many insect species, males and females are morphologically indistinguishable at larval or nymphal stages. This can be problematical for physiological or biochemical studies that require the separation of the sexes in early or middle stages of pre-adult development. For example, the brown planthopper, *Nilaparvata lugens*, shows a wing dimorphism (long wing and short wing forms) that is induced by environmental stimuli during nymphal stages. Interestingly, there is a sex-related difference in response to the stimuli (Kisimoto, 1956; Watanabe, 1967; Morooka et al., 1988). However, a detailed molecular analysis of the sex differences in responsiveness to environmental stimuli is hindered by the difficulty of distinguishing male and female nymphs at the critical developmental stages. In order to analyze the effect of the stimuli in nymphs, we need reliable sex discrimination methods in pre-adult stages.

DNA-based sex identification has proven to be effective in mammals (Handyside et al., 1990), birds (Griffiths et al., 1998; Bello and Sanchez, 1999), fish (Kovacs et al., 2000), and insects (Douglas et al., 2004). Molecular sexing in these species is based on polymerase chain reaction (PCR) amplification of DNA sequences on the sex chromosomes. Comparative random amplified polymorphic DNA (RAPD) assays are often used

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Abbreviations used: AFLP = amplified fragment length polymorphisms; bp = base pair; EST = expressed sequence tag; RAPD = random amplified polymorphic DNA; SCAR = sequence characterized amplified region.

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for detecting sex-specific DNA sequences. Likewise, AFLP makers are reproducible and reliable (e.g., Vos et al. 1995; Griffiths and Orr, 1999). In their construction of a genetic map of barley, Mano et al. (2001) devised a simple and efficient method to analyze AFLPs that makes use of small slab gels and a discontinuous Tris-glycine buffer system.

Sex-specific DNA sequences have been identified in several insect species, such as *Drosophila melanogaster* (Williams and Furnier, 1987; Hennig, 1990), the Mediterranean fruit fly *Ceratitis capitata* (Zhou et al., 2000), the malaria mosquito *Anopheles gambiae* (Holt et al., 2002, Krzywinski et al., 2004), and the silkworms *Bombyx mori* and *B. mandarina* (Abe et al., 1998). The sex chromosome system of *C. capitata* is XX in the female and XY in the male. Males are discriminated by amplifying repetitive A-T-rich DNA sequences from the Y chromosome. In *B. mori*, the sex chromosomes are ZW in the female and ZZ in the male. Female-specific DNA sequences, derived from the W chromosome, are detected using RAPDs (random amplified polymorphic DNAs) (Abe et al., 1998).

N. lugens has XX chromosomes in the female and XY in the male (Noda and Tatewaki, 1990). To date, however, Y-specific DNA sequences have not been identified. In this report, we used a modified AFLP system to detect male-specific DNA fragments in *N. lugens* and have developed a rapid PCR-based sexing technique that has proved to be reliable for a newly established strain of this species.

MATERIALS AND METHODS

Insects

Two geographical strains of the brown planthopper *N. lugens* were used. The Izumo strain was collected in the Shimane Prefecture of Japan in 1987 and the Nagasaki strain in Nagasaki Prefecture in 2000. Successive generations were reared on rice seedlings under a 16L:8D photoperiodic regime at 25°C.

DNA Extraction

Genomic DNA for AFLP analysis was purified from 15 planthoppers using the GenomicPrep Cells and Tissue DNA isolation kit according to the manufacturer's instructions (Amersham Biosciences), starting with a volume of 360 µl cell lysate buffer. The DNA was further purified by extraction with phenol/chloroform and precipitated with isopropanol. DNA concentrations were measured using a spectrophotometer and the DNA was dissolved at a concentration of 50 ng/µl. Genomic DNA for the population molecular sexing screen were purified from adult or nymphal insects using the same DNA isolation kit but starting with a volume of 60 µl of cell lysate buffer. The DNAs recovered were dissolved in 80 µl of water.

AFLP

AFLP analysis was mainly performed using the method of Kikuchi et al. (2003). A total of 250 ng of genomic DNA was completely digested with restriction enzymes (1.25 U EcoRI and 1.25 U MseI) in a volume of 25 µl for 3 h at 37°C. EcoRI adaptor and MseI adaptor were ligated to the digested genomic DNA using T4 DNA ligase (Takara) at 25°C for 3 h. The digested and ligated DNA was preamplified with EcoRI universal primer (E: 5′-GACTGCGTACCAATTC-3′) and MseI universal primer (M: 5′-GATGAGTCCTGAGTAA-3′). The preamplification profile included an initial denaturation for 2 min at 94°C, followed by 20 cycles of a 30-sec denaturation at 94°C, a 1-min annealing at 56°C, and a 1-min extension at 72°C, followed by a final 10-min extension at 72°C. Selective amplification was carried out using the preamplified products with randomly selected primer sets from one of EcoRI selective primers (E-AAG, E-ACC, E-ACG, E-AGA, E-AGC, E-AGG, E-CAG, E-CCA, E-CCT, E-CGA, E-CTC, E-GAA, E-GAG, E-GGT, E-TAC, E-TAG, E-TAT, E-TCG, E-TCT, E-TGA, E-TGC, E-TGG) and one of the MseI selective primers (M-AAG, M-AAT, M-ACT, M-AGA, M-AGC, M-AGG, M-ATC, M-ATT, M-CAA, M-CAG, M-CCC, M-CCG, M-CTA, M-GAA, M-GCG, M-GGA, M-GGG, M-GTA,

M-GTG, M-GTT). This reaction was performed for 40 cycles with the following cycle profile: a 30-s DNA denaturation step at 94°C, a 30-s annealing step (see below), and a 1-min extension step at 72°C. The annealing temperature in the first cycle was 65°C; this was reduced at each cycle by 0.7°C for the next 17 cycles, and was continued at 56°C for the remaining 23 cycles. All amplification reactions were performed in a PTC-200 thermocycler (MJ Research, Inc). Products of a PCR reaction were analyzed on a polyacrylamide denaturing gel (1-mm thick, 160 × 160 mm, 15 lanes). The gel consisted of 6% stacking gel (20 mm) containing 125 mM Tris-HCl (pH 7.0) and 8.5 M urea, and 7% separating gel (100 mm) containing 375 mM Tris-HCl (pH 9.2) and 8.5 M urea. Running buffer consisted of 25 mM Tris and 192 mM glycine. Sixteen microliters of each sample was loaded on the gel. The samples were electrophoresed at 150 V for 30 min and at 270 V for 2 h at 37°C in the incubator. The gel was stained with a GelStar nucleic acid gel stain (Cambrex).

Cloning and Sequencing of the AFLP Fragment and Its Proximal 5′ Region

AFLP markers of interest were excised from the polyacrylamide gels and the piece of gel containing the DNA band was ground with a pellet pestle in a tube with 30 µl TE (10 mM Tris-HCl, 1 mM EDTA). The AFLP fragment was subcloned into pCR II-TOPO cloning vector using TOPO TA cloning Kit (Invitrogen) and sequenced in both directions.

The proximal 5′ region of the AFLP fragment M3 was isolated by PCR using an LA PCR in vitro cloning kit in accordance with the manufacturer's instructions (Takara). The sequence of M3 was used to design two fragment-specific antisense primers: PM3R1, 5′-AGAAGGTTGTTTCGCAGCTC-3′ and PM3R2, 5′-CCTCATACACTGGCAGGTCA-3′. These were employed to amplify the upstream region of M3 of male genomic DNA. Template genomic DNA was digested with one of the restriction enzymes, EcoRI, HindIII, PstI, SalI, or XbaI. Amplified DNA fragments produced by PCR were each subcloned into pCR II-TOPO cloning vector and sequenced.

Several primers for SCAR marker construction were designed. A primer set of PM3F (5′-GGC AGTTGTCAGGGCAGTAG-3′) and PM3R3 (5′-AGA AGGTTGTTTCGCAGCTC-3′) amplified a DNA band of 1.2 kbp from female genomic DNA. The DNA fragment produced was subcloned and sequenced. The sequence was compared with male-specific sequence (M3n) by the CLASTAL-W program (Thompson et al., 1994).

Production of a Male-Specific Genomic DNA Fragment

PCR primers were designed to amplify the male-specific sequence (PM3nF and PM3nR), and also the sequence common to both sexes (PM3femaleF and PM3femaleR) as a positive control (see Fig. 2). PCR was performed on 1 µl of genomic DNA in 30 µl of reaction mixture (10 mM Tris-HCl, pH 8.3/50 mM KCl/2.5 mM $MgCl_2$/500 µM of each dNTP/1 µM of each oligonucleotide primer/0.5 U of *Taq* DNA polymerase; Invitrogen). Forty cycles of amplification were performed, each of which consisted of denaturation for 30 s at 94°C, annealing for 30 s at 56°C, and extension for 30 s at 72°C. Samples of the PCR reaction were analyzed on 2% agarose gels.

Establishment of a New Strain for Molecular Sexing

Last instar nymphs of the Nagasaki Strain were collected and reared individually in the test tube with rice seedlings. Newly emerged males and females were paired and individually mated in test tubes. After mating for two days, males were collected and DNA extracted. The presence or absence of the M3n sequence was determined by PCR using PM3nF and PM3nR primers as described above. Eggs deposited by females mated with M3n-positive males were collected and reared for successive generations on rice seedlings under a 16L:8D photoperiodic regime at 25°C in order to create a colony in which all the males were positive for the male-specific sequence.

RESULTS

Identification of Male-Specific AFLPs

To identify male-specific AFLPs, 42 EcoRI-MseI primer combinations were screened in male and female genomic DNA of the *N. lugens* Izumo strain. On average, approximately 30 fragments were resolved for each primer pair by polyacrylamide gel electrophoresis. Four male-specific bands were detected and designated as M1, M2, M3, and M4 (Fig. 1). These bands were gel-purified, cloned, and sequenced. AFLP M1, amplified with selective primers E-GGT (5′-GACTGCGTACCAATTCGGT-3′) and M-CCC (5′-GATGAGTCCTGAGTAACCC-3′), consisted of 613 bp. AFLPSs M2 and M3, amplified with selective primers E-TAC (5′-GACTGCGTACCA ATTCTAC-3′) and M-GCG (5′-GATGAGTCCTGA GTAAGCG-3′) were 431 and 294 bp, respectively. AFLP M4 amplified with selective primers E-GTA (5′-GACTGCGTACCAATTCGTA-3′) and M-AGA (5′-GATGAGTCCTGAGTAAAGA-3′) was 225 bp. GenBank searches using BLASTN for nucleotide sequence similarities and BLASTX for deduced amino acid (aa) sequence similarities revealed that the M1, M2, and M4 sequences were not homologous to any reported sequences from other species, whereas the amino acid sequence of M3 showed 70% homology with the human E3 ubiquitin protein ligase URE-B1 (HSPC272).

To convert these AFLPs into sequence characterized amplified region (SCAR) markers (Paran and Michelmore, 1993), which are amplified as a single band without any additional PCR band, several primers for PCR were designed using the sequence data. For all four AFLP fragments, the internally designed primers amplified PCR products of the expected size from template DNA of both males and females (data not shown). As we were unable to obtain the male-specific SCAR marker from these AFLPs sequences, we used LA-PCR in vitro cloning to amplify the 5′ flanking region of M3. The 5′ flanking region of M3 was isolated from male genomic DNA using PstI. The single amplified fragment (≈1.2 kbp) was gel-purified, cloned, and sequenced. The combined segment, consisting of 5′ flanking region and the original M3 fragment, was designated M3n (gene accession no. AB247939). This segment was 1,493 bp in length and contained several incomplete amino acid coding regions.

Many primers designed to amplify the internal sequence of M3n produced amplification products from both male and female genomic DNA. This suggests that the female genome contains a sequence similar to M3n. A pair of primers designed for each end of M3n (PM3F 5′-GGCAGTTGT CAGGGCAGTAG-3′ and PM3R3 5′-AGAAGGTTG TTTCGCAGCTC-3′) gave a product of the expected size from both sexes. However, comparison of the florescence-intensities of the bands on agarose gels suggested that the amount of DNA amplified from the female was considerably smaller than from the male. The product amplified by PCR of female DNA was cloned, sequenced, and compared with the M3n sequence (M3female, GenBank accession no. AB259 832). M3n and M3female shared 90.56% nucleotide sequence identity. Newly-designed two M3n specific (male-specific) primers PM3nF (5′-GAG CTGGAGTGTGTTGATGT-3′) and PM3nR (5′-CAA GTTGATTGAAACAGACT-3′), were identified as good candidates for a SCAR marker (Fig. 2). PCR

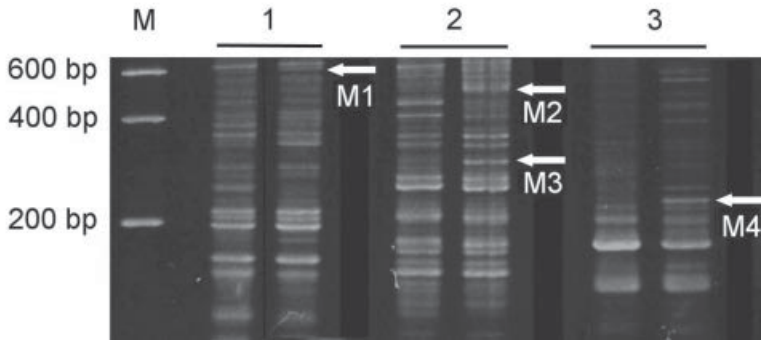

Fig. 1. Male-specific AFLP patterns of genomic DNA from males (right) and females (left). The arrows indicate the male-specific AFLPs. Lane M, 200 bp DNA molecular size marker (Takara); 1, E-GGT and M-CCC primers; 2, E-TAC and M-GCG primers; 3, E-GTA and M-AGA primers.

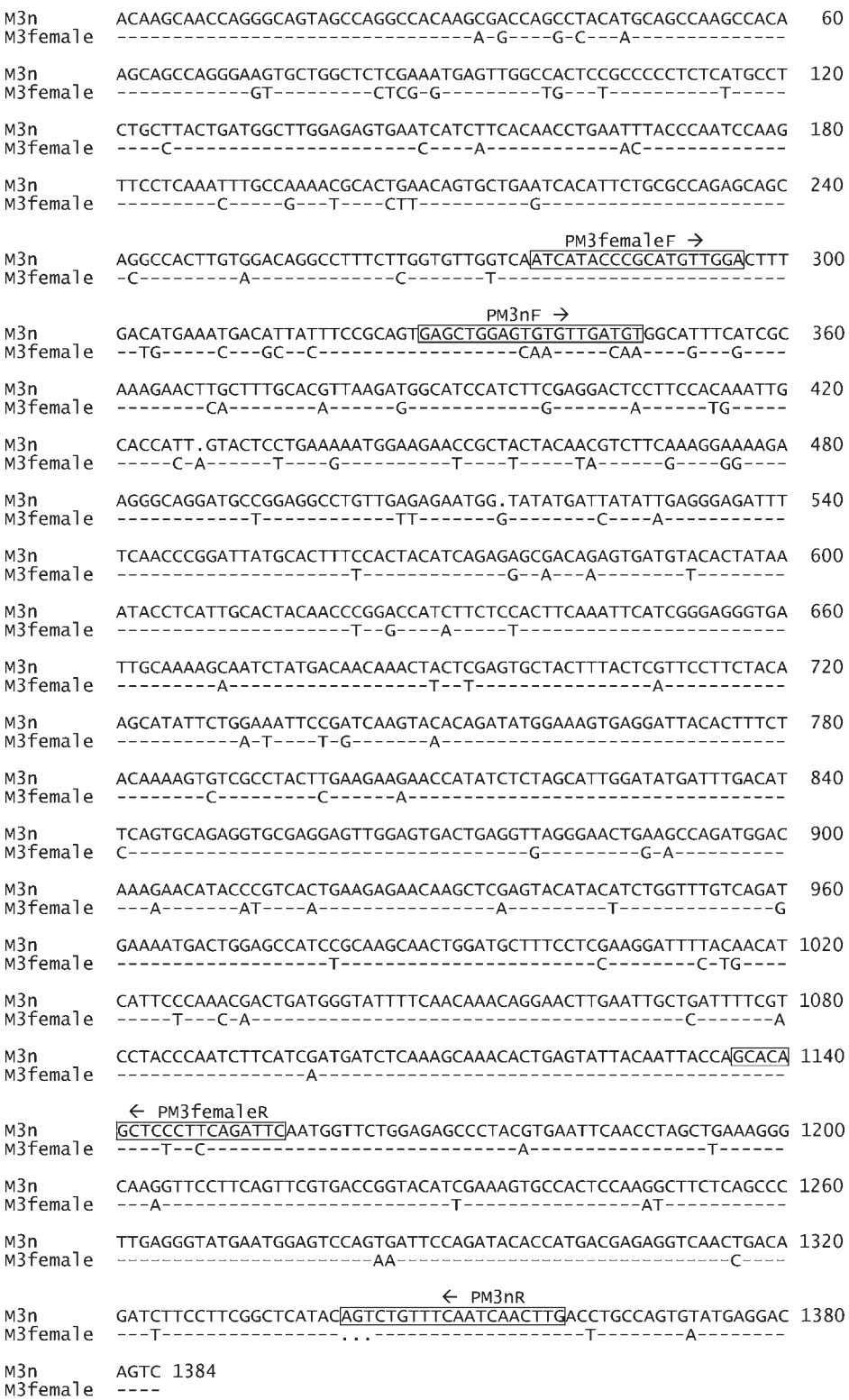

Fig. 2. Comparison of the male-specific M3n sequence and the similar nucleotide sequence detected in females, M3female. A dash (–) indicates the same nucleotide as above and a dot (•) indicates no corresponding nucleotide. Boxes indicate the locations of primers used in this study.

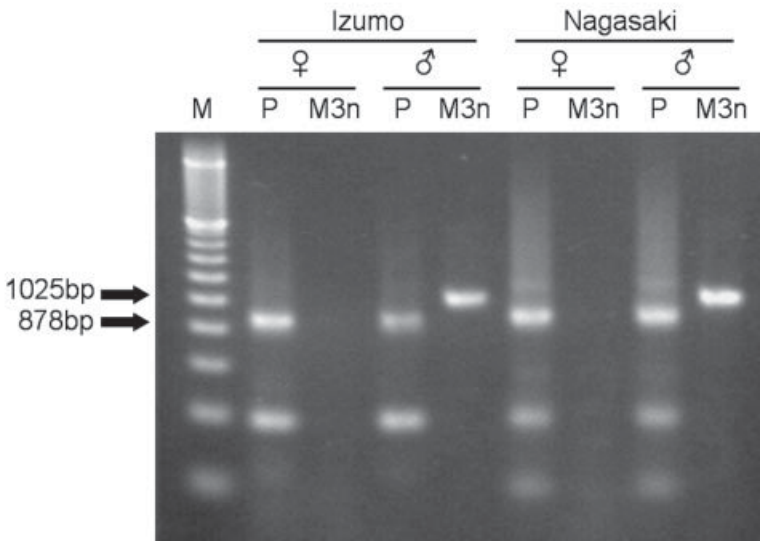

Fig. 3. PCR amplification patterns of genomic DNA from different geographic populations of *N. lugens*; Izumo strain (left) and Nagasaki strain (right). Lane P, positive PCR control using the primer pair PM3femaleF (5′-ATCATACCCGCATGTTGGAC-3′) and PM3femaleR (5′-GAATCTGAAGGGAGCTGTGC-3′). Lane M3n, DNA fragment amplified using the male-specific primer pair PM3nF (5′-GAGCTG GAGTGTGTTGATGT-3′) and PM3nR (5′-CAA GTTGATTGAAACAGACT-3′). Lane M, 200-bp DNA molecular size marker (Takara).

using this primer pair produced a unique band of 1,100 bp only in males in two planthopper strains (Fig. 3). We also designed a set of primers, PM3-femaleF (5′-ATCATACCCGCATGTTGGAC-3′) and PM3femaleR (5′-GAATCTGAAGGGAGCTGTGC-3′), which amplified a sequence in both sexes and thereby could be used to ensure that the lack of amplified fragments in female with the PM3nF and PM3nR primers was not caused by the failure of the PCR procedure. The PM3femaleF and PM3-femaleR primer set produced a clear band of 850 bp in both sexes, and also an unexpected, weaker band of 390 bp (Fig. 3).

Population Screen Using Primers PM3nF and PM3nR

The PM3nF and PM3nR primer pair was tested for its specificity in discriminating males in a laboratory population of *N. lugens*. Genomic DNA was extracted from adults of the Izumo strain and subjected to PCR amplification. The primer set produced the expected size of DNA fragment in only 50% (3 of 6) of the adult males and none of the adult females, whereas the primer set of PM3-femaleF and PM3femaleR produced 850-bp bands in every individual of both sexes. A similar result was obtained in the Nagasaki strain. Amplification was observed only in 37.8% (45/119) of adult males (data not shown) whereas the control primers of PM3femaleF and PM3femaleR produced DNA of the expected size in all individuals. The PM3nF/PM3nR primer set was not sufficiently specific for use in identifying male DNAs as it did not amplify the expected PCR product in all males.

Establishment of a New Laboratory Strain Amenable to Molecular Sexing

As described above, male planthoppers showed heterogeneity for the M3n sequence. We selected offspring of males that had the M3n amplification product. These offspring should possess the M3n sequence or one that is very similar. To confirm the validity of the molecular sexing method, genomic DNA was extracted from offspring in the new strain once they had reached the adult stage and PCR was performed using the PM3nF and PM3nR primers. These primers produced a DNA fragment of about 1,100 bp in all of the adult males tested (24 of 24) but in none of the adult females (Fig. 4). The primer combination PM3-femaleF and PM3femaleR produced 850- and 390-bp bands in every individual of both sexes.

Genomic DNA was extracted from abdomen of the 3rd instar nymphs and used for PCR amplification. The PM3n/PM3nR primers produced a DNA fragment in about half of the nymphs (326 of 688, 47.4%) (Fig. 5) whereas the primer combination of PM3femaleF and PM3femaleR produced 850- and 390-bp bands in every individual.

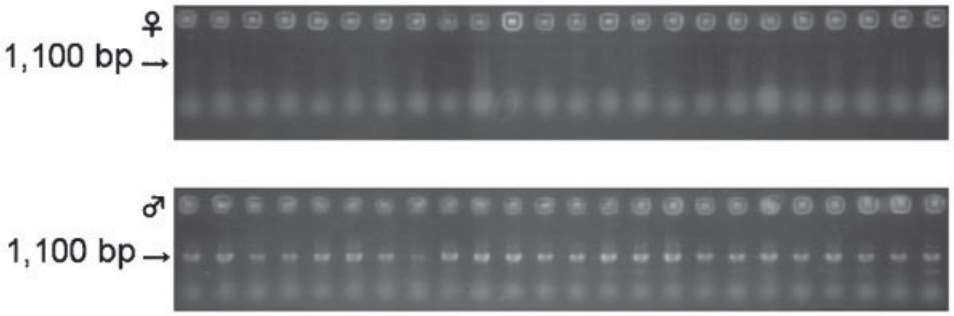

Fig. 4. PCR amplification using the male-specific primers PM3nF (5′-GAGCTGGAGTGTGTTGATGT-3′) and PM3nR (5′- CAAGTTGATTGAAACAGACT-3′) on genomic DNA from adults of the newly established planthopper colony. Twenty-four females (above) and 24 males (below) of the new strain were tested.

DISCUSSION

Molecular genetic methods for discriminating the sexes require different approaches in different species. In *Ceratitis capitata*, ribosomal DNA arrays are localized on the X and Y chromosomes (Bedo and Webb, 1989) and polymorphisms of the ITS1 region of the ribosomal DNA have been reported (Douglas, 2001). Additionally, an Y114 A-T rich repetitive DNA sequence element was identified from the Y chromosome (Zhou et al., 2000). Two molecular sexing methods have been described in *C. captita*: the first is based on the restriction fragment length polymorphisms (RFLP) obtained after enzymatic digestion of a PCR amplification product from the ITS1 ribosomal DNA sequence; the second screens for PCR amplification of the Y114 sequence of the Y chromosome (Douglas et al., 2004). The RAPD assay has been used to identify sex-linked DNA sequences in several species. In *Bombyx mori*, Abe et al. (1998) identified RAPD nucleotide sequences on the W chromosomes; they converted these sequences into a SCAR marker. The RAPD protocol is simple, using short primers in a low-stringency PCR to generate randomly primed products that are separated and compared on an agarose gel. However, the RAPD assay has been criticized because variation in the number and concentration of the products may result from small changes in the reaction conditions. Griffiths and Orr (1999)

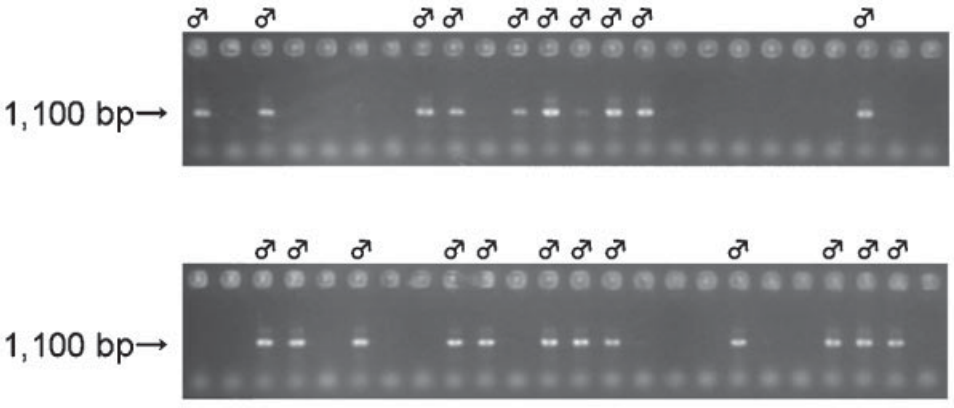

Fig. 5. Typical PCR amplification results using the male-specific primers PM3nF (5′-GAGCTGGAGTGTGTTGATGT-3′) and PM3nR (5′-CAAGTTGATTGAAACAGACT-3′) on genomic DNA from 3rd instar nymphs. Forty-eight individuals from the new strain are shown. The presence of the 1,100-bp band PCR product shows that this individual possesses the male-specific DNA sequence.

used the AFLP approach to isolate a sex-linked marker in the ostrich (*Struthi camelus*) whose W and Z sex chromosomes show little genetic divergence. The AFLP reaction is stable across a 1,000-fold dilution in template quantity and the intensity of the AFLP bands do not greatly vary.

N. lugens has a diploid chromosome number of 30 and the sex-determining system is XX in the female and XY in the male (Goh et al., 1988, Noda and Tetewaki, 1990). In this report, we have identified and determined a male-specific nucleotide sequence, termed M3n, in *N. lugens*. PCR primers designed from this sequence amplified a male-specific band from the genomic DNAs of two different geographic populations of *N. lugens*. Our results suggest that M3n is derived from the Y chromosome since the other chromosomes are obviously shared in both sexes. This is the first report to identify sequences from the Y chromosome of this species. The presence of a male-specific sequence supports the conclusion of the previous cytological reports that male brown planthopper *N. lugens* has an XY sex chromosome system. Noda and Tatewaki (1990) reported that two other rice planthopper species, the small brown planthopper *Laodelphax striatellus* and the whitebacked planthopper *Sogatella furcifera*, do not possess Y chromosomes, that is, the sex determination system of theses species is XX in the female and XO in the male. The reason for the presence of a Y chromosome and its function in the brown planthopper, *N. lugens*, remain unknown.

We detected only four male-specific AFLPs in about 1,200 fragments produced using 42 primer pairs. Moreover, PCR primers designed using these AFLPs proved not to be male-specific. This suggests that the Y chromosome of *N. lugens* shares similar sequences with autosomes or the X chromosome. In the domestic silkworm *Bombyx mori*, Abe et al. (1998) found only four RAPDs on the W chromosome using about 700 arbitrary primers. They pointed out that difficulty in finding a RAPD marker on the W chromosome may arise because the greater part of the W chromosome of *B. mori* consists of sequences similar and homologous to dispersed DNA sequences on the autosomes and on the Z chromosome. The male-specific AFLP M3n of *N. lugens* was similar to an EST clone of the same strain (Izumo) of this species. However, the sequences of M3n, M3female, and the EST clone were not identical. The slight difference among the three indicates that *N. lugens* would appear to have a number of DNA sequences in its genome that resemble M3n. The male-specific band was detectable in two strains of *N. lugens* collected in different years and at different locations (Izumo strain and Nagasaki strain). We also tested another strain (Oita strain collected in 2005) and observed the male-specific band in six of eight males. Accordingly, we conclude that the M3n sequence is widespread in this species. Although, unfortunately, we could not develop a SCAR marker that is applicable to field populations of the planthopper, our purpose is not sex discrimination of the various field populations. We aim at obtaining an experimental colony in which we can easily distinguish sex in premature stages, and we could have established the colony amenable to molecular sexing.

Our method of molecular sexing is based on the presence or absence of the products of PCR amplification of a SCAR marker. Absence of the sex-specific DNA fragment results from either the lack of Y chromosomal DNA in the sample or from failure of amplification. To ensure that the results are reliable, it is desirable to perform a co-amplification of a DNA sequence that originates from the autosome or the X chromosome. We designed a set of primers, PM3femaleF and PM3femaleR, to test for amplification failure. Samples from which the PCR products are amplified by these primers can reliably be used for molecular sexing. The method we describe here for molecular sexing will be a useful tool for the analysis of sex-specific patterns of gene expression.

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