韓應昆誌 43(4): 311~315 (2004)
Korean J. Appl. Entomol.

Isolation and Characterization of Microsatellites in the Brown Planthopper, *Nilaparvata lugens* Stål

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벼멸구(*Nilaparvata lugens*)에서 마이크로새털라이트 마커의 분리 및 특성검정

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ABSTRACT : The brown planthopper, *Nilaparvata lugens*, is among the most serious insect pests of rice. It is widely distributed in Asia, Australia and Pacific islands. An earlier mitochondrial DNA study revealed that there exist significant genetic differences between populations north and south of the Red River Delta region in Vietnam. However the mitochondrial DNA was not sufficiently variable to examine the sources of immigration. For a more detailed analysis of geographic population structure of *N. lugens*, we developed microsatellite markers. Thirty-seven putative microsatellite loci were isolated using a magnetic biotin method, and five primer pairs designed from the flanking regions of sequenced microsatellite clones were labeled with fluorescent. Of these five primer sets, two have proven to be useful across all the samples we used in this study. We used variation at these two microsatellite loci to test the hypothesis that *N. lugens* biotypes (1, 2, and 3) sampled from laboratory selection constituted distinct genetic units. Allele frequency differences among the three major biotype categories were not significantly different at one locus (27035). However, the other (7314) did show differences among the major three biotypes. The methods we describe here will be useful for studying population structure of crop pest and for tracking the patterns of migratory pest like the rice planthoppers.

KEY WORDS : Brown planthopper, Microsatellite loci, Biotypes, Population structure

초 록 : 벼멸구(*Nilaparvata lugens*)는 벼에 가장 큰 피해를 주는 해충 중의 하나로서, 마이토콘드리아 DNA를 분석한 선행 연구결과에 의하면 북 베트남의 홍하유역을 중심으로 남쪽과 북쪽의 개체군이 유전적으로 뚜렷한 차이를 보이고 있다. 그러나 이러한 마이토콘드리아 DNA의 변이로는 좀 더 상세한 지역간 개체군의 유전적 변이를 검정할 수 없으므로, 마이크로새털라이트 마커를 이용할 수 있는 방법을 모색하였다. 총 37개 마이크로새털라이트 위치를 분석한 결과 5개 위치에서 성공적으로 라벨을 할 수 있었으며, 그 중 2개 위치에서 유용한 개체군 변이정보를 얻을 수 있었다. 이러한 두 위치에서 벼멸구의 생태형(1, 2, 3형)에 따른 변이를 검정할 수 있는지의 여부를 검정한 결과, 두 위치 중에서 한 곳(27035)에서는 생태형간의 차이를 나타내지 않았으나, 다른 한 곳(7314)에서는 생태형 간에 차이를 보였다. 따라서 마이크로새털라이트 마커를 이용하면 좀 더 상세한 벼멸구 지역 개체군의 차이를 검정하여 이동과 분산의 근원과 경로를 알아내는데 유용한 방법이 될 것으로 생각된다.

검색어 : 벼멸구, 마이크로새털라이트, 생태형, 개체군 구조

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The brown planthopper, *Nilaparvata lugens* Stål, is known as a long-distance migratory rice pest with biotypes adapted to feeding on different pest resistant varieties of rice (Pathak, 1969; Kisimoto, 1976; Uhm *et al.*, 1988). As a migratory pest, population development of newly arrived planthoppers in rice fields is influenced by many factors, such as the size of migrating cohorts and their place of origin, the seasonal timing of migration, and the spatial pattern of migratory colonization. The geographic environmental relationships between sources of migratory pest and the areas they impact have attracted much research interest. Population geneticists have worked to understand the structure of tropical source populations to enable more accurate prediction and management of *N. lugens* outbreaks in temperate rice growing regions of Northern China, Korea, and Japan (Kisimoto, 1995; Song, 1995; Sogawa, 1997).

Three biotypes of *N. lugens*, designated biotype 1, 2, and 3, can be identified by their ability to feed and infest rice varieties with different resistance genes (Saxena and Barrion, 1985; Tanaka, 1999). These virulent biotypes of *N. lugens* have proven especially useful for fine-scaled investigations into the geographic source areas for immigrating populations. Previous biotypes and mitochondrial DNA studies of *N. lugens* immigrant to Korea revealed that there are significant genetic differences between potential source populations north and south of the Red River Valley in Vietnam (Mun *et al.*, 1999). However, the data were not sufficient to definitively determine the source of immigration. Therefore, more detailed analysis of molecular markers are necessary for understanding geographic population structure and the virulence variability in these planthoppers (Mun *et al.*, 1999; Roderick, 1996).

Microsatellite markers, or simple sequence repeats, are a class of genetic markers found in all prokaryotic and eukaryotic genomes that vary in the number of tandemly repeated motifs of simple DNA sequences. They are present in coding and non-coding regions and are usually characterized by a high degree of length polymorphism. Because of their general occurrence and variability, microsatellite markers are routinely used to investigate the genetic structuring of populations (Zane *et al.*, 2002).

We isolated microsatellite loci using a streptavidin magnetic bead method. This method is relatively fast and simple. The method relies on the extremely efficient digestion-ligation reaction shared in common with the amplified fragment length polymorphism (AFLP) protocols (Vos *et al.*, 1995; Zane *et al.*, 2002).

Here we specify microsatellite loci useful for more detailed analyses of geographic population structure in *N. lugens*. We demonstrate the variability of these loci and use these new taxon-specific markers to estimate genetic differences among laboratory-selected biotypes of *N. lugens*.

Materials and Methods

N. lugens collection and biotype strain preparation

Four individuals were collected from each field population in Taiwan (Caiyi, May 2000), Malaysia (Penang, August 1997), Thailand (Bangkok, August 1997), Indonesia (West Java, February 1998), and Japan (Kumamoto, September 2000). Laboratory selected biotype populations were substantially evaluated by measuring honeydew excretion of females and selected on rice varieties based on carrying *N. lugens* resistance gene (Table 1).

Table 1. Collection localities and resistant host strain selection of *N. lugens*

N. lugens Strain	Country, Locality	Collected date	Individuals tested	Rice Variety	Seedling generation
Field population					
	Taiwan, Caiyi	May 2000	4		
	Malaysia, Penang	Aug. 1997	4		
	Thailand, Bangkok	Aug. 1997	4		
	Indonesia, West Java	Feb. 1998	4		
	Japan, Kumamoto	Sep. 2000	8		
Lab population with no resistant selection*					
Nishigoshi-1991 strain	Kumamoto	Sep. 1991	8	Reiho	119
Isahaya-1997 strain	Nagasaki	Jul. 1997	8	Reiho	40
Isahaya-1998 strain	Hinohikari	Aug. 1998	8	Reiho	25
Lab population with resistant selection**					
Bph 1-selected strain	Kumamoto	Sep. 1991	30	Mudgo	9
Bph 2-selected strain	Kumamoto	Sep. 1991	30	ASD 7	9
Bph 3-selected strain	Kumanoto	Sep. 1996	30	Norin PL10	12

* The japonica varieties (Reiho, Hinonikari, Shinrei and Mochiminori) have no known resistance genes to *N. lugens*.

**In the three selected strains, the population size decreased to ca. 50 individuals (including females and males) at the early generation of selection.

DNA extraction and microsatellite

In order to develop polymorphic microsatellite makers for *N. lugens*, total genomic DNA was extracted by the phenol/chloroform method from 10 female individuals of laboratory strains, which had been stored in 95% ethanol.

Total extracted DNA was digested randomly with *RsaI* using 10-25 μg of genomic DNA, five units of *RsaI* (New England Biolabs). The resulting DNA fragments were ligated with oligonuclotide adapter linkers ADP1 (5′-ctcttgcttacgcgtggact-3′) and ADP2 (5′-tagtccacgcgtaagcaagagcaca-3′) with 5′ phosphorylation. These adapters include *MluI* restriction sites. These adapters were ligated under the following conditions: 5 μM of adapter linker, 3 μg of *RsaI* digested DNA, and 15 units of T4 ligase, in a total volume 75 μg. The mixture was incubated overnight at 14°C. This linker ligated DNA was amplified by asymmetric PCR using ADP1 as a primer.

Approximately 250-500 ng of the PCR amplified linker-ligated DNA was denatured with denaturation buffer (Boehringer Mannheim). At the same time, approximately 50-80 pmol of biotinylated oligonucleotide mixture was prehybridized at 55°C. This oligo mixture contained the series of di-, tri-, and tetra-nucleotide repeats motifs; TA_8, $GATA_6$, ACT_{14}, $CTAG_6$, $GACA_6$, CT_{15}, CTT_{14}, AGA_{14}, CAC_8, $CCCT_6$, ACA_8, $CCCT_6$, ACA_{14}, CAT_{14}, GAC_{11}, CT_{10}, CAG_{10}, CA_{20}, GG_8. The denatured DNA and prehybridized oligonucleotide repeats were hybridized overnight incubation at 60°C, 73°C and 83°C. Hybridization products were captured by shaking for two hours with streptavidin-covered magnetic beads (Dynal Inc.), washed with TE and then with hybridization buffer. The bound DNA was washed with hybridization buffer separately three times at room temperature, 55°C and at 60°C. The repeat enriched DNA created by this process was eluted in 300 μl of ddH_2O by heating at 95°C for 10 minutes.

The enriched DNA were amplified by PCR with ADPI primer and cleaned by QiaQuick columns (QIAgen). Cleaned PCR products were digested with *MluI* and cleaned by 30% of PGE for vector ligation. These DNA fragments were ligated to a variant of the plasmid vector pUC19 and transformed into 25 μl of *E. coli* cells (DH5X, Max efficiency-life tech 18258013) by heat shock at 42°C. After adding 200 μl of SOC medium, the transformations were incubated at 37°C for 90 minute. The transformed mixture was spread on LB plate contain ampicillin and X-gal and grown at 37°C overnight. White colonies were screened by PCR using vector primers (pUC+ and pUC-) and the PCR products with insert >250 bp were cleaned with QiaQuick columns and sequenced on an ABI 377 automated sequencer (Applied Biosystems).

The sequences that contained repeat sequences were used for designing microsatellite primer with Oligo (MBI) and the primers were tested for amplifications against all planthopper individuals of field population. Ones successfully amplifying planthopper sequence with repeat region were labeled with fluorescent dyes and used for AFLP analysis on an ABI 377.

To genotype individuals of *N. lugens*, 25 μl PCR reactions were performed using 20-40 ng genomic DNA, 8 mM dNTPs, 10 umol forward and reverse fluorescently labeled primers (Sigma, Genosis), $MgCl_2$ (Applied Biosystems), 10X buffer (Applied Biosystems) and one unit of Taq ploymerase (Applied Biosystems). All amplifications were performed on an iCycler (Bio-Rad) thermocycler. For the AFLP runs, 1.5 μl of PCR product was mixed with 0.4 μl of Temra size standard (75-500 bp), 0.2 μl of blue dye, 0.9 μl of formamide and separated on an ABI 377. Fragment sizes were scored using GeneScan Analysis v2.5 (Applied Biosystems).

Genetic variability

Genetic diversity within population was measured as the allele diversity calculated for each locus. Population structure of field and lab populations including three biotypes of *N. lugens* was tested for statistical significance using exact tests, and quantified using Wright's (1931) *Fst* based on allele frequencies. Statistics were calculated using the program Arlequin v. 2.0 (Schneider *et al.*, 2001).

Results and Discussion

Approximately 200 white colonies were amplified by PCR, of which 30% contained inserts greater than 250 bp. Of these 60, only 37 (18.5%) contained repetitive motifs with flanking region suitable for designing PCR primers. Thirty-seven experimental primer pairs were designed. Of these, only five pairs (2.5%) were successful in amplifying targeted microsatellite loci for individual specimens (Table 2). The low percentage of working primer pairs was likely due to priming site were too close to repeat regions and primer mismatch causing amplifications non targeted regions.

Confirmed microsatellite loci were reamplified using fluorescently labeled primers to score their variability across samples. Two of the five fluorescently labeled primer pairs successfully amplified the targeted products across all for individuals of *N. lugens*. Therefore we used the variation found by these microsatellite loci to test the hypothesis that *N. lugens* biotypes (1, 2, and 3) were distinct genetic units.

Fourteen and eight alleles were detected using primer 7314 and 27305, respectively. Genetic diversity

Table 2. Characteristics of microsatellite loci of *N. lugens*

Marker name	Forward Primer (5′-3′)	Reverse Primer R (5′-3′)	Expected Size of allele (bp)	Repeat structure	No. of alleles
6020	cagtccagtcgcaatagtg	cttcgttcaatagtataatcc	210	$(ACT)_{10}$	−*
7314	gacagtctgacagtggatc	acgatttccatgtgtgtat	220	$(CA)_{11}$	14
26009	aatttcaatacgtttcaagg	tgtgggataaaatgatgtgg	175	$(GTCT)11(CT)_{12}$	−*
27305	atgacggcgatgtagataaa	gcagctccaaacactcaaat	160	$(TGT)10TG(CCCT)_6$	8
28305	tgtggtagattatgaagggag	agttttctatacgtttcaagg	200	$(CTG)_9$	−*

* Not survey for allele size variation

Table 3. Gene diversity for each locus of *N. lugens* in field populations and laboratory strains (equivalent to expected heterozygosity, with a sample size correction). Divergence among population was assessed statistically using exact test and Wright's (1931) *Fst*

Locus	Population	Field Pop.	Strain 1	Strain 2	Strain 3	Strain 4	Total	Differentiation among all population	
								P(exact test)	*Fst*
	Sample size	23	21	30	30	30	134		
7314	No. of allele	8	5	5	6	8	14		
	Genetic diversity (*Fst*)	0.873	0.749	0.418	0.745	0.732		0.000	0.202
	Sample size	22	24	30	30	30	136		
27305	No. of allele	3	4	2	5	3	8		
	Genetic diversity (*Fst*)	0.402	0.377	0.096	0.428	0.446		0.000	0.031

based on allele frequencies was higher at the 7314 locus, *Fst* 0.418-0.873 than at the 27305, *Fst* 0.0096-0.446. Allele frequency differences among biotypes were not significantly different using these two microsatellite loci. However, *Fst* value in locus 7314 was 0.202 among all the populations suggesting that there are differences among three biotypes (1, 2, and 3) (Table 3).

Results of this experiment using two microsatellite loci were inconclusive for documenting different categories of biotypes. However, one microsatellite locus proved discriminating among three biotype strains, showing that this approach is promising for the development of genetic makers to track the migration patterns of the *N. lugens*.

Acknowledgement

Our work is supported by grants from Korean Science Foundations, US National Science Foundation, US Department of Agriculture, California Department of Food and Agriculture, and the Agricultural Experiment Station of the University of California.

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(Received for publication 16 November 2004;
accepted 14 December 2004)