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Assessment of genetic diversity of rice brown planthopper populations using molecular markers

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ABSTRACT

Random primers were used to assess the genetic variability among brown planthopper (BPH) populations from four locations of Odisha and five locations of Andhra Pradesh. A total of 137 bands were amplified by 17 random primers, of which 116 are polymorphic. Thirty four unique bands were identified, which can be useful for developing diagnostic markers. Genetic similarity between BPH populations varied from 0.346 to 0.72 with an average of 0.556, indicating that wide genetic variation exists between BPH populations at molecular level. All the populations could be uniquely distinguished from each other and grouped into two major clusters at 38% level of genetic similarity. The BPH population from Pipili, Odisha was found to be different from others. Further study with host differentials can ascertain its biotype status.

Key words:*rice , brown planthopper, genetic variability, RAPD*

Brown planthopper (*Nilaparvata lugens* Stal.) is one of the most destructive pest of rice in tropical and temperate Asia causing "hopper burn" (Sogaya and Cheng, 1997). Use of resistant varieties seems to be the best alternative for the management of BPH. Host-plant resistance is likely to be more durable if it employs an array of resistance genes encoding diverse mechanisms of resistance using marker-assisted selection (MAS) breeding approach. Durability of resistance depends on the population structure and mobility of the insect. The resistance is more likely to breakdown if the pest population is genetically diverse or if rates of mutation or migration are high. Brown planthopper is highly migratory resulting rapid gene flow between populations that may lead to high degree of genetic variability among them. Populations of BPH are categorized into 4 biotypes; biotype 1 in the Philippines, biotype 2 widely distributed in the Philippines, Vietnam, China and Indonesia, biotype 3 is laboratory bred in Philippines and biotype 4 in Indian subcontinent (Khush and Brar, 1991).

Morphological markers are routinely used to assess genetic diversity of various insect populations (Loxdale *et al*., 1996). However, these markers are not reliable because many characters of interest have low heritability and much influenced by environment. Various molecular marker techniques have been employed for quick and reliable assessment of genetic variation and population structure of different insect pests. The PCR based randomly amplified polymorphic DNA (RAPD) technique is simple, quicker and cost effective (Welsh *et al*., 1990; Williams *et al*., 1990). The technique has been used for estimation of genetic diversity among and between insect populations, genetic structure of populations, phylogeny, geographical origin and understanding of evolution of insect pests (Zhou *et al*., 2000; Kumar *et al*., 2001; Gadelhak and Enan, 2005; Kartik *et al*., 2010). The precise characterization of genetic diversity of the pest is one of the basis in resistance breeding programs for any particular geographical region. Identification of resistance genes that show incompatible reaction with local pest population could be promising strategy to reduce losses due to pests.

There is no report on characterization of BPH populations of India at DNA level, inspite it being a major rice pest. Therefore, the present investigation was carried out to assess the level of genetic variability among morphologically indistinguishable populations of BPH from various hotspot locations of Odisha and

Andhra Pradesh where high-intensity BPH infestation is favoured by multiple rice cropping, cultural practices and local climatic conditions. We have used RAPD marker technique to provide insight into genetic diversity among BPH populations of Odisha and Andhra Pradesh at molecular level.

MATERIALS AND METHODS

The adult females of brown planthoppers were collected from four different locations of Odisha (Cuttack, Jagatsinghpur, Pipilli and Salepur) and five different locations of Andhra Pradesh (Nellore, Bapatla, Warangal, Maruteru and Srikakulum) and preserved in 95% ethanol at 4^0C for genomic DNA isolation. Genomic DNA was isolated from female insects following a modified method (Anonymous, 1993). Single adult female was soaked in 50 μl of extraction buffer {50 mM Tris-HCl (pH 8.0), 25mM EDTA, 50mM NaCl, 1% SDS} for 10 minutes, and then homogenized in 1.5ml of eppendorf tube with sterilized polypropylene pestle gently and washed with 350 μl of extraction buffer. Ten microliter of Proteinase-K (10 mg ml^{-1}) was added, mixed well and incubated at 37^0C for 1 hr followed by addition of 400μl of equilibrated phenol. The samples were emulsified and centrifuged at 12,000 rpm for 10 minutes. The upper layer was carefully transferred to another tube and an equal volume containing 200μl of phenol and 200μl of chloroform: isoamyl alcohol (24:1) was added, mixed well and the solution was centrifuged at 12,000 rpm for 10 minutes. Then the supernatant was transferred to a new tube and ten microliter of RNase (10 mg ml^{-1}) was added and incubated for 1 hr at 37^0C. Four hundred microlitre of chloroform: isoamyl alcohol (24:1) was added, mixed well and centrifuged at 12,000 rpm at 4^0C for 10 minutes. The upper aqueous layer was carefully transferred to another eppendorf tube and the ten mirolitre of NaCl (5M) and twice the volume of ice cold absolute alcohol was added to it. An overnight incubation was carried out at -20^0C followed by centrifugation at 12,000 rpm for10 minutes. The DNA pellet was washed with 100μl of 70% ethanol and dried at room temperature. It was dissolved in 50 μl of TE buffer. The quality and quantity of genomic DNA was estimated by using spectrophotometer and agarose gel electrophoresis. The samples were diluted in $T_{10}E_1$ buffer to get final concentration of 12ng μl^{-1} and stored at -20^0C for further analysis. DNA from ten individual insects of each location was pooled to prepare bulk DNA.

Seventeen random decamer primers were used for DNA amplification. These primers were from commercially available RAPD primer kits (Operon Technologies, Almeda California, USA). The PCR amplification was performed in a 20 Ml reaction mixture volume containing 24ng of DNA, 1X PCR buffer {75 mM Tris-HCl (pH 9.0), 50mM KCl, 20 mM $(NH_4)_2SO_4$}, 200µM dNTP mix (MBI Fermantas, Lithuania, USA), 5 pmol of primer, 2 mM of magnesium chloride and 1U of *Taq* (*Thermus aquaticus*) DNA polymerase enzyme (Biotools, Spain). A single primer was included in each PCR reaction. The reaction mixture was overlaid with one drop of mineral oil to prevent evaporation. The PCR amplification was carried out using following parameters: initial denaturation at 93^0C for 2 min, denaturation at 93^0C for 1min, annealing at 36^0C for 1 min and extension at 72^0C for 2 min for 44 cycles and final extension at 72^0C for 5 min. The amplified products were size fractionated on 1.5% agarose gel containing ethidium bromide (10 µg ml^{-1}) and photographed using gel documentation system (Fluor Chem™ 5500, Alpha Innotech, USA) to detect polymorphism. The size of amplified fragments was determined by using size standards (100bp plus DNA ladder, MBI Fermentas, Lithuania).

The amplified bands were scored as present (1) or absent (0) for each primer population combination. The data was entered into a binary matrix (0/1) and subsequently analysed using the computer package NTSYS-pc Version 2.02 (Rolf, 1998). The total number of bands per primer, percentage of polymorphic bands, low frequency bands, high frequency bands and unique bands were calculated to assess diversity of bands of a RAPD primer. In order to find the efficiency of RAPD primers for differentiation of genotypes/ populations, the discriminating power (D) of each primer was calculated following formula,

$$Dj = 1-Cj = 1-\sum Pi\ (NPi-1)/(N-1)$$

where Dj is discriminating power of jth primer, Pi is frequency of ith band, Cj confusion probability of jth primer (Tessier *et al.*, 1999) and the minimum number of primer required to identify and differentiate genotypes from each other primer was worked out using the formula, Xj = {N (N-1)/2}Cj.

Jaccard's similarity coefficients were calculated and used to assess the genetic relationship among the nine rice brown planthopper populations (Ni and Li, 1979). A dendrogram was constructed using unweighted pair group method using arithmetic averages (UPGMA). The Cophenetic correlation coefficient (Lapointe and Legendre, 1992) was calculated to measure the goodness of fit of clusters. The average similarity index for all pair wise comparisons ($\overline{X}_D$) were calculated and used to estimate the probability of DNA fingerprints of two BPH populations being identical by chance as described by Ramakrishna *et al.* (1994) employing formula $(\overline{X}_D)^n$ where, $\overline{X}_D$= average similarity index and n = the average number of amplified alleles per population.

RESULTS AND DISCUSSION

All the seventeen random primers successfully amplified a total of 137 bands, of which 116 (84.67%) are polymorphic (Table 1). The number of bands per primer ranged from two (OPH13, OPN1, OPN15) to fifteen (OPN16) with an average of 8.06 bands per primer. The size of the amplified bands varied from 250bp (OPN8, OPN16) to 3200bp (OPN7). Fourteen primers were between populations while three primers,

Table 1. Amplification pattern of bands produced by random primers

RAPD Primer	Total No of bands	No of polymorphic bands	No of common bands	Size of bands (bp)	D*
OPH3	9	8	5	550-1900	0.722
OPH5	9	9	4	500-1500	0.784
OPH13	2	0	2	500-800	0
OPN1	2	0	2	900-1010	0
OPN4	14	13	9	375-1550	0.746
OPN5	9	8	3	530-1250	0.815
OPN6	10	9	5	300-1100	0.653
OPN7	12	12	5	350-3300	0.868
OPN8	7	6	4	250-1500	0.69
OPN10	7	7	5	390-1800	0.655
OPN11	9	9	3	390-2000	0.735
OPN12	7	6	3	640-1700	0.635
OPN15	2	0	2	500-900	0
OPN16	15	15	10	250-2000	0.759
OPN18	10	6	6	350-1800	0.492
OPN19	10	7	7	450-1600	0.447

*D - discriminating power of a primer

OPH13, OPN1 and OPN15 were monomorphic. Five primers, OPH5, OPN7, OPN10, OPN11 and OPN16 amplified bands, all being polymorphic. The amplification pattern with highly polymorphic primer, OPN7 is shown in the figure 1.

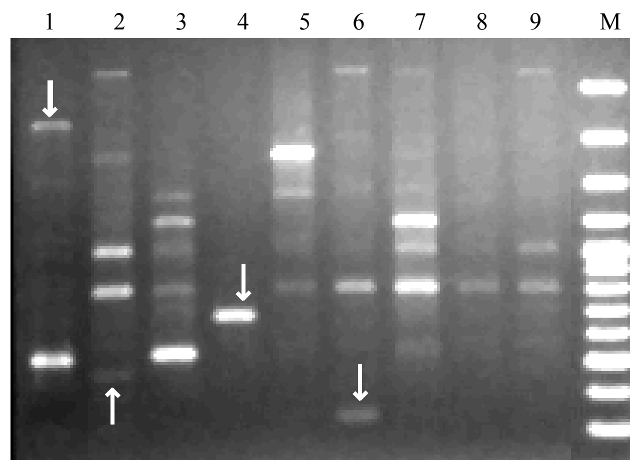

Fig. 1. Amplification pattern of genomes of BPH populations obtained with random primer, OPN7 Numbers on the top of the gel indicate the BPH populations given in the Table 1.
M- 100bp plus DNA ladder, arrows indicate the unique bands amplified in the different BPH populations.

Seventy eight (56.93%) bands were found to be common. A band that was observed in > 30% of all the BPH populations studied was considered to be common / high frequency/ abundant band. All the primers amplified at least one common/ high frequency band. Number of common bands varied from 2 (OPH13, OPN1 and OPN15) to 10 (OPN16). Twenty two bands were amplified in all the BPH populations. Such common bands amplified with different primers could represent species-specific markers and their additional characterization would be useful to determine their potential in distinguishing from other planthopper species. Nineteen bands were amplified only in two populations. Two bands of 1250bp (OPH5) and 1350bp (OPN8) were amplified in all the BPH populations from Andhra Pradesh while one band of 1200bp (OPN16) was amplified in all the populations from Odisha.

A total of thirty four (25.8%) unique bands were amplified by thirteen primers (Table 2). Three

Table 2. Unique bands amplified in different rice BPH populations

Name of RAPD primer	No. of unique bands	Population along with size of unique bands (bp)
OPH3	2	Salepur (550), Pipili (600)
OPH5	2	Cuttack (850), Pipili (1500)
OPN4	2	Pipili (990), Pipili (1550)
OPN5	2	Cuttack (530), Salepur (1250)
OPN6	3	Pipili (340), Pipili (490), Maruteru (1090)
OPN7	4	Bapatla (350), Salepur (410), Jagatsinghpur(700), Pipili (2100)
OPN8	2	Salepur (250), Pipili (900)
OPN10	2	Pipili (800), Salepur (850)
OPN11	4	Pipili (350), Pipili (600), Pipili(2000), Jagatsinghpur (900)
OPN12	4	Cuttack (640), Pipili (1030), Pipili (1200), Pipili (1700)
OPN16	3	Pipili (250), Pipili (450), Pipili (630)
OPN18	2	Maruteru (350), Salepur (550)
OPN19	2	Salepur (1220), Nellore (1300)
Total	34	

primers, OPN7, OPN11 and OPN12 amplified four unique bands each followed by three bands by primers, OPN6 and OPN16. Eighteen, seven, three, two, two, one and one unique bands were amplified in BPH populations from Pipili, Salepur, Cuttack, Jagatsinghpur, Maruteru, Bapatala and Nellore, respectively. No unique band could be detected from populations of Srikakulam and Warangal. These unique bands could be developed into diagnostic markers to identify a particular population of BPH.

Genetic similarity estimated on the basis of all the bands amplified by seventeen primers ranged from 0.346 to 0.72 with an average of 0.556, indicating wide genetic variation among BPH populations. Warangal population showed highest genetic similarity with Srikakulam population (i.e. 0.753) while Pipili population showed least similarity with Warangal population (i.e. 0.346). Cluster analysis based on UPGMA provided a clear resolution of relationships among the nine rice BPH populations (Fig. 2). Two major clusters were observed at 38% level of genetic similarity. The Cophenetic correlation coefficients (r = 0.977) revealed the reliability and stability of clustering. First major cluster contained only one population, Pipili. Second major cluster was further sub-grouped into three sub-clusters, IIA, IIB and IIC at 55% of genetic similarity. Sub-cluster, IIA contained only Salepur population while sub-cluster, IIB contained Cuttack and Jagatsinghpur populations. All the five populations, Srikakulam, Maruteru, Bapatla, Nellore and Warangal from Andhra Pradesh were included in the sub-cluster, IIC with an average genetic similarity of 0.673 (Table 4).

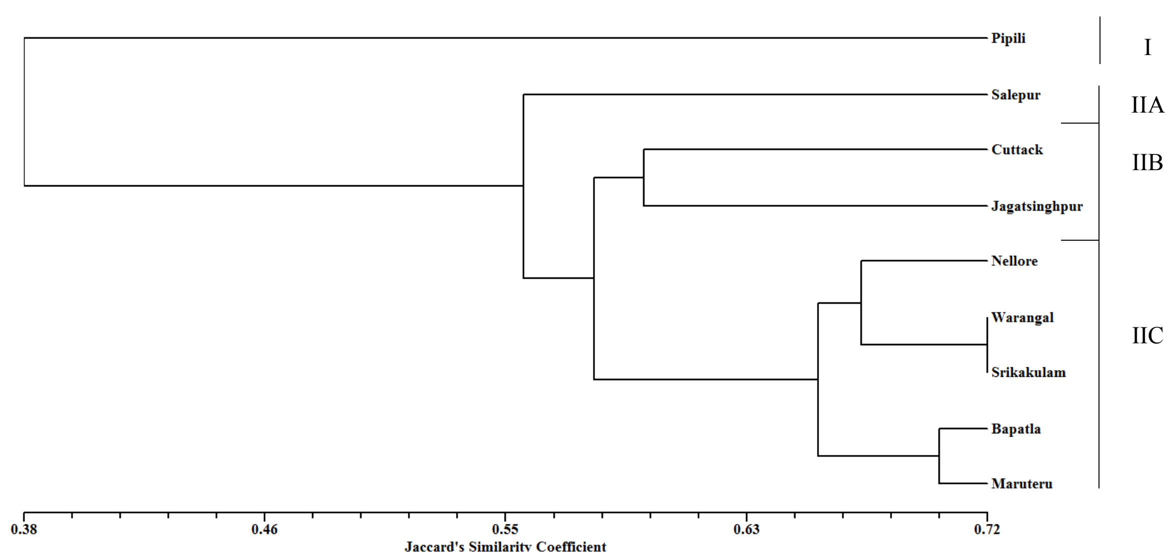

Fig. 2. Dendrogram showing genetic relationships between BPH populations
Scale on the bottom indicates the Jaccard's coefficient of similarity. The major clusters and sub-clusters are indicated on right margin.

With exception from BPH population from Pipili, the genetic similarity between populations is independent of geographic distance, suggesting that gene flow between populations is independent of geographic distance and appears to be unrestricted. The populations from Andhra Pradesh are genetically more similar than those of Orissa populations and varied from 0.6 to 0.72 with an average of 0.6. The genetic similarity between BPH populations of Odisha varied from 0.375 to 0.597 with an average of 0.462.

The continuity of land cover under the rice crop would allow migration of adults, resulting in unrestricted gene flow between populations due to sexual reproduction. The local rice varieties may also be playing important role in structuring the local pest populations. The BPH population from Pipili, though morphologically indistinguishable from the rest, clustered to the major cluster I at relatively low genetic similarity of 38%. The BPH populations from Cuttack, Jagatsinghpur and Salepur are closer to Pipili. There is no geographical barrier that could preclude migration of adults from these places to Pipili. These observations suggest that the population from Pipili could be genetically differentiated form and there appears to be some kind of reproductive barrier preventing free gene flow.

All the individual populations of BPH included in the study could be distinguished precisely from each other. The discriminating power of random primers varied from 0 (OPH13, OPN1 and OPN15) to 0.868 (OPN7), with an average of 0.54, indicating that fourteen primers are efficient in differentiating populations of BPH (Table 1). On the basis of discriminating power, the minimum number of RAPD primers required to differentiate all the BPH populations in the present study was found to be two (i.e. OPN7 and OPN5). The combination of all the polymorphic and non-polymorphic bands obtained with all the 17 RAPD enabled development of DNA fingerprint/profile data set for nine BPH populations (data not shown), which would be very useful for future reference. The probability of identical match was found to be 2×10^{-4} based on the DNA profiles/fingerprints generated by the two most discriminating RAPD primers (i.e. OPN7 and OPN5), suggesting that 10^{4} rice BPH populations can be distinguished by using these RAPD primers. Inclusion of all the 17 RAPD primers provided a very high resolution power enabling nearly 10^{17} populations to be precisely identified (Table 3). Several workers have been demonstrated the utility and power of RAPD technique in distinguishing insect populations (Dowdy *et al*., 1996; Kumar *et al*., 2001).

Table 3. Analysis of DNA fingerprinting using different sets RAPD primers

Attributes	Two primers	17 primers
Average bands for each population(n)	6.89	66.56
Average similarity index ($\overline{X}_D$)	0.311	0.556
Probability of identical match by chance ($\overline{X}_D$)^n	3.2×10^{-4}	1.08×10^{-17}

The present study has revealed that the RAPD-PCR technique is extremely useful for rapid identification of genetic polymorphisms in BPH populations. The unique bands generated by RAPD primers could be used to develop diagnostic markers. The low similarity index between populations is a reflection of the high genetic diversity which is obvious because planthoppers are highly migratory, frequency of recombination and selection is high. The present study indicated that populations of BPH could be distinguish using only a small number of RAPD primers. Further, our study revealed that BPH population of Pipili is different from others, indicating that there may be different biotypes of BPH.

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