SRAP analysis of brown planthopper (*Nilaparvata lugens*) populations maintained on differential rice host varieties

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Abstract. *Chaerani, Dadang A, Fatimah, Husin BA, Sutrisno, Yunus M. 2021. SRAP analysis of brown planthopper (*Nilaparvata lugens*) populations maintained on differential rice host varieties. Biodiversitas 22: 4266-4272.* Brown planthopper (*Nilaparvata lugens* Stål) biotypes differ in virulence to rice varieties carrying different *Bph* resistance genes. These biotypes are reported can be genetically discriminated against using DNA markers. Four brown planthoppers (BPH) populations, which displayed two virulence phenotypes, have been produced by selection and adaptation on four differential host varieties. Sequence-related amplified polymorphism (SRAP) marker preferentially amplifies the coding regions in the genome and, thus, can discriminate the observed virulence variations among those populations. This study aimed to analyze the genetic variation of four developed BPH populations using SRAP markers. Genetic analysis of a total of 40 BPH females with 18 polymorphic primers revealed equal genetic diversity parameter values among populations (Na: 1.1 to 1.4, Ne: 1.2 to 1.3, I: 0.22 to 0.29, He: 0.14 to 0.18, and UHe: 0.15 to 0.19). Analysis of population structure by AMOVA indicated low genetic variation among populations (9%). Still, pairwise PhiPT population values between all pairs of the population revealed the presence of moderate genetic differentiations (PhiPT ranged from 0.57 to 0.133, *P*<0.01). Two partial clusters in plots of PCoA were corresponded to two virulence groups, indicating the ability of SRAP markers to discriminate virulence phenotype. Further selection and adaptation are expected can form four desired virulence patterns with complete genetic separation among the population before its application as resistance screening agents of rice lines.

Keywords: Brown planthopper, DNA marker, genetic differentiation, rice, virulence

INTRODUCTION

Brown planthopper (*Nilaparvata lugens* Stål, Hemiptera: Delphacidae) is Indonesia's most destructive rice (Baehaki and Mejaya 2015). Recurrent outbreaks of brown planthopper (BPH) in Indonesia are caused by continuous rice cultivation, extensive use, and frequent insecticides (Baehaki and Mejaya 2015). This pest is a typical monophagous vascular feeder of rice (Cheng et al. 2013). Feeding activities by both nymphs and adults on the basal portion of plants result in rapid wilting and complete drying, which is observed as a hopper-burn symptom in rice fields (Bottrell and Schoenly 2012; Cheng et al. 2013; Bao and Zhang 2019). In addition, brown planthopper (BPH) is also a vector of viral diseases. Hence, even at low population densities, *N. lugens* can cause significant losses in rice production when they transmit these viruses (Cheng et al. 2013; Bao and Zhang 2019).

BPH outbreak in tropical Asia was triggered by rice intensification production during the green revolution era in the 1970s and 1980s (Bottrell and Schoenly 2012). Sequential release and continuous succession monoculture planting of resistant rice with *Bph1* and *bph2* resistance genes from the International Rice Research Institute (IRRI) throughout tropical Asia to control BPH promoted the selection of local populations capable of feeding on these varieties after only a few years after first adopted by farmers (Bottrell and Schoenly 2012; Horgan et al. 2017).

Field BPH adaptation to these genes occurred within 10 to 20 generations of BPH life cycle (Cruz et al. 2011; Horgan et al. 2017).

Rice breeding centers in Asia have been using the resistance screening methods and its modification developed at IRRI in the 1970s to BPH biotypes with varying virulence to different BPH resistance (*Bph*) genes (Bottrell and Schoenly 2012). BPH biotypes are numbered according to *Bph* genes: cultures of BPH not virulent to all *Bph* resistance genes are designated 'biotype 1', whereas those infectious to *Bph1* or *bph2* are termed as 'biotype 2' or 'biotype 3', respectively (Bottrell and Schoenly 2012). Biotype 4 is virulent to *Bph1* and *bph2* but not *Bph3* or *Bph4* (Bottrell and Schoenly 2012; Kobayashi et al. 2014). 'Biotype 3' was initially produced by continuous adaptation of laboratory-reared colonies on rice variety carrying *bph2* (Cheng et al. 2013). Biotype 4 is the most destructive in South Asia and occurs predominantly on the Rathu Heenati variety (Cheng et al. 2013). BPH biotypes do not differ significantly in morphology and biochemistry, overlap in cytological characters and acoustic behavior, display slight genetic divergence among biotypes based on morphological characters, and have no mating barriers (Kobayashi 2016).

In Indonesia, field populations with 'biotype 3' virulence patterns were dominant in 2006, and 'biotype 1' has extinct, but 'biotype 4' virulence phenotypes emerged in 2010 in few provinces of Indonesia (Baehaki 2012a).

Later surveys indicated that field populations with multiple virulences by overcoming *bph4*, *bph5*, *Bph6*, *bph7*, *Bph9*, or *bph2+Bph3* genes were distributed in Java and South Sulawesi (Chaerani et al. 2016). A similar trend was also observed in other countries of Asia where *bph5*, *bph7*, *Bph8*, *Bph9*, *Bph10*, and *Bph18* genes are also ineffective against field BPH population (Cruz et al. 2011; Horgan et al. 2015).

Because of rapid adaptation of field BPH virulence in Indonesia, stringent resistance screening of promising rice lines has been obliged before its registration and release by the use of BPH biotype 1, 2, and 3, as well as a representative field population as the screening agents (Sasmita et al. 2019). However, during laboratory rearing, the virulence characteristics of BPH biotypes may be unstable (Manzila et al. 2000; Baehaki and Munawar 2008; Chaerani et al. 2016), and the field population must be replaced with a new one after few generations of rearing (Baehaki 2012b). To address these issues, virulence selection and adaptation on appropriate host variety resulted in four BPH populations (populations T, M, A, and R) with four biotype virulence patterns (Chaerani et al. 2017; Chaerani et al. 2021). However, these populations still displayed two virulence patterns, indicating partial host adaptation.

BPH biotype is merely a population with increased or decreased frequencies of virulence alleles through selection and sympatric speciation (Kobayashi 2016). Therefore, phenotypic plasticity shown by BPH population indicates the change in allele frequencies of genes associated with host adaptation. In several insects, including BPH, DNA marker analysis has been employed to study the divergence of biotypes species to understand the basis, i.e., geographic or host plant-associated differentiation (Wang et al. 2020). However, the first genetic study using random amplified polymorphic DNA (RAPD) markers in BPH did not indicate genetic divergences among BPH biotypes (Shufran and Whalon 1995). Still, the later genetic study suggested that the markers can partially discriminate biotypes 1 and 2 resulted from the purification of contaminated biotype stocks (Bahagiawati and Rijzaani 2005). A more recent study could completely separate four BPH biotypes analyzed with expressed sequence tags–simple sequence repeat (EST-SSR markers) (Jing et al. 2012).

A PCR-based marker system similar to RAPD called sequence-related amplified polymorphism (SRAP) is designed to strengthen the coding regions or the interval between genes and their non-coding flanking regions and tightly links to actual genes (Li and Quiros 2001; Li et al. 2014). The SRAP technique uses a 17-bases forward primer and an 18-base reverse primer which amplifies the open reading frames (ORFs) (Li and Quiros 2001). A single forward primer can be combined with numerous, interchangeable reverse primers, resulting in distinct and polymorphic SRAP loci (Robarts and Wolfe 2014). This inexpensive marker is highly reproducible, has less technical efforts, and produces a significantly greater number of variable fragments per locus and a higher rate of polymorphism relative to inter simple sequence repeat (ISSR), RAPD, and SSR markers (Li and Quiros 2001;

Robarts and Wolfe 2014). In addition, SRAP was reported to be more potent than RAPD analysis for detecting the genetic polymorphism among closely related species (ElAzim et al. 2019). SRAP markers have gained popularity in studies of genetic diversity, genetic linkage map construction and comparative genetics in various crops (Li et al. 2014; Robarts and Wolfe 2014) and to a lesser extent in studies of genetic diversity of plant-pathogen (Tripathi and Dubey 2015; Longya et al. 2020), insect (Wang et al. 2020) and animal (ElAzim et al. 2019).

A genetic mapping study of biotype 2 virulence genes employed some SRAP markers and found 106 polymorphic markers. Furthermore, one of the SRAP and an SSR marker flanked an interval of 1.1 cM on chromosome 14 where a quantitative trait locus (QTL) with a significant effect (24%) on BPH growth rate was detected (Jing et al. 2014). Based on this result, we were interested in testing the SRAP marker's ability to distinguish the virulence variation among the four advanced BPH populations. The objective of this study was thus to analyze the genetic differentiation among the four developed BPH populations using SRAP markers and to relate it with the virulence phenotypes observed among the populations.

MATERIALS AND METHOD

Brown planthopper population

The genetic material used in this study consisted of populations T, M, A, and R adapted on differential rice host varieties TN1 (carries no *Bph* gene), Mudgo (*Bph1*), ASD7 (*bph2*), and Rathu Heenati (*Bph3*, *Bph17*), respectively. These populations resulted from three to four virulence selection process combined with one selfing in each cycle on appropriate variety from three field populations (Chaerani et al. 2017; Chaerani et al. 2021). Population T and A were at the 2nd and 3rd generation of adaptation, respectively, whereas populations M and R were at the 4th generation after the final election cycle. Thus, population R displayed the desired phenotype characteristics by damaging all four differential varieties. Meanwhile, population T, M, and A exhibited deviation from the expected biotype 1, 2, and 3 virulence character, respectively, i.e., population T and M showed biotype 4 virulence character, and population A have the same virulence characteristics to that of population R.

Polymerase chain reaction

Genomic DNAs from 10 females per population available from a previous study (Chaerani et al. 2021) were used in the current study. A total of 89 primer combinations of the forward primer Me (Me01 to Me25) and the reverse Em primers (Em01 to Em16, Em18 to Em27, and Em30 to Em36) were selected from the list of 106 polymorphic SRAP primers in the cross of BPH biotype 1 and biotype 2 (Jing et al. 2014). Polymorphism tests of the primers were performed on a DNA pool made from equal concentration (10 ng/μl) of randomly selected five female samples per population. PCR amplification was performed on a thermal cycler (Bio-Rad) in 10-μl reaction

volumes containing 1x PCR buffer, 1 mM of dNTP, 0.3 M of each forward and reversed primer, 0.5 U of Taq DNA polymerase (DreamTaq), and 10 ng/µl of pooled DNA. The PCR cycling profile is as described in Jing et al. (2014), wherein the first few cycles, low-temperature annealing (~ 35C) was employed, and a higher annealing temperature (~50C) was used in the following processes. PCR products were fractionated by electrophoresis on 4% agarose gels in 0.5% TBE for 4 h at a constant current of 100 V. DNA bands were visualized under a UV trans-illuminator (GelDoc Bio-Rad) after being stained with 0.5 µg/l EtBr solution. Primers yielded polymorphic bands were further used for individual PCR amplification of a total 40 females. PCR product separation was performed by electrophoresis on 8% non-denaturing polyacrylamide gels in 1% TBE for 80 minutes at a constant current of 90 V. Gels were stained in EtBr solution before visualization under a UV trans-illuminator.

Data analysis

DNA bands with the same mobility were scored dominantly by eye as "1" for the presence or "0" for absence. Differences in band intensity were ignored. The distinguishing ability of SRAP markers in assessing genetic diversity was evaluated based on polymorphic information content (PIC) values calculated using PowerMarker V 3.25 (Liu and Muse 2005). Genetic diversity parameters, including the number of observed alleles (Na), number of effective alleles (Ne), Shannon's information index (I), and population heterozygosity as measured by observed heterozygosity (Ho), expected heterozygosity (He), and unbiased heterozygosity (UHe) was estimated using GenAlex 6.5 (Peakall and Smouse 2012). This software was also employed to assess genetic differentiation among and within populations by analyzing of molecular variance (AMOVA) based on 999 random permutation and pairwise PhiPT population values. In contrast, genetic relatedness among populations was assessed by multivariate principal coordinate analysis (PCoA).

RESULTS AND DISCUSSION

Marker diversity

Eighty nine SRAP primer combinations were employed for polymorphism tests. Among these primers, 24 (27%) yielded polymorphic bands, 10 (11%) produced monomorphic bands, 25 (28%) primers yielded inconsistent and unclear bands, and the remaining primers failed to amplify DNA. Of the polymorphic primers, 18 (20%) primers derived from the combination of 11 forward and 9 reverse primers produced precise amplification (Table 1), and hence they were used in genotyping of 40 BPH samples.

The observed low primer polymorphism rate could be partly explained by the use of 4% agarose gels for primer screening, rather than polyacrylamide gels which can provide a more satisfactory resolution of DNA bands and hence more number of scoreable bands. SRAP fingerprint results are primarily determined by PCR amplification conditions, electrophoresis systems, and primer combination choice (Li et al. 2014). SRAP fractionation on acrylamide gels is preferred to analyze amplicons of ~100–1000 bp (Li et al. 2014).

The polymorphic SRAP primer combinations amplified multiple polymorphic fragments, with the total number of polymorphic DNA fragments was 265 and ranged from 10 (primer Me21Em09) to 21 (primer Me19Em13) and an average of 15 bands per primer (Table 1). An example of SRAP amplification is depicted in Figure 1. On average, 15 polymorphic fragments per primer were amplified. In addition, a wide range of band sizes was observed, from 95 to 625 bp (Table 1). SRAP primers quickly generate polymorphic fragments because of variations in primer binding sites in exon and intron regions among different individuals (Jing et al. 2014). However, despite causing a large number of polymorphic fragments, these polymorphic SRAP markers were, on average, not informative (PIC <0.25), and only two markers (Me19EM20 and Me21Em09) were slightly informative (0.25<PIC<0.50) (Botstein et al. 1980). The resulting low PIC values could be explained by the selection of primers or the narrow genetic differences among the material studied (Alzahib et al. 2021).

Population genetic parameter

The mean percentage of polymorphic markers among populations was moderate (61%), with the lowest (49%) and highest (73%) percentages were observed in populations R and A, respectively (Table 2). The values of genetic diversity parameters were almost equal among the populations (the range of Na values were 1.1 to 1.4, Ne 1.2 to 1.3, I 0.22 to 0.29, He 0.14 to 0.18, and UHe 0.15 to 0.19).

Table 1. Features of polymorphic SRAP markers used in genetic analysis of four brown planthoppers (*Nilaparvata lugens* Stål) populations adapted on differential host rice varieties

Primer combination	N polymorphic band	PIC	Range of allele size (bp)
Me19Em20	13	0.31	110–412
Me21Em09	10	0.26	102–597
Me16Em13	11	0.24	129–454
Me22Em05	14	0.23	111–527
Me10Em07	15	0.23	141–542
Me22Em12	20	0.21	105–465
Me25EM11	15	0.20	121–481
Me04Em10	15	0.19	120–553
Me08Em05	18	0.19	106–591
Me03Em07	16	0.18	96–515
Me11Em13	11	0.18	109–387
Me19Em13	21	0.18	138–486
Me11Em05	15	0.18	95–510
Me10Em12	12	0.17	129–596
Me19Em12	14	0.17	117–429
Me22Em04	18	0.16	130–625
Me14Em05	16	0.15	105–568
Me04Em13	14	0.14	115–521
Mean	15	0.20	

Note: PIC is polymorphic information content, an average of 40 female insects

Nevertheless, there is a tendency that populations R and A showed the lowest and highest value of genetic diversity parameters, respectively, according to the percentage of polymorphic markers. In general, the importance of genetic diversity parameters did not represent the respective virulence characteristics of each population. For instance, populations A and R with higher virulence characteristics than populations T and M showed contrasting genetic diversity values.

All populations had private alleles, i.e., allele unique to a single population. Being low in frequency and occurring in one or more populations, private alleles can be used to identify populations by combining some genetic loci (Alzahib et al. 2021). The number of private alleles in our studied population was high and equal between population T and R (8 and 9, respectively) and between population M and A (17 and 16, respectively). Therefore, private alleles in our populations do not represent a population identity but may be the result of slippage and proofreading errors during DNA replication that primarily changes the arrangement of DNA nucleotides (Alzahib et al. 2021).

Population structure

AMOVA indicated that only 9% of the total variation was attributed to differences among populations (Table 3). This rate among-population genetic variation is similar (11%) to that obtained from AMOVA of the same four populations using SSR markers (Chaerani et al. 2021). Both values are much lower than the genetic variation among biotypes 1, 2, 3, and Y, which accounted for 25.5% of the total variation (Jing et al. 2014). Thus, the low genetic divergence among populations could result from early host adaptation of individuals selected from a common source of field populations. Nevertheless, the overall PhiPT value was greater than zero (0.088) and significant (*P*<0.001) (Table 3), indicating the presence of some genetic divergences among populations (Xu et al. 2016).

Further analysis based on pairwise PhiPT population values, which provide estimates of genetic distances between pairs of populations, obtained a range of PhiPT values from 0.057 to 0.133. The highest genetic differentiation (PhiPT 0.133) was observed between population T and R, and the lowest (PhiPT 0.057) was between population T and M (Table 4). These values indicate moderate differentiation among the population based on the threshold of $0.05<$PhiPT≤0.15 (Wang et al. 2020). However, between all pairs of populations, there was significant genetic differentiation (*P*=0.01) (Table 4). Therefore, genetic adaptation on the host may have partially occurred and been observed as two virulence characters among the four populations.
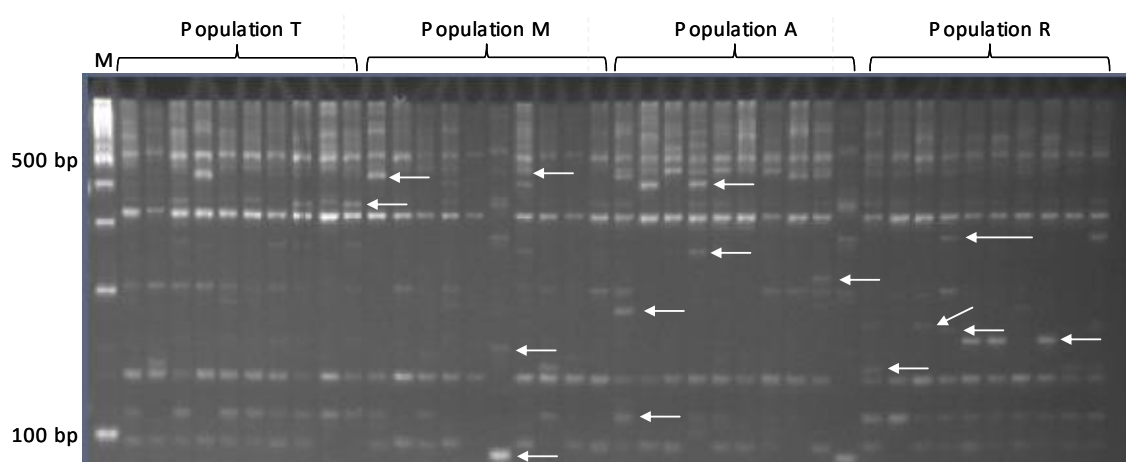
Figure 1. An example of PCR products of four rice brown planthopper (*Nilaparvata lugens* Stål) populations (*n*=10) generated by SRAP primer combination of Me03 and Em07 separated on 8% non-denaturing polyacrylamide gel. Population T, M, A, and R were adapted on TN1 (carries no *Bph* gene), Mudgo (*Bph1*), ASD7 (*bph2*), and Rathu Heenathi (*Bph3*, *Bph17*), respectively. Arrows indicate polymorphic DNA bands. M = 100 bp size marker.

Table 2. Population genetic parameters for brown planthopper (*Nilaparvata lugens* Stål) populations adapted on differential host rice varieties based on 268 loci derived from 18 polymorphic SRAP markers

Population (host variety and resistance gene)	P (%)	Na	Ne	I	He	UHe	Pa
T (TN1 [carries no *Bph* gene])	53.9	1.1	1.2	0.23	0.15	0.16	8
M (Mudgo [*Bph1*])	68.0	1.4	1.3	0.26	0.16	0.17	17
A (ASD7 [*bph2*])	73.1	1.5	1.3	0.29	0.18	0.19	16
R (Rathu Heenathi [*Bph3*, *Bph17*])	49.2	1.1	1.2	0.22	0.14	0.15	9
Mean	61.0±5.7	1.3±0.03	1.3±0.01	0.25±0.01	0.16±0.01	0.17±0.01	12.5±4.65

Note: Values for each population are mean from 10 females. P: polymorphic locus, Na: number of different alleles, Ne: number of effective alleles, I: Shannon's information index.

Genetic relationship

Results from PCoA are three bi-plots with the first, second, and third axis accounted for 24.7%, 20.6%, and 18.1% of the total variation explained, respectively (Figure 1). Two partial clusters were observed: one group consisted of most individuals from populations T and M, whereas the other cluster contained most members of populations A and R. Most individuals from the same population tended to aggregate. This partial clustering corresponds to two virulence patterns observed among the populations, i.e., biotype 4 (population T and M) and the highly virulent phenotype (population A and R).

Virulence phenotype results from complicated interactions between herbivores and plant hosts or between defense and virulence mechanisms (Horgan 2018). BPH virulence adaptation is mediated by the endosymbiont yeast-like symbiont and bacteria, which compensate for the antifeedant effect in the host and provide the nutrition necessary for BPH reproduction and egg development (Chen et al. 2011; Ferrater et al. 2013; Fan et al. 2015). During the early virulence adaptation on resistant variety, abundant and high diversities of microbial endosymbionts are present in BPH females feeding on a resistant rice variety compared to that feeding on a susceptible variety, but once BPH has overcome the host resistance, endosymbiont density is reduced (Chen et al. 2011; Ferrater et al. 2015). Therefore, endosymbionts have an essential role in the adaptation and survival but do not determine BPH virulence. The complementary interaction between BPH and its endosymbionts has been proved by genome sequencing of BPH, which reveals that missing genes essential in biochemical pathways for its survival on the nutritionally imbalanced sap diet are present in the genomes of its microbial endosymbionts (Bao and Yang 2019).

Table 3. Results of analysis of molecular variance (AMOVA) for 40 females of four brown planthoppers (*Nilaparvata lugens* Stål) adapted on differential host rice varieties based on 268 loci derived from 18 SRAP markers

Source	df	SS	MS	Estimates of variance components	Molecular variance (%)
Among Pops	3	171.700	57.233	2.812	9
Within Pops	36	1047.900	29.108	29.108	91
Total	39	1219.600		31.921	100
Stat	Value	P(rand >= data)			
PhiPT	0.088	0.001			

Table 4. Pairwise population PhiPT values among four brown planthopper (*Nilaparvata lugens* Stål) populations adapted on differential host rice varieties.

Population (host variety and resistance gene)	T	M	A	R
T (TN1 [carries no *Bph* gene])	…	0.01	0.01	0.01
M (Mudgo [*Bph1*])	0.057	…	0.01	0.01
A (ASD7 [*bph2*])	0.090	0.063	…	0.01
R (Rathu Heenathi [*Bph3*, *Bph17*])	0.133	0.117	0.069	…

Note: PhiPT values are below diagonal, whereas probability values based on 999 permutations are shown above diagonal
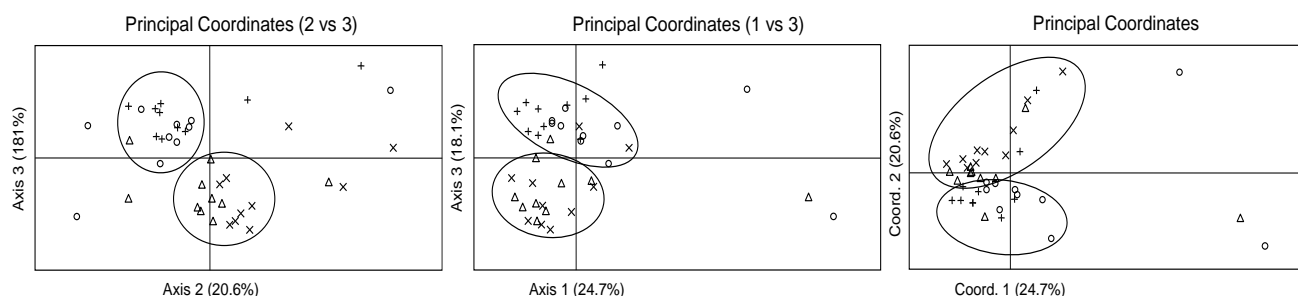
Figure 2. Bi-plots of principal coordinate analysis (PCoA) of brown planthopper population T ('+'), M ('○'), A ('Δ'), and R ('×') adapted on differential rice host varieties TN1 (no *Bph* gene), Mudgo (*Bph1*), ASD7 (*bph2*), and Rathu Heenathi (*Bph3*, *Bph17*), respectively.

Two independent genetic mapping studies on inbred populations of biotype 1 and 2 identified a significant, recessive virulence gene associated and three QTLs explaining considerable phenotypic variation on virulence and BPH growth rate, respectively (Jing et al. 2014; Kobayashi et al. 2014). In addition, molecular analysis of two BPH biotypes revealed differential expression profiles of genes related to digestion, saliva secretion, detoxification, metabolisms of lipid, carbohydrate, amino acid, and nitrogen; signaling pathways of defense, stress, and immunity responses (Yu et al. 2014), as well as variations in nucleotide and amino acid substitutions in mitochondrial protein-coding genes (Lv et al. 2015).

Regarding population genetics, differences between BPH biotype populations reflect differences in allele frequencies of virulence genes associated with host adaptation (Kobayashi 2016). A BPH biotype consists of individuals who differ in virulence. Increased or decreased proportion determines the population's virulence phenotypes associated with the host adaptation through selection and sympatric speciation (Kobayashi 2016). Thus, virulence selection and adaptation can change the virulence character of a BPH population. However, virulence adaptation can be partial or complete (Horgan 2018). In leafhopper, partial transformation can occur as little as 5-6 generations of selection (Vu et al. 2014). Our BPH populations, which were in the 2nd to 4th generation after the final virulence selection cycle or the 5th to 7th generation after the first election cycle, were at the state of partial virulence adaptation on its host as revealed by the presence of two, instead of four, virulence phenotypes. Interestingly, SRAP analysis partially clustered the four BPH populations into two PCoA plots that corresponded with the two virulence groups. Therefore, apart from its analogy to the morphological character (Robarts and Wolfe 2014), SRAP markers also can elucidate markers with inherent biological significance, including phenotypes (Liao et al. 2016).

Preexisting virulence or avirulence genes at low frequencies could be removed from the population selection (Kobayashi 2016). Kobayashi (2016) reviewed that genotypes can lose their virulence to *Bph1* or *bph2* genes by removing virulent individuals to *Bph1* for two generations or to *bph2* for three generations of selection, respectively. For our case, a longer adaptation period would be needed to remove virulent individuals from populations T, M, and A, intended to have biotype 1, 2, and 3 virulence characteristics. Our populations were developed from field populations that potentially have high proportion virulence alleles (Kobayashi 2016). The selection process is critical because mixtures or contamination in the population can generate conflicting results in subsequent investigation using different virulence test methods, i.e., standard seedbox screening tests, honeydew excretion, and body weight measurement (Kobayashi 2016). On the contrary, more virulent phenotypes can be developed by continuous host adaptation without an artificial virulence selection process, similar to that observed in the field. Laboratory and field experiments showed that 5 to 33 generations of constant transformation on a single variety could generate a virulent population that was previously unable to damage-resistant types (Nemoto and Yokoo 2002; Cruz et al. 2011; Jing et al. 2012).

The current study revealed low genetic variation with minor but significant genetic differentiation among the four BPH populations. The 18 polymorphic SRAP markers employed have been proved for their utility in separating the populations into two clusters concordant with the two virulence phenotypes present among populations. Concerning virulence phenotype, population R can represent a field population used in the resistance screening of rice lines. However, these four populations are not ready to be used as resistance screening agents because of incomplete genetic separation. Continued virulence selection and adaptation on the host is necessary to result in the desired four virulence phenotypes with complete genetic differentiation.

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