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Molecular screening of traditional rice varieties using *tsv1*-linked simple sequence repeat markers

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Abstract

Genebanks offer vast amounts of traditional germplasm with potential sources of novel genes against biotic and abiotic stresses. In order to utilize the germplasm in rice breeding programmes, there should be a fast screening approach such as the use of molecular markers. Thus, the current study aimed to evaluate the use of *tsv1* resistance-linked simple sequence repeat markers (SSR) for the preliminary screening of Philippine traditional rice germplasm against rice tungro spherical virus (RTSV). The *tsv1* resistance-linked SSR markers consisted of two to four repeat motifs with 5–24 base repeats. Expected sizes ranged from 123 to 465 base pairs (bp) with polymorphism information content ranging from 0.23 to 0.73. Genetic analysis showed six major clusters at 50%: Clusters A, B and C had individual accessions, Cluster D had three accessions, Cluster E had 55 and Cluster F had 42 accessions. The study showed the germplasm with alleles linked to *tsv1* but should be validated in the future with induced screening. In general, the material consisted of selected germplasm showing the presence of alleles linked to the *tsv1* gene. These rice accessions could be a source of resistance to RTSV following further validation. Furthermore, molecular markers provide a useful tool to accelerate the screening of genetic resources for biotic and abiotic stress tolerance.

Introduction

Rice is one of the most important staple foods in the world and is grown mainly in South and Southeast Asia. Diseases and abiotic stresses coupled with climate change are major constraints to increasing rice production. Farmers lose an estimated average of 0.37 of rice crop due to major pests and diseases every year. Among these pests, rice tungro disease (RTD) can cause total crop failure, especially in several countries in South and Southeast Asia (Bajet *et al.*, 1986; Hibino, 1996). This disease is caused by rice tungro spherical virus (RTSV), which acts as a helper virus for the transmission of rice tungro bacilliform virus. Rice tungro spherical virus has a polyadenylated single-stranded RNA genome of about 12 kb that is encapsidated within isometric particles. The genome encodes a single large open reading frame of 3473 amino acids, and although RTSV strains cannot be distinguished serologically, they do differ significantly at the molecular level (Azzam and Chancellor, 2002).

There are several approaches and methods employed in addressing these challenges: use of resistant or tolerant varieties, adjustment of planting strategies and changes in area planted, etc. Development of resistant varieties is one of the top approaches in coping with various stresses (Gregorio *et al.*, 2002; Das and Rao, 2015; Ali *et al.*, 2017; Kumar *et al.*, 2017; Shamim and Singh, 2017). This involves backcrossing the source parent to susceptible varieties (recurrent parent) that have desirable properties and selecting resistant plants. These donor parents could come from existing sources or could be from the vast germplasm being stored in genebanks, which depends on the availability of information. This information could be the basis for breeders to determine the possible source of resistant traits in the next breeding programme. Hence, it is important to evaluate genetic resources using faster and more reliable preliminary processes. Molecular marker technology has been developed to study the molecular characterization of a range of crops (Das and Rao, 2015; Bhagwat *et al.*, 2017; Shamim and Singh, 2017).

Morphological markers were used first to study the diversity of species and then isozyme markers were used. However, with the development of modern biotechnology in recent years, DNA molecular markers have gradually become the main tool for studying genetic diversity (Yun *et al.*, 1998; Ravi *et al.*, 2003; Nybom, 2004; Li *et al.*, 2009; Huang *et al.*, 2015; Chen *et al.*, 2017). A range of molecular approaches is used to study the diversity in conserved germplasm (Ashraf *et al.*, 2016). Molecular markers are also important for the characterization of germplasm and offer opportunities to delineate genetic differences at the molecular level among the available diversity. Among the molecular markers, microsatellite markers, also

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Table 1. List of rice accessions used in the current study

Number	Variety name	Number	Variety name	Number	Variety name
1	Binaka	36	Minantika	70	Binutiti
2	Burdagol	37	Ceres	71	Bomitog
3	Dinalores	38	Badaje	72	Bontotan
4	Macaraniag	39	Binorsige	73	Catorse
5	Mimis	40	Baning	74	Colot
6	Minantika	41	Kinandangpula	75	Daliket
7	Minerva	42	Dinolores	76	Dinarunan2
8	Caluban	43	Bayudan	77	Dinominga
9	Dayoyo	44	Dinorado	78	Ifugaorice
10	Ligantong	45	Binakayo	79	Kasaya
11	Binato	46	Inday	80	Kinalansing
12	Kanting	47	Sablay	81	Kinalumpang
13	Linawang	48	Señorita	82	Lipunan
14	3Dakot	49	Magay	83	Magsanaya
15	Buwa	50	Binarit	84	Nato
16	Bumaro	51	Waraywaray	85	Pakaronon
17	Handurayan	52	Lubag	86	Pangasinan1
18	Binutuen	53	Makalpo	87	Pinilik
19	Lawlaw	54	Pula	88	Poloytinawon
20	Immaramang	55	Balatinaw	89	Provincial
21	Saigorot	56	Maguetay	90	Sagang
22	Redtoner	57	Makalpo	91	Azucena
23	Blackrice	58	Isla	92	Davao
24	Tambayangan	59	Londran	93	Macanbinundok
25	Illek	60	Pdlikwet	94	Putanitim
26	Bangkitan	61	Sanglay	95	Domsofid
27	Sampayna	62	Murado	96	IR64
28	Pulupot	63	Binernal	97	Moggo
29	Kalanipon	64	Gininto	98	Katibong
30	Sanpedro	65	Binangkuro	99	Laila
31	Crimas	66	Binagsang	100	Dalakirik
32	Salimpopoy	67	Alimuran	101	Binisaya
33	Buayan	68	Tresmarias	102	Kananoy
34	Awot	69	Banggi	103	Allilik
35	Lampiket				

known as simple sequence repeats (SSR), are most useful in the characterization of cultivars due to their reproducibility, multi-allelism, co-dominant inheritance and good genome coverage, allowing genetic discrimination even in related cultivars (Guichoux *et al.*, 2011). These markers have demonstrated a potential to detect genetic diversity, management of plant genetic resources and molecular screening of germplasm against biotic stresses (Virk *et al.*, 2000; Song *et al.*, 2003; Sabar *et al.*, 2016). They have also used successfully in the selection of distinct genotypes for breeding through marker-assisted selection, in order to increase the number of recombination events, resulting in new allelic combinations and a greater chance of obtaining superior genotypes (Sabar *et al.*, 2016). The current study presented a fast-molecular screening approach using gene-specific SSR markers linked to the *tsv1* gene, which confers resistance to RTSV and is one of the viruses that cause RTD. The study aimed to evaluate the use of *tsv1* resistance linked SSR for the preliminary screening of Philippine traditional rice germplasm against RTSV.

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Table 2. Primers used in the current study linked to *tsv1* genes in rice

Locus ID	Motif	Number of repeats	Forward primer	Reverse primer	Expected marker length	PIC
RM21796	AT	12	CTCATTCCTCAGTCCCTTCTTGG	GGTCTTACCTAACCTTGTCAGTTGC	337	0.73
RM21797	ATCC	5	GCTGACACAGCACTCCTATCTGC	CTCCATCGATCATCAACTCAAACC	195	0.32
RM21798	ACG	7	GAGCAAGTTGTTTGGCGGTAGG	ATCACGAGGCACACGATCACC	324	0.51
RM21800	AT	15	GTGAAATTTGCCTCGCTGTAACG	CATCTAACCCTGCTTTGGACTGG	360	0.34
RM21801	AG	10	GCGCACAGCATGTCGAAGTCC	AAACCCGAGGCAAATACGAAACG	192	0.52
RM21808	AC	11	TCCGATCGCTCCAGTATTCAGTGC	ACAGCTGCGCATGCAACAACG	123	0.63
RM21809	AGG	7	CGATCTCCTCGATGAGGTTGTCC	CCGCCTACGACTGCCAAATCC	465	0.07
RM21810	AG	19	CTAGTTGCCAAGAGGAGGCATCG	CTGTAGCTGAGGCACCAGAATGG	188	0.23
RM21822	AG	12	AGTGATGAGCTCAAGAACAAGC	GTCTCAACTCATCTCATCTTCTCC	460	0.45
RM336	AAG	18	GTATCTTACAGAGAAACGGCATCG	GGTTTGTTTCAGGTTCGTCTATCC	153	0.49
RM5495	AG	24	GGACGCACTTGTCATCTCTCTCC	GGATAGGATCTCGATGGCAGAGG	280	0.51

PIC, polymorphism information content.

Materials and methods

Plant materials

Accessions (103) were chosen randomly from the traditional rice varieties (TRVs) in the PhilRice-Genebank (Table 1). Leaf samples were collected in all accessions that were grown in the field in 2015 dry season in $1 \times 1.5\ m^2$ without replication at the Philippine Rice Research Institute, Maligaya, Muñoz, Nueva Ecija, the Philippines (N,15°40′E, 120°53′, 64.5 m a.s.l.).

Fresh leaf samples were used to extract the genomic DNA with the use of the modified Cetyl Tri-methyl Ammonium bromide (CTAB) method (Murray and Thompson, 1980). The quality and concentration of DNA were estimated by using Nanodrop spectrophotometer (Thermo Scientific, Inc., Waltham, MA, USA) and DNA was diluted in Tris-ethylenediaminetetraacetic acid (Tris-EDTA) buffer and stored at −4 °C prior to polymerase chain reaction (PCR) analysis.

Polymerase chain reaction analysis

Eleven SSR primers, linked with the tsv1 gene that confers resistance to RTSV (Lee *et al*., 2010), were used from chromosome number 7. All primers were synthesized by 1st Base (Singapore) according to sequence information obtained from Gramene (http://www.gramene.org, Table 2). The PCR amplifying procedure used the protocol of McCouch Laboratory (Cornell University, Ithaca, NY, USA) with slight modifications: (i) initial denaturation at 94 °C for 5 min; (ii) denaturation at 94 °C for 1 min; (iii) annealing at temperature from 55 to 60 °C (based on specified annealing temperature) for 1 min; (iv) extension at 72 °C for 2 min; (v) repeat steps (ii) to (iv) 34 times; and (vi) then final extension at 72 °C for 7 min. The amplified products were stored at 4 °C. The DNA amplification was carried out with a total of 4.1 µl reaction containing 0.2 µM of each primer, 20 µM of deoxyribonucleotide triphosphate (dNTPs), 1.5 µ 10X PCR buffer, 1.5 mM magnesium chloride ($MgCl_2$), 50 ng of DNA template and 0.5 unit of i-Taq DNA polymerase (Intron Biotechnology, Inc., South Korea). The amplified products and standard molecular weight markers 1Kb Plus (ThermoFisher Scientific, Massachusetts, US) were run on 8% non-denaturing polyacrylamide gel at 100 V, 500 mA for 75 min. The gel was stained with GelRed™ according to the manufacturer's protocol (Biotium, Inc., California, USA) and then observed under an ultraviolet transilluminator (Bio-Rad Laboratories, UK).

Data analysis

The polymorphic SSR bands for each accession were scored individually for the presence or absence of the expected bands. This resulted in binary data of 1s and 0s. The polymorphism information content (PIC) was determined for each SSR locus using GenAlEx 6.5 (Peakall and Smouse, 2012). Both matrices were then analysed using the Numerical Taxonomy and Multivariate Analysis in the statistical package NTSYSpc version 2.2 (Rohlf, 2005). The data matrices were used to calculate genetic diversity based on Nei's coefficients, and one dendrogram displaying relationships among the 103 rice accessions was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) (Sokal and Michener, 1958). Principal coordinate analysis (PCA) was done to visualize individual and/or group differences using GenAlEx 6.5 (Peakall and Smouse, 2012).

Results

Markers used in the study consisted of two-, three- and four-nucleotide repeat motifs ranging from 5 to 24 repeats (Table 2). Allele sizes ranged from 123 to 465 with PIC values ranging from 0.07 to 0.73 for the 11 SSR markers. The lowest PIC value was observed for RM21809 (0.07) while the highest was recorded for RM21796 (0.73). The average PIC value was 0.43. Informative markers with PIC values >0.5 were RM21796, RM21798, RM21801 and RM5495, with a range of 0.51–0.73 (Table 2).

All 11 markers showed polymorphism between 103 TRVs. A total of 30 bands were scored. The study revealed that primers RM336 and RM337 had four alleles each compared with the rest of the markers used (Fig. 1). Cluster analysis was used to group the varieties and construct a dendrogram. The Nei's coefficient from the similarity matrix was based on the UPGMA algorithm. A total of six major clusters at a 50% genetic distance coefficient were generated. Three clusters (Clusters D, E and F)

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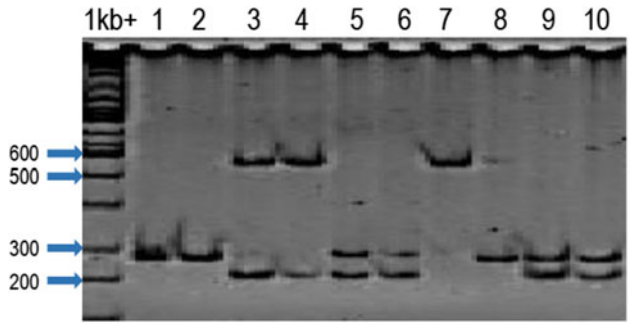

Fig. 1. Representative picture of a non-denaturing 8% polyacrylamide gel with RM336 showing four alleles with the 1 kb+ ladder as control, with some accessions showing similar banding patterns such as 1, 2 and 8 (253 bp); 3 and 4 (550 bp/201 bp); 5, 6, 9 and 10 (253 bp/201 bp). The bands represent the alleles with the corresponding sizes.

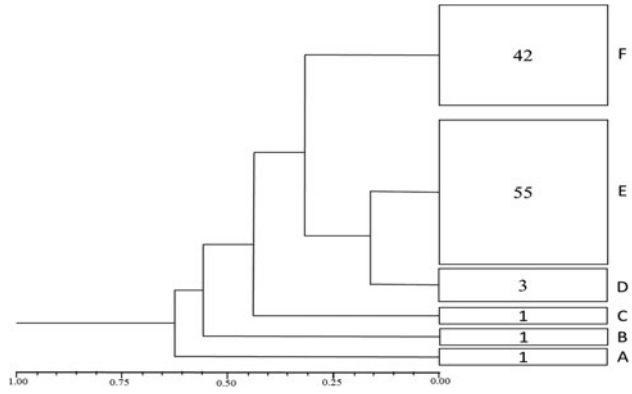

Fig. 2. Simplified UPGMA clustering of 103 *O. sativa* TRVs based on 11 *tsv1*-linked SSR markers.

had individual accessions, one (Cluster C) had three accessions while another cluster (Cluster B) had 56 accessions and lastly, one cluster (Cluster A) had 41 accessions (Fig. 2). There is no conclusive revelation among the germplasm in this cluster. The clustering revealed variation among the accessions in terms of the presence and absence of alleles based on the 11 *tsv1*-linked loci. From six major clusters, the PCA showed only three major clusters which could mean that these TRVs could be resistant, moderately resistant and susceptible (Fig. 3). The highest similarity was revealed between Buwa and Binakayo (100%) and the least between Binorsige and Binaka (<10%).

Discussion

High similarity was revealed between Buwa and Binakayo (100%), which could be a possible duplicate, but since low-coverage SSR markers were used, based only in the 200 kb region, no conclusions about their relationship can be drawn. However, it could be stated from the current molecular analysis that these two TRVs are similar in their resistance to RTSV.

Lee *et al.* (2010) observed RM336 is expected to have five alleles based on the recombination that occurred at that locus. However, the current study found four alleles only for RM336 and RM337, which could be due to differences in the accessions used. Lee *et al.* (2010) used TN1 and Utri Merah crosses while the current study selected Philippine TRVs, which revealed more alleles than the TN1 × Utri Merah crosses. According to Schiff *et al.* (2001), Lindhout (2002) and Ghosh *et al.* (2010), most disease resistance traits are controlled by multiple genes. These traits could involve many genes and environmental factors. Resistance indicated by molecular analysis still needs to be correlated with phenotypic evaluation (Keka *et al.*, 2008).

In order to dissect the different genes, it is imperative that there should be a preliminary screening of germplasm in genebanks that is fast enough to support breeding new varieties with improved traits (2016 National Rice R&D Highlights). Various studies on genetic diversity in rice have found medium to high diversity in genebanks (Fuentes *et al.*, 1999; Chakravarthi and Naravaneni, 2006). In the current paper, information from the analysis will be used to provide relevant information on the resistant germplasm stored in the genebank and its use in breeding for RTSV-resistant varieties. There have been several studies that have used molecular markers with emphasis on determining genetic diversity (Ni *et al.*, 2002; Ravi *et al.*, 2003; Chakravarthi and Naravaneni, 2006; Thomson, *et al.*, 2007; Rahman *et al.*, 2010; Sajib *et al.*, 2012; Ashraf *et al.*, 2016). On one hand, there are studies that have looked into genetic diversity in terms of responses of different rice cultivars to abiotic stress such as salinity (Kanawapee *et al.*, 2011), iron toxicity (Onaga *et al.*, 2013), drought (Anupam *et al.*, 2017) and aerobic condition tolerance (Singh and Sengar, 2015). On the other hand, very few studies have utilized molecular markers to showcase diversity such as bacterial leaf blight (Sabar *et al.*, 2016) and blast-linked markers (Anupam *et al.*, 2017). The current study looked into the diversity using *tsv1*-linked markers that are involved with resistance to RTSV.

Ravi *et al.* (2003) reported that SSR analysis resulted in a more definitive separation of clusters of genotypes, indicating a higher level of efficiency of SSR markers for the accurate determination

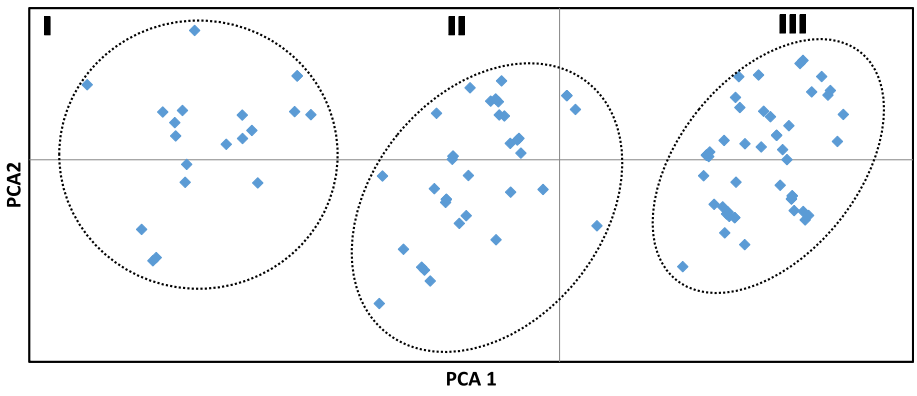

Fig. 3. Principal coordinate analysis of 103 TRVs based on 11 tsv*1*-linked SSR markers showing distinct groupings.

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of relationships between accessions. Chakravarthi and Naravaneni (2006) supported this finding in their paper on genetic diversity and DNA fingerprinting of 15 elite rice genotypes using 30 SSR primers: distinct polymorphism was found among the cultivars studied, indicating the robust nature of microsatellites in revealing polymorphism. This polymorphism gives precise information about the extent of genetic diversity which helps in the development of an appropriate breeding programme. Sajib *et al.* (2012) found PIC values ranging from 0.14 (RM510) to 0.71 (RM163) in all loci, with an average of 0.43, similar to the findings of the current study (0.70–0.73). Rice genetic improvement depends mainly on the extent of variability present in the population. Molecular markers are useful tools in the assessment of genetic variation in terms of non-genic and genic markers. The current study presented the ability to use molecular markers linked to a specific gene of interest. The information could provide a fast and robust preliminary evaluation of rice germplasm using *tsv1*-linked SSR markers. The availability of these data will provide guidance to future breeding activities for RTSV resistance varieties. The study explored and was successful in the application of SSR markers for screening of the presence and diversity of resistance genes present in the germplasm. Such information will also provide a faster data turn-over to the database containing relevant information on the germplasm kept in genebanks.

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Conflict of interest. None.

Ethical standards. Not applicable.

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