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Genetic diversity of *Oryza sativa* 'Dahanala' traditional red rice and molecular markers associated with trichome density on adaxial surfaces

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Abstract: *Oryza sativa* 'Dahanala' is a traditional red rice variety acknowledged for its thrips resistance. This study focused on the genetic diversity assessment of Dahanala accessions in Sri Lanka. Twenty-six Dahanala accessions conserved at the genebank of the Plant Genetic Resources Centre and six accessions from the Rice Research and Development Institute, Batalagoda, were analyzed using seven seed morphology characters followed by molecular characterization with 31 simple sequence repeat markers, showing a significant genetic variation of the accessions. Accessions 003924/003327 and 0010160/006165 were reported as two potential pairs of duplicates. The polymorphic information content values varied between 0 (RM255) and 0.697 (RM412). Genetic distance ranged within 0.0 and 0.94 revealing considerable genetic variance. Sixteen closely related accessions were selected as a representative set of Dahanala, including accessions 003924, 003327, 006376, 010160, 006165, 006378, 004968, 003304, 006739, 005386, 004507, 003149, 003131, 627, 626 and 629. To assess the variation of trichome phenotypes among Dahanala accessions, leaf trichome density as well as RM277 and RM279 markers, which carry a putative relationship with thrips resistance, were used as morphological and molecular markers, respectively. Results revealed a variation of trichome density from 6.7 to 30.83 trichomes/mm^2. According to the molecular marker analysis, both markers revealed polymorphism in thrips-resistant Dahanala accessions and susceptible *Oryza sativa* 'Suduru Samba' accessions, but no clear linkage between the markers and trichome phenotypes within the selected Dahanala accessions could be found. Further studies are needed to dissect the relationship between trichome phenotype and thrips resistance in red rice.

Keywords: Dahanala, Genetic diversity, Rice, Simple sequence repeat markers, Trichome density

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Introduction

As the oldest domesticated crop since more than 10,000 years, rice (*Oryza sativa*) has become central to the lives of billions of people around the world and is rated as the world's second most important cereal crop following only corn (Gramene; Shahbandeh, 2022). In 2019, with a production volume of over 209Mt, China was ranked as the world's largest rice producer followed by India and Indonesia. Though the total world rice production reached nearly 496Mt in 2020, the supply is not sufficient to fulfil the rising demand of an increasing global population and decreasing cultivable land (Shahbandeh, 2022). Most of the lands used for rice cultivation in those countries, including Sri Lanka, are occupied with new improved varieties (NIV), while traditional varieties are cultivated to a minor extent. Despite its lower productivity, emerging knowledge of traditional rice health benefits h as c ontributed to

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increasing its market value (Wickramasinghe and Noda, 2008). Beneficial qualities include antioxidant and indigenous medicinal properties, good nutritional values and good quality attributes of the grains (Suriyagoda *et al*, 2011). Most prominently, resistance towards biotic and abiotic stresses has been shown for traditional varieties whereas many of the NIV remain susceptible to those stress conditions (Wickramasinghe *et al*, 2007).

Oryza sativa 'Dahanala' is a traditional red rice variety conserved at the Plant Genetic Resources Centre (PGRC) genebank, Gannoruwa, Sri Lanka, and is acknowledged for its indigenous medicinal value. Furthermore, the characterization of national rice germplasm has been able to identify traditional rice varieties such as Dahanala and Wanni Dahanala as donors of thrips resistance (Kudagamage, 1977; Nugaliyadde and Heinrichs, 1984). The thrips resistance of some traditional rice varieties may be attributed to a higher density of non-glandular trichomes on the adaxial surface of the leaves, which contributes to the plant's antixenosis ability by preventing thrips from landing on the plant surface and inhibiting their movements and feeding (Panda and Khush, 1995).

Simple sequence repeats (SSR) are defined as a small sequence of DNA that contains repeat motifs ranging in size between one and six base pairs. Unlike other DNA markers, such as restriction fragment length polymorphism (RFLP), random amplification of polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP), microsatellite SSR markers have been preferred by scientists due to their ability to represent the polymorphic behaviour of an individual by producing comparatively higher informative bands along with co-dominant nature and reproducibility of the results (Korzun, 2002).

A considerable collection of highly reproducible microsatellite markers (SSR), dispersed throughout 12 chromosomes of the rice genome, is available (McCouch *et al*, 2001). Since their development in 1985, microsatellites have been used for characterizing populations, diagnosing certain genotypic characters through DNA fingerprinting and screening different types of individuals (Jeffreys *et al*, 1985).

Within a breeding programme to develop thrips-resistant improved rice cultivars, microsatellites markers linked to thrips resistance in rice were identified using bulk segregant analysis of F_2 progeny derived from a cross between thrips resistant Dahanala and thrips susceptible Suduru Samba rice varieties (Gimhani, 2010). According to the findings, a putative association of thrips resistance with RM279 and RM277 markers and evidences for a possible major QTL responsible for thrips resistance close to the RM279 marker locus were discovered (Gimhani, 2010). Moreover, a significant association between markers RM277 and RM279 and higher trichome density on the adaxial surface of the leaf was also found.

In Sri Lanka, a total of 32 Dahanala accessions are conserved in genebanks, including 26 accessions at the PGRC genebank, Gannaoruwa, and 6 accessions at the Rice Research Development Institute (RRDI), Batalagoda. As these accessions are conserved by the vernacular names given by farmers (Wanni Dahanala, Dhana Hala, Dhanala, Danahala and Dahanala), the possibility of conserving duplicates of the same cultivar under different names or, conversely, different cultivars under the same name, is high.

The objective of the present study was to analyze the genetic diversity of 32 accessions of the Dahanala traditional rice variety conserved in Sri Lanka, using both morphological and molecular parameters. Furthermore, the study scope was targeted on the evaluation of trichome densities and the presence of RM277 and RM279 marker loci in Dahanala accessions as putative correlation with thrips resistance, thus providing information on a potentially valuable breeding resource.

Materials and methods

Plant materials

Thirty-two Dahanala accessions were included in this study, listed in Table 1. Seeds of 26 accessions were obtained from the PGRC genebank, Gannoruwa, Sri Lanka, and six accessions from RRDI, Batalagoda, Sri Lanka. Accession 006739 is the oldest Dahanala line available in Sri Lankan genebanks. The advanced breeding line *Oryza sativa* Bg 360 was used as reference variety during the analysis.

Morphological seed characterization

Five replicated seeds of each Dahanala accession were evaluated with seven qualitative and quantitative characters (i.e. seed shape, colour and pubescence of lemma and palea, seed coat colour, sterile lemma colour, grain length and grain width), using a modified rice seed evaluation list based on the International Plant Genetic Resources Institute rice descriptors (Bioversity International, IRRI and WARDA, 2007). See Supplemental Data 1 for a detailed description of seed morphological characters.

Molecular characterization

DNA extraction

Genomic DNA of each accession was extracted from 3-week-old immature leaves using a modified cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson, 1980) with some optimizations at PGRC, Gannoruwa.

About 20–30 seeds per accession were cleaned well and allowed to soak in distilled water in Petri dishes for 24 hours to enhance germination. The soaked seeds were then transferred onto wet tissues inside the labelled Petri dishes for germination. Distilled water and Albert's solution (1g/450ml) were added from time to time to maintain the moisture and nutrient level required by the seedlings. Seedlings were inspected for any fungal contamination and infected

Table 1. Details of plant material used in the study. PGRC, Plant Genetic Resources Centre, Gannoruwa, Sri Lanka; RRDI, Rice Research Development Institute, Batalegoda, Sri Lanka; CRBS, Central Rice Breeding Station (presently named RRDI), Batalagoda, Sri Lanka; CARI, Central Agricultural Research Institute, Sri Lanka; IRRI, International Rice Research Institute, Los Baños, the Philippines.

No.	Accession Number	Conserved Location	Cultivar Name	Origin
1	002049	PGRC Genebank	Dahanala	CARI
2	002050	PGRC Genebank	Dahanala	CARI
3	002053	PGRC Genebank	Dahanala	Unknown
4	003131	PGRC Genebank	Dahanala 2014	Unknown
5	003149	PGRC Genebank	Dahanala 37 YM 2014	Unknown
6	003386	PGRC Genebank	Dahanala	Unknown
7	003540	PGRC Genebank	Dahanala	Unknown
8	003917	PGRC Genebank	Dahanala	Unknown
9	003924	PGRC Genebank	Dahanala	Unknown
10	003971	PGRC Genebank	Dahanala	Unknown
11	004030	PGRC Genebank	Dahanala	Unknown
12	004968	PGRC Genebank	Dahanala	Unknown
13	006165	PGRC Genebank	Dahanala	Unknown
14	006376	PGRC Genebank	Dahanala	Unknown
15	006377	PGRC Genebank	Dahanala	Unknown
16	006378	PGRC Genebank	Dahanala	Unknown
17	006739	PGRC Genebank	Dahanala	IRRI
18	010160	PGRC Genebank	Dahanala	Unknown
19	014122	PGRC Genebank	Dahanala	Unknown
20	015533	PGRC Genebank	Dahanala	Unknown
21	006357	PGRC Genebank	Wanni Dahanala	Unknown
22	006358	PGRC Genebank	Wanni Dahanala	Unknown
23	003304	PGRC Genebank	Danahala	Unknown
24	003327	PGRC Genebank	Danahala	Unknown
25	004507	PGRC Genebank	Dhana Hala	Unknown
26	005386	PGRC Genebank	Dhanala	CRBS
27	592	RRDI	Wanni Dahanala	CRBS
28	626	RRDI	Dahanala	CRBS
29	627	RRDI	Dahanala	CRBS
30	629	RRDI	Dahanala	CRBS
31	1214	RRDI	Dahanala	CRBS
32	1246	RRDI	Dahanala	CRBS
33	Advanced breeding line	RRDI	Bg 360	
34	003333	PGRC Genebank	Suduru Samba	Unknown
35		RRDI	Suduru Samba	Unknown

ones were discarded. About one week after germination, seedlings were transferred to pots filled with mud at the plant house. Two weeks after transplanting, juvenile immature leaves were harvested for DNA extraction. Two grams of fresh leaf samples were ground well with liquid nitrogen using mortar and pestle until a fine powder was formed. The powdered sample was transferred into Oak Ridge centrifuge tubes with 4ml of preheated (65°C) 2% CTAB extraction buffer. Subsequently, 1.2μl of 0.2% β-mercaptoethanol was added to each tube. The tubes were incubated at 65°C for 30 minutes in a water bath. After that, an equal amount (4ml) of chloroform:isoamyl alcohol (24:1) was added to each tube. The tubes were slowly shaken for 10 minutes in a shaker.

All the tubes were centrifuged at 8,000rpm for 15 minutes. The supernatant was transferred into a new tube without disturbing the interface. Then an equal volume of chloroform:isoamyl alcohol (24:1) was added to each tube. After that, the tubes were centrifuged at 8,000rpm for 15 minutes. The supernatant was transferred into a new vial and an equal volume of chloroform:isoamyl alcohol (24:1) was added again. After the repeated centrifugation step (8,000rpm) pelleted DNA was spooled out, transferred to a centrifuge tube and washed by adding 70% ethanol followed by centrifugation at 10,000rpm for 5 minutes.

DNA pellets were air-dried for 3–4 hours and stored in TE buffer at -20°C. DNA integrity checking and quantification were carried out using 0.8% Agarose gel electrophoresis.

Microsatellite marker characterization

Thirty-one microsatellite markers dispersed broadly over the 12 rice chromosomes were selected from the published sequence database (Gramene Microsat). The primer sequence information is provided in Table 2. In addition, markers RM277 and RM279, previously shown to be correlated with trichome density and thrips resistance, were tested on selected accessions.

Primer optimization

Prior to genotyping of each accession, the annealing temperature for each primer was optimized using the temperature gradient method to avoid non-specific PCR amplification. PCR amplification was carried out for all 31 primer pairs using an annealing temperature range between 51°C and 61ºC. Amplified PCR products were confirmed by using 1.5% agarose gel electrophoresis in 0.5x TBE buffer at 90V for 45 minutes to 2 hours, based on the product size. The annealing temperature with minimum non-specific products was selected for each primer for PCR amplification (Table 2).

Molecular assessment of DNA bulks

To reduce costs, DNA samples were bulked for initial genotyping of Dahanala using the selected 31 SSR primers. PCR amplifications were performed using Applied Biosystems 9902 thermal cycler. The initial denaturation step was performed at 94°C for 4 minutes, followed by 35 cycles of denaturation at 95 °C for 1 minute, primer annealing at optimized temperatures (55ºC/57ºC/59ºC/61ºC) for 1 minute, extension at 72°C for 2 minutes, and final extension at 72°C for 5 minutes. Amplified PCR products were confirmed by 1.5% agarose gel electrophoresis.

Confirmed amplified PCR products of all 31 SSR primers were resolved using 8% non-denaturing polyacrylamide gel (acrylamide:bisacrylamide ratio of 29:1) electrophoresis (PAGE) using 1x TBE buffer and DNA bands were visualized by the Bio-Rad gel documentation system with the assistance of Quantity One software. Where necessary, the DNA bulks were resolved to separate divergent markers.

Trichome density analysis

A total of 297 rice seedlings were transplanted representing all 32 accessions of Dahanala along with the reference variety (Bg 360) into pots filled with mud at the PGRC plant house. Trichome density was determined 30 days after transplanting, using the third leaves of three randomly selected plants for each accession. The trichome count was acquired by observing the first $\frac{1}{4}$ area of the adaxial surface of the leaf blade from the tip of each sample under a light microscope keeping a paper strip with a $2\times3mm^2$ square hole on it. Nine readings per individual were recorded. The average trichome density (trichome number/mm^2) per accession was calculated from the mean of three counts per sample (Wickramasinghe *et al*, 2007).

Data scoring and analysis

Morphological characterization: Cluster analysis of morphological data was carried out through the average linkage method and Euclidean distance using Minitab version 15 (Minitab, 1991).

Molecular characterization: Gel images were scored manually by visual observation as presence (1) and absence (0) of every allele for all 31 pairs of SSR markers. DNA bands of expanded bulks were scored individually along with non-expanded bulks. Scored data were analyzed by Power Marker version 3.25 (Liu and Muse, 2005) calculating major allele frequency, polymorphic information content (PIC) value and Nei's genetic distance (Nei *et al*, 1983) across the 32 accessions to identify the prevailing genetic diversity. The phylogenetic tree was constructed based on Nei's genetic distance (Nei *et al*, 1983) according to neighbour-joining method using MEGA 6.06 software (Tamura *et al*, 2013).

Trichome density analysis: The trichome density distribution of the population was assessed through the Kruskal-Wallis test at 0.05 significance level by using Minitab version 15.

Results

Morphological characterization of 32 Dahanala accessions

Considerable variation in seed morphology was observed among the 32 Dahanala accessions (Table 3, Figure 1). Grain shapes were either half-spindle shaped (6–7mm) or spindle shaped (7–8mm) whilst vast variation was identified in lemma and palea colour. The majority of accessions had brownish-black lemma and palea colour, only one had brownish-black furrows on straw background (Acc. No. 003971). All seeds had red pericarp and straw-coloured sterile lemma, showing no diversity in these traits. The nature of the pubescence varied between short hairs and hairs on the upper portion. Grain width ranged from 2.858mm to 3.508mm while grain length varied from 9.314mm to 6.946mm.

In a dendrogram based on the seed morphological characters, the 32 accessions clustered into eight groups at 91.18% similarity level showing their potential closer relationship (Figure 2). Accessions 003971, 002049, 002050 and 014122 clustered independently and distantly denoting morphological deviations from the rest of the accessions.

Molecular assessment of Dahanala accessions using SSR microsatellite markers

To reduce costs, the 32 DNA samples were pooled into eight bulks at 91.18% similarity level, based on the dendrogram resulting from seed morphological

characterization (Figure 3). All DNA bulks were able to produce amplified fragments with all selected 31 SSR primers, of which 15 primers with uniform bands without any heterogenic banding patterns. (Figure 3 and Supplemental Figure 1).

DNA bulks (B3, B4 and B6) displaying heterogenic banding patterns or unusual stutter bands for some markers (e.g. RM515, Supplemental Figure 2) were expanded and PCR products resolved again on 8% PAGE (Figure 4; Supplemental Figure 3 and 4) along with other non-expanded bulks amplified with the same primer. DNA bulks displaying a single banding pattern were not expanded as all DNA samples contained in those bulks were assumed to be homozygous for that marker.

Allelic diversity

Three to six alleles per locus were amplified with the mean value of four by 31 SSR primers across the 32 Dahanala accessions resulting in a total of 124 alleles. Except for monomorphic primer RM255, which was discarded from further analysis, the other 30 markers produced a range of alleles among the bulks and individual accessions. RM202 primer had the highest polymorphism, yielding six alleles per locus. The lowest major allele frequency was observed for marker RM515 (0.303). Genetic diversity ranged between 0.169 (RM216; RM236) and 0.744 (RM515) with a mean value of 0.475. The PIC varied between 0.161 (RM216; RM236) and 0.697 (RM412) (Table 4).

Table 2. Microsatellite primer details (Gramene Microsat). Ch. No., Chromosome number; AT, optimized annealing temperature (°C). For the location of RM277 and RM279 on the chromosomes, see Supplemental Figure 5.

Primer	Forward	Reverse	Product size (bp)	Ch. No.	AT (°C)
RM20B	atcttgtccctgcaggtcat	gaaacagaggcacatttcattg	114–144	11	57
RM25	ggaaagaatgatcttttcatgg	ctaccatcaaaaccaatgttc	120–124	8	57
RM84	taagggtccatccacaagatg	ttgcaaatgcagctagagtac	118–124	1	57
RM201	ctcgtttattacctacagtacc	ctacctcctttctagaccgata	136–150	9	57
RM202	cagattggagatgaagtcctcc	ccagcaagcatgtcaatgta	166–186	11	59
RM207	ccattcgtgagaagatctga	cacctcatcctcgtaacgcc	110–132	2	57
RM208	tctgcaagccttgtctgatg	taagtcgatcattgtgtggacc	164–176	2	57
RM213	atctgtttgcaggggacaag	aggtctagacgatgtcgtga	126–150	2	59
RM215	caaaatggagcagcaagagc	tgagcacctccttctctgtag	146–160	9	59
RM216	gcatggccgatggtaaag	tgtataaaaccacacggcca	132–154	10	61
RM217	atcgcagcaatgcctcgt	gggtgtgaacaaagacac	114–144	6	57
RM219	cgtcggatgatgtaaagcct	catatcggcattcgcctg	184–204	9	55
RM220	ggaaggtaactgtttccaac	gaaatgcttcccacatgtct	100–130	1	61
RM224	atcgatcgatcttcacgagg	tgctataaaaggcattcggg	124–142	11	61
RM228	ctggccattagtccttgg	gcttgcggctctgcttac	100–122	10	61
RM236	gcgctggtggaaaatgag	ggcatccctctttgattcctc	190–196	2	57
RM237	caaatcccgactgctgtcc	tgggaagagagcactacagc	124–138	1	57
RM241	gagccaaataagatcgctga	tgcaagcagcagatttagtg	124–142	4	57
RM255	tgttgcgtgtggagatgtg	cgaaaccgctcagttcaac	132–154	4	57
RM259	tggagtttgagaggaggg	cttgttgcatggtgccatgt	148–176	1	59
RM270	ggccgttggttctaaaatc	tgcgcagtatcatcggcgag	210–224	12	57
RM277	cggtcaaattcatcacctgac	caaggcttgcaagggaag	118–124	12	55
RM279	gcgggagagggatctcct	ggctaggagttaacctcgcg	148–174	2	55
RM412	cacttgagaaagttagtgcagc	cccaaacacacccaaatac	176–200	6	57
RM418	tcgcgtatcgtcatgcatag	gagcacatatgccacgtacg	245–290	7	59
RM440	catgcaacaacgtcaccttc	atggttggtaggcaccaaag	161–217	5	59
RM480	gctcaagcattctgcagttg	gcgcttctgcttattggaag	199–221	5	61
RM515	taggacgaccaaagggtgag	tggcctgctctctctctctc	211–219	8	57
RM518	ctcttcactcactcaccatgg	atccatctggagcaagcaac	158–180	4	55
RM536	tctctcctcttgtttggctc	acacaccaacacgaccacac	220–230	11	61
RM539	gagcgtccttgttaaaaccg	agtagggtatcacgcatccg	249–289	6	61
RM560	gcaggaggaacagaatcagc	agcccgtgatacggtgatag	224–240	7	59
RM571	ggaggtgaaagcgaatcatg	cctgctgctctttcatcagc	182–192	3	61

Table 3. Variation of seed morphology of 32 Dahanala accessions. Grain shape: 2, semi-round shape; 3, half-spindle shape; 4, spindle shape. Lemma and palea colour: 0, straw; 3, brown furrows on straw; 4, brown; 9, black; 11, brownish-black; 18, brownish-black furrows on straw background. Seed coat color: 5, red. Sterile lemma color: 1, straw. Pubescence of lemma: 3, hair on upper portion, 4, short hairs. For the complete list of modified descriptors, see Supplemental Data 1.

Accession No.	Grain shape	Lemma and palea colour	Seed coat colour	Sterile lemma colour	Pubescence of lemma	Grain width [mm]	Grain length [mm]
002049	4	0	5	1	3	3.218	9.314
002050	3	0	5	1	4	3.264	8.366
002053	4	3	5	1	4	3.186	8.336
003131	4	11	5	1	3	3.204	8.618
003149	3	11	5	1	3	3.084	8.250
003386	4	3	5	1	4	3.230	8.878
003540	4	3	5	1	3	3.480	8.466
003917	2	0	5	1	4	2.858	7.126
003924	3	9	5	1	4	3.386	7.912
003971	3	18	5	1	3	3.508	8.388
004030	4	3	5	1	4	3.304	8.584
006165	3	9	5	1	3	3.010	7.790
004968	3	11	5	1	3	2.866	7.158
006376	4	9	5	1	3	3.106	8.156
006377	3	0	5	1	3	3.346	6.946
006378	4	11	5	1	4	3.266	7.848
006739	3	11	5	1	3	3.204	7.880
010160	3	9	5	1	3	3.126	8.148
014122	4	4	5	1	4	3.366	9.620
015533	4	4	5	1	4	3.386	8.446
006357	4	3	5	1	4	2.880	8.772
006358	4	3	5	1	3	3.074	8.688
003304	3	11	5	1	3	3.454	8.654
003327	3	9	5	1	4	3.166	8.188
004507	3	11	5	1	4	3.094	8.364
592	3	3	5	1	3	3.252	8.580
626	3	11	5	1	4	3.256	8.848
627	3	11	5	1	4	3.436	8.340
629	3	11	5	1	3	3.198	8.082
1214	3	4	5	1	4	3.148	8.602
1246	3	4	5	1	3	3.204	8.126

Cluster analysis

A phylogenetic tree was constructed from Nei's genetic distances and showed significant genetic diversity within the population by grouping all accessions into six major clusters (Figure 5). Some variation of the clustering pattern was observed compared to previously classified eight bulks based on morphology characterization. Bulks 1, 2 and 8 from morphological characterization, along with control Bg 360 clustered together, also bulks 6 and 7 formed a single cluster. On the other hand, bulk 4 is separated into two sub-clusters based on molecular characterization. Furthermore, phylogenetic results revealed accessions 003924/003327 and accessions 0010160/006165 as two sets of putative duplicates among the 32 Dahanala accessions, suggesting the possibility of having duplicated samples among conserved accessions.

Trichome analysis

The 32 Dahanala accessions were characterized for their trichome morphology and density as a proxy for potential thrips resistance. A significant variation in trichome density within the population was identified ($P<0.05$) (Table 5). The highest and the lowest density values were recorded from accession 003386 (30.83 trichomes/mm^2) and accession 006357 (6.70 trichomes/mm^2), respectively. The Suduru Samba variety, which is highly susceptible to thrips, had no trichomes (Figure 6) while moderately thrips susceptible Bg 360 showed medium trichome density (8 trichomes/mm^2) (Table 5).

Besides the density variation, differences in nature and the placements of the trichomes on the adaxial surfaces of the rice leaves were observed under the light microscope (Figures 7 and 8).

Figure 1. Seed morphological characters of Dahanala red rice accessions. Left: hulled seeds; Right: dehulled seeds. Acc. No.006739, reported to be the oldest line of Dahanala variety conserved at IRRI, is highlighted as a reference accession for morphological comparison with seeds of other accessions.

Evaluation of the presence of RM277 and RM279 in selected Dahanala accessions

Based on previous studies of Gimhani (2010), RM277 and RM279 primers, selected for having a putative relationship with thrips resistance and trichome density, were used for PCR amplification of selected accessions. Ten accessions were selected to represent all 32 accessions of Dahanala based on the calculated trichome density values and significantly different morphology of the trichomes. These included two accessions with long trichomes (Acc. No. 003924; Acc. No. 003304), one accession with short trichomes (Acc. No. 002049), two accessions with the highest and lowest trichome density (Acc. No. 003386 and 006357, respectively), one accession with comparatively bigger gaps in between two trichomes (Acc. No. 592), and one accession which deviated in the cluster analysis (Acc. No. 003971). Moderately thrips-susceptible Bg 360 and highly thrips-susceptible Suduru Samba (two accessions, see Table 1) were used as reference samples.

No polymorphism was observed in amplified PCR products of RM277 between the Bg 360 sample and the selected Dahanala accessions with lengthy trichomes (Acc. No. 003924; Acc. No. 003304), the accession with the lowest trichome density (Acc. No. 006357), the accession with the highest trichome density (Acc. No. 003386), the accession with short trichomes (Acc. No. 002049), the accession with comparatively higher gaps between two trichomes (Acc. No. 592), and the

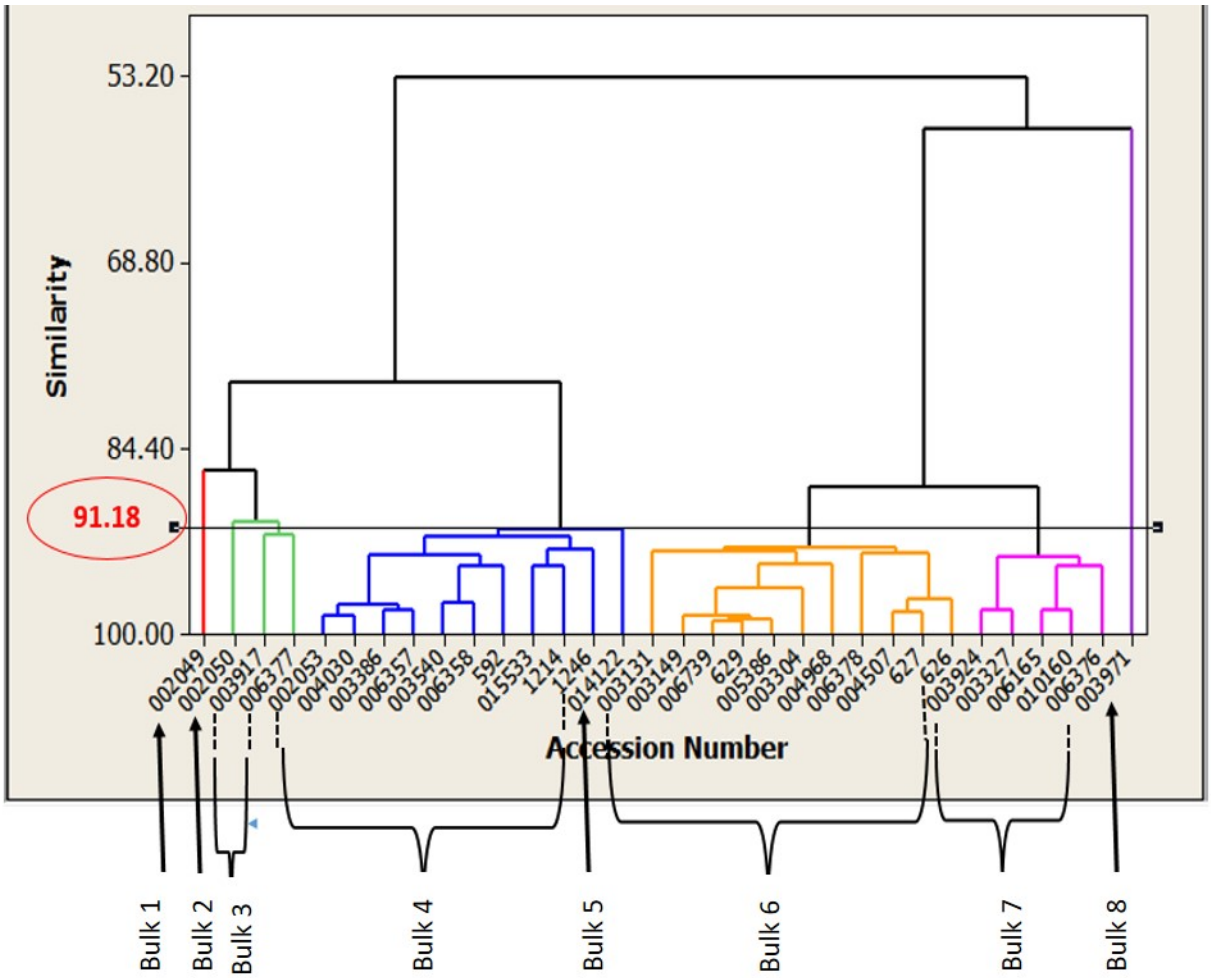

Figure 2. Dendrogram of 32 Dahanala rice accessions based on seed morphological characterization. Accession numbers are as in Table 1. The grouping at 91.18% similarity level used to generate DNA bulks for molecular characterization is outlined on the bottom.

accession that revealed to be a deviated accession in cluster analysis (Acc. No. 003971) (Figure 8). However, the highly susceptible Suduru Samba variety (zero trichomes) was polymorphic in this marker compared to other samples.

Discussion

In this study, we analyzed the genetic diversity among 32 accessions of the traditional rice variety Dahanala using seed morphology and 31 SSR markers, followed by a molecular and phenotypic evaluation of trichomes characters potentially related to thrips resistance. Since the morphological characterization was based on only seven seed traits, comparatively, a molecular diversity assessment using 31 loci present in the genome can provide more accurate results with higher reliability than morphological markers (Semagn, 2002).

The majority of the markers showed allelic diversity with a relatively high PIC value around 0.5, suggesting they can be used in future genetic diversity assessments of rice germplasm (Table 4). Interestingly, a previous study using the same SSR markers (Manatunga *et al*, 2019) also had PIC values ranging from 0.00 (RM518 and RM237) to 0.72 (RM515) denoting high genetic diversity but for different markers.

Similar to other Sri Lankan traditional varieties such as Pachchaperumal, Murungakayan, Pokkali, Kuruluthuda and Kaluheenati, Dahanala germplasm showed a significant genetic variation among the available 32 accessions, ranging from 0.0 to 0.94 compared to varieties Murungakayan (0.00–0.76) and Pokkali (0.33–0.77) (Siriwardhana *et al*, 2016; Warusawithana *et al*, 2017; Manatunga *et al*, 2019).

Our study found two pairs of putative duplicate accessions. Studies of Murungakayan, Pokkali and Kaluheenati varieties have found four sets of duplicates with the highest genetic similarities in their respective germplasms (Siriwardhana *et al*, 2016; Thotago-dawatta *et al*, 2017; Manatunga *et al*, 2019). Concurrently, Karunadasa and Samarasinghe (2017) also identified one set of genetically similar duplicates in Kuruluthuda.

Since the morphological characterization was based on only seven traits, comparatively, molecular diversity assessment can provide more contrasting results with higher reliability because DNA markers cover a

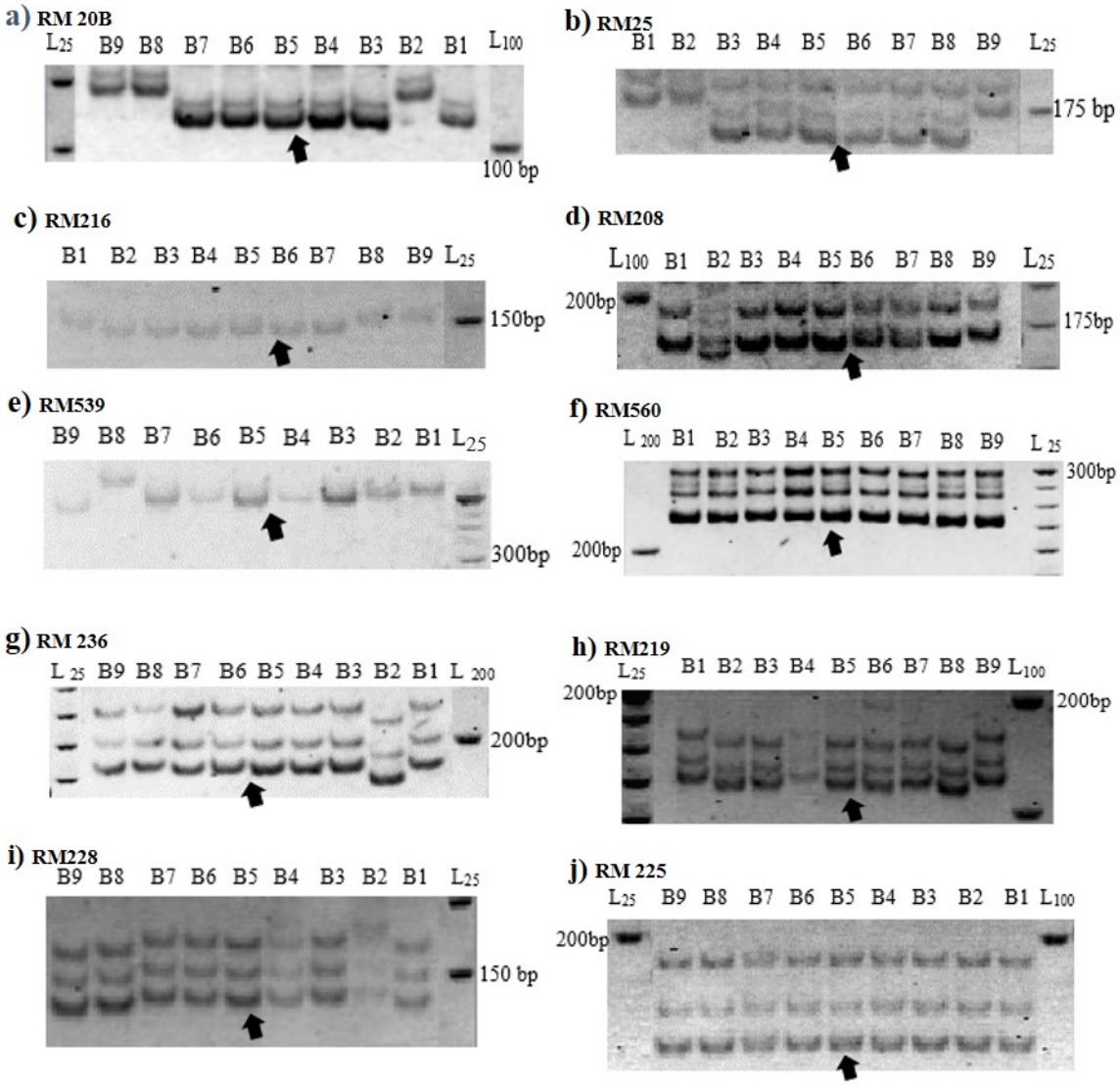

Figure 3. Polyacrylamide gel profiles (8%) showing the amplification of DNA bulks using 10 SSR primers. The arrowheads indicate corresponding scored alleles. B1–B8, DNA bulks; B9, Bg360; L25, L100, L200, size markers; bp, basepairs. For additional SSR primers see Supplemental Figure 1.

major portion of the genome than morphological markers (Semagn, 2002).

According to the constructed phylogenetic tree, unexpectedly, the reference sample Bg 360 clustered with three Dahanala accessions (003971, 002050 and 002049) indicating a closer relationship of these samples respective to other accessions. Separate clustering of these three accessions was also observed under morphological characterization. Similar results were reported from the assessment of 23 Murungakayan accessions, where the Bg 360 reference sample clustered separately with Acc. No. 003495, exhibiting a genetic deviation from other accessions (Manatunga *et al*, 2019). Matsui and Kagata (2003) suggest that higher genetic variation could exist among individual accessions of the same cultivar because of cross-pollination along with temperature stress and some mutagenic characteristics of floral organs. Additionally, the accidental mixing of seed samples during the process of sample collection and storage in genebank may cause some deviation.

In the present study, 16 out of 32 accessions were selected as a representative set of Dahanala, which group together in Cluster 1 with the highest similarity and carry the highest distance from the reference sample Bg 360. These include PGRC accessions 003924, 003327, 006376, 010160, 006165, 006378, 004968, 003304, 006739, 005386, 004507, 003149, 003131 along with three RRDI accessions 627, 626, 629. Most importantly, those 16 accessions clustered together with the Acc. No. 006739, which is considered the oldest line of Dahanala found in Sri Lanka (Figure 6). Similarly, in previous studies, 11 out of 20 accessions and 8 out of 23 accessions were selected as representative sets of Pachchaperumal and Murungakayan, respectively (Warusawithana *et al*, 2017; Manatunga *et al*, 2019).

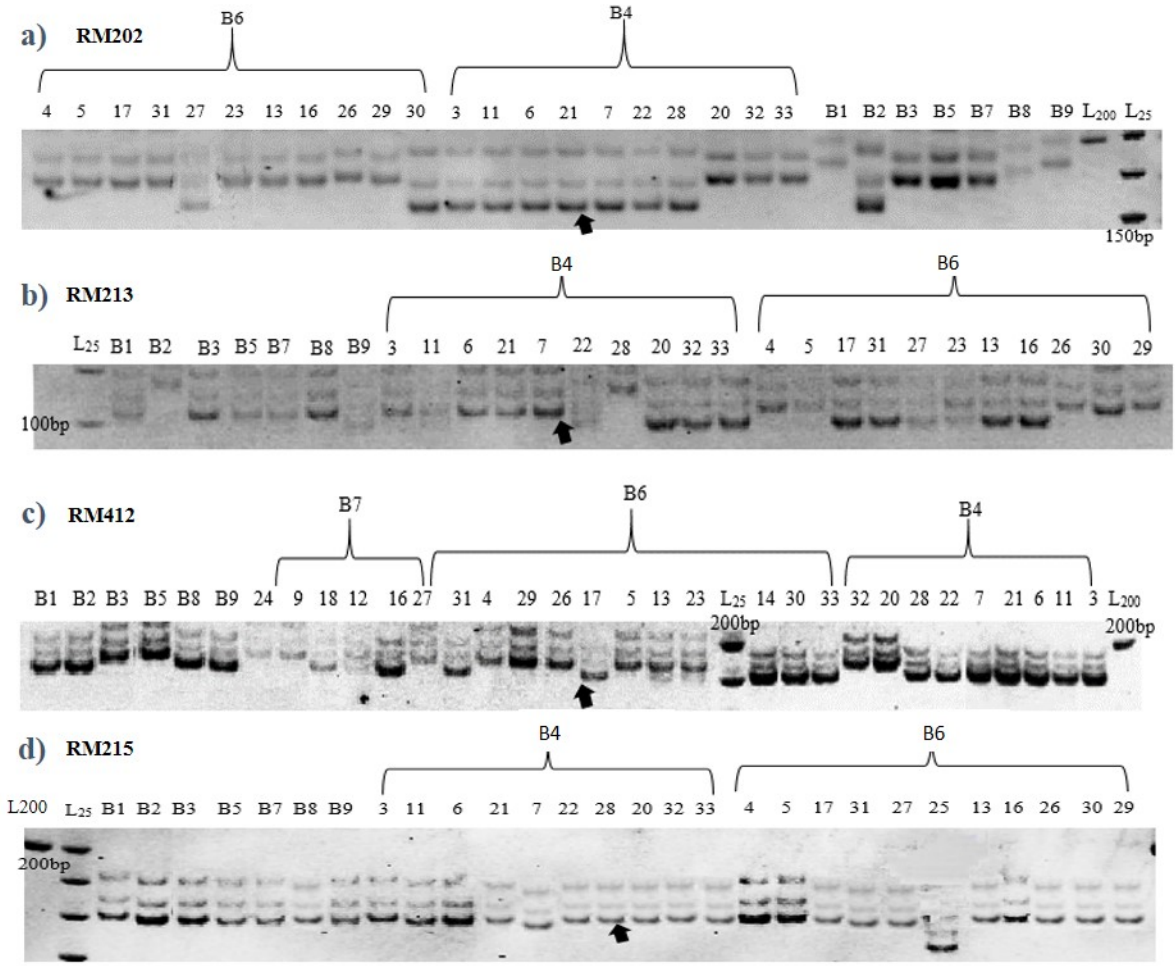

Figure 4. Amplification of individual accessions of expanded DNA bulks using four SSR primers. PCR products were separated on 8% PAGE along with other bulks. B, bulks; L25, L100, L200, size markers; bp, basepairs. Numbers indicate individual accessions within bulks (as in Table 1). Additional markers are included in Supplemental Figures 3 and 4.

As the morphological characterization is based on only seven seed morphology related traits, comparatively, the molecular diversity assessment, which carries an assessment of 31 loci present in the genome, provided more accurate results with higher reliability. Present results can be useful in selecting suitable accessions of Dahanala as donor parents in future rice breeding programmes. However, with respect to conserving germplasm of the Dahanala traditional rice variety, further studies should be conducted to verify the identity of this representative sample of Dahanala via amplification of a higher number of loci or sequencing the genome.

Trichome analysis

Since previous studies have revealed a relationship of thrips resistance with the presence of non-glandular trichomes on the adaxial surface of the leaves (Nugaliyadde and Heinrichs, 1984), and thrips reported to be fed on the mesophyll cells of the young leaves through the adaxial surface (Gimhani, 2010), our study focused on inspection of the trichome density on the adaxial surface of Dahanala, which is known as a thrips-resistant variety.

Even though all examined accessions were conserved in genebanks under the same variety name, we detected a significant variation among trichome densities as well as in the trichomes' nature and locality among accessions (Figures 6 and 7).

In a first attempt at identifying QTLs for thrips resistance in rice, Gimhani (2010) identified both thrips resistance and trichome density as quantitative traits based on bulk segregating analysis followed by a screening of an F_2 population between thrips-resistant Dahanala and susceptible Suduru Samba. Gimhani (2010) found a significant negative correlation between thrips damage and the trichome density values ($r = -0.378$, $P<0.05$), revealing the damage score is low when the trichome density is high. They also showed that 14.3% of phenotypic variation (thrips resistance) in F_2 segregants could be explained by a factor of presence of trichomes, suggesting that the presence of trichomes could play a significant role in defending the plants against thrips, in agreement with the findings of Nugaliyadde and Heinrichs (1984), who reported that morphological features of the foliage and the presence of allomones in resistant plants contribute to their defence against thrips. In addition, Ananthakrishnan (1979)

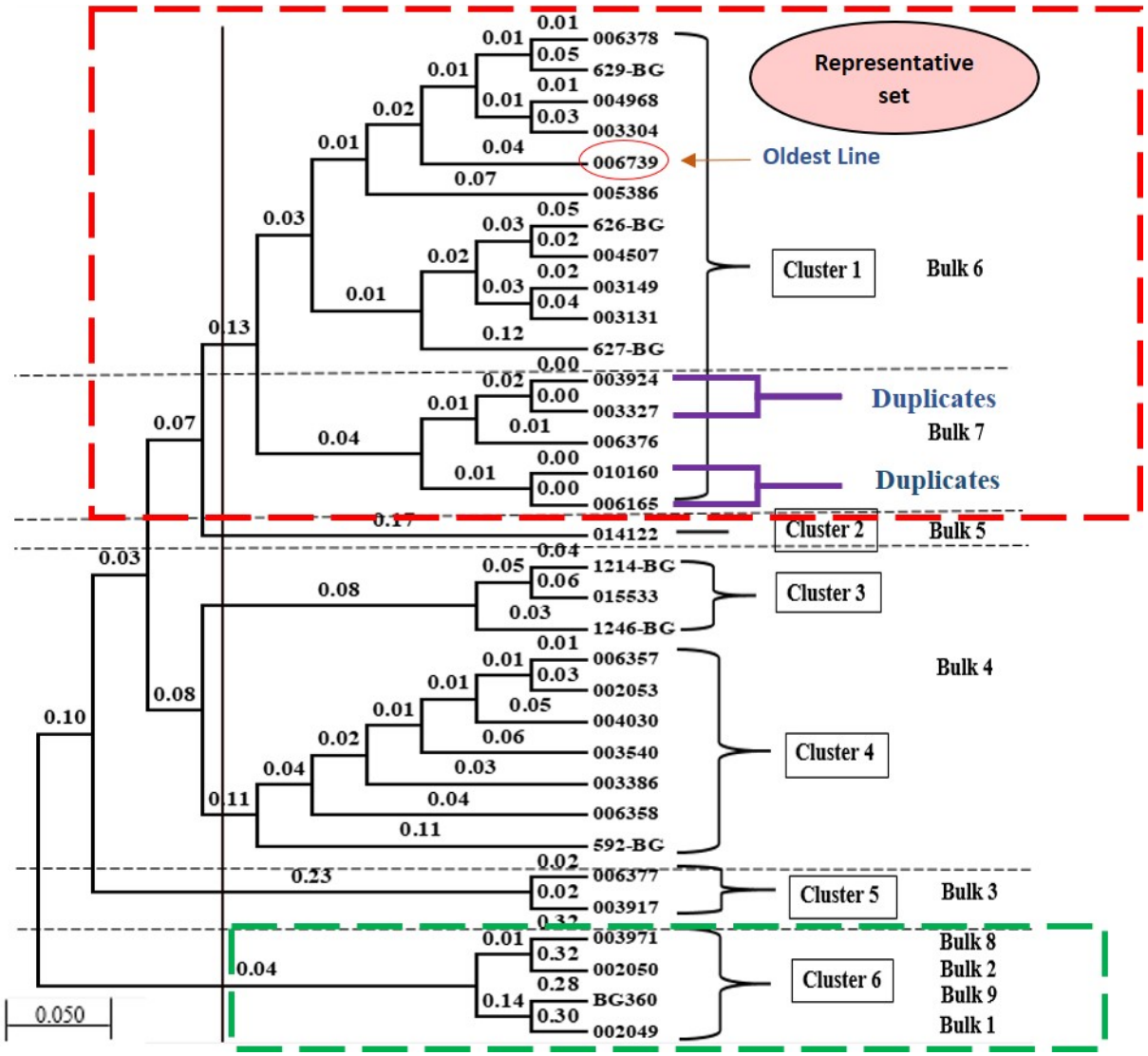

Figure 5. Dendrogram of Dahanala accessions based on analysis of simple sequence repeat (SSR) markers. Branch lengths are indicated by decimal values on the branches. Bulks based on morphological clustering are indicated. Duplicates and reference accessions are indicated. The representative set of Dahanala accessions is indicated by a red-dotted rectangle. The deviated bulks are indicated by the green-dotted rectangle. BG, Batalagoda accessions.

found that leaf age and thickness, and the nature and distribution of trichomes and silica cells in the substrate are some important biophysical factors that influence thrips oviposition. On the other hand, Wickramasinghe *et al* (2007), showed that the removal of trichomes on the adaxial surface of the leaf of Dahanala seedlings did not result in a highly susceptible reaction. This indicates that besides trichome density there might be several other factors influencing thrips resistance. Based on the analysis of this study, the nature along with the position of trichomes on the leaf can also be one of those factors.

Although an association of trichrome density with RM277 and RM279 markers was identified previously (Gimhani, 2010), the results of the current project were not able to provide clear evidence of this relationship, as the Dahanala accessions used in this study were not polymorphic for RM277 and the allelic patterns for RM279 of both accessions with the highest (003386; 30.83 trichomes/mm^2) and lowest (006357; 6.70 trichomes/mm^2) trichome density were identical. Additionally, as our current study only analyzed selected representative samples of Dahanala using RM279 and RM277 putative markers, the results are insufficient to predict a clear association. Hence additional studies will be required to further test this hypothesis.

Although marker RM277 did not show polymorphism between selected Dahanala accessions and the Bg 360 variety, the highly thrips-susceptible Suduru Samba varieties with zero trichomes were polymorphic for this marker. Therefore, RM277 can be suitable for use in future analysis to detect Suduru Samba against the Dahanala variety. Additionally, since significant allelic variation was discovered within all selected Dahanala accessions as well as among Dahanala, Suduru Samba, and Bg 360 varieties with RM279 marker, this evidence can be used to select among these groups in future studies.

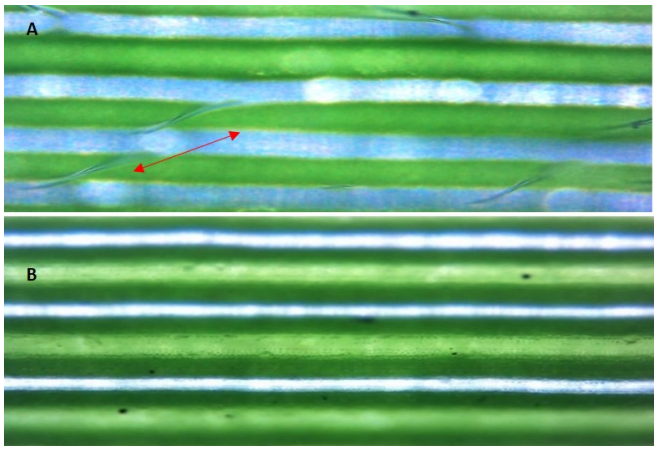

Figure 6. Microscopic images of trichomes on adaxial surfaces of rice leaves of reference samples. A, Bg 360, moderately susceptible to thrips; B, Suduru Samba, highly susceptible to thrips (without any trichomes on the adaxial surface; zero trichomes). The red arrow indicates the presence of trichomes.

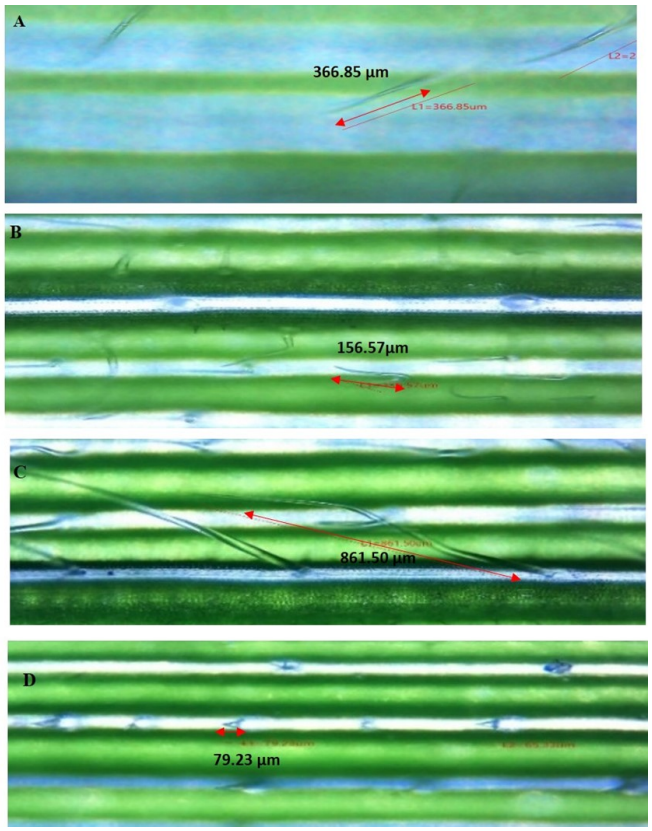

Figure 7. Microscopic images of trichomes on adaxial surfaces of Dahanala rice leaves denoting variance among accessions. A, Acc.No. 006357, the accession with the lowest trichome density (7.0 trichomes/mm^2); B, Acc. No. 003386, the accession with the highest trichome density (31.0 trichomes/mm^2); C, Acc. No. 003304, the accession with the longest trichomes (~860μm); D, Acc.No. 002049, the accession with the shortest trichomes (~79μm). The red arrows indicate the presence of trichomes.

Table 4. Allelic diversity of 31 SSR markers on 32 traditional red rice accessions. PIC, polymorphic information content.

Marker	Major Allele Frequency	Allele No.	Gene Diversity	PIC
RM20B	0.879	3	0.219	0.204
RM25	0.909	4	0.171	0.166
RM84	0.667	4	0.517	0.481
RM201	0.515	4	0.608	0.537
RM202	0.394	6	0.722	0.677
RM207	0.758	5	0.408	0.386
RM208	0.530	4	0.538	0.435
RM213	0.515	4	0.612	0.544
RM215	0.485	5	0.623	0.553
RM216	0.909	3	0.169	0.161
RM217	0.636	4	0.541	0.497
RM219	0.606	3	0.500	0.401
RM220	0.515	5	0.630	0.572
RM224	0.606	5	0.579	0.538
RM228	0.576	3	0.533	0.442
RM236	0.909	3	0.169	0.161
RM237	0.606	3	0.533	0.458
RM241	0.879	4	0.222	0.214
RM255	1.000	1	0.000	0.000
RM259	0.515	4	0.553	0.456
RM270	0.515	3	0.551	0.452
RM412	0.333	5	0.740	0.698
RM418	0.697	5	0.490	0.467
RM440	0.515	5	0.669	0.631
RM480	0.879	3	0.220	0.209
RM515	0.303	4	0.744	0.696
RM518	0.364	5	0.736	0.692
RM536	0.576	5	0.604	0.560
RM539	0.879	4	0.222	0.214
RM560	0.545	3	0.544	0.448
RM571	0.788	5	0.365	0.348
Mean	0.639	4	0.475	0.429

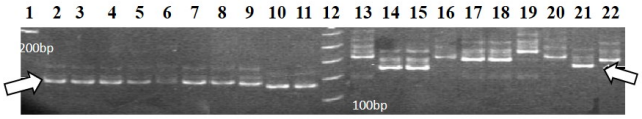

Figure 8. Allelic variation for RM277 and RM279 primers in selected Dahanala red rice accessions. Lanes 2–11, PCR product amplified with RM277; Lanes 1 3–22, PCR product amplified with RM279; 1, 200bp ladder; 2, Acc. No. 003924; 3, Acc. No. 006357; 4, Acc. No. 003386; 5, Acc. No. 002049; 6, Bg360 (moderately susceptible); 7, Acc. No. 592; 8, Acc. No. 003304; 9, Acc. No.003971; 10, Suduru Samba Batalegoda variety; 11, Suduru Samba PGRC variety Acc. No 003333; 12, 25bp ladder; 13, Acc. No. 003304; 14, Suduru Samba PGRC variety Acc. No. 003333; 15, Suduru Samba Batalegoda variety; 16, Acc. No. 003924; 17, Acc. No. 006357; 18, Acc. No. 003386; 19, Acc. No. 002049; 20, Bg 360 (Moderately susceptible); 21, Acc. No. 003971; 22, Acc. No. 592.

Table 5. Trichome density observed for 32 Dahanala traditional red rice accession. Accessions were grouped as follows: A, 31–35 trichomes/mm^2; B, 26–30 trichomes/mm^2; C, 21–25 trichomes/mm^2; D, 16–20 trichomes/mm^2; E, 11–15 trichomes/mm^2; F, 5–10 trichomes/mm^2; G, 0–5 trichomes/mm^2.

Accession No.	Cultivar Name	Density (trichomes/mm^2)	Group
3386	Dahanala	31	A
15533	Dahanala	26	B
626	Dahanala	26	B
2049	Dahanala	25	C
3131	Dahanala 2014	23	C
3149	Dahanala 37 YM	23	C
3924	Dahanala	23	C
10160	Dahanala	23	C
14122	Dahanala	23	C
5386	Dahanala	22	C
3917	Dahanala	21	C
6377	Dahanala	21	C
3304	Danahala	21	C
3327	Danahala	21	C
6376	Dahanala	20	D
3540	Dahanala	19	D
6378	Dahanala	19	D
6739	Dahanala	19	D
2053	Dahanala	18	D
3971	Dahanala	18	D
4968	Dahanala	18	D
629	Dahanala	18	D
4507	Dhana Hala	17	D
627	Dahanala	17	D
1214	Dahanala	16	D
2050	Dahanala	15	E
4030	Dahanala	15	E
592	Wanni Dahanala	14	E
1246	Dahanala	14	E
6165	Dahanala	13	E
6358	Wanni Dahanala	11	E
6357	Wanni Dahanala	7	F
	Bg 360	8	F
	Suduru Samba	0	G

One reason for not detecting a strong association between trichome density and RM279 or RM277 markers in this research might be some limitations associated with the study, such as the low number of accessions studied. Therefore, before selecting a donor parent for thrips resistance among Dahanala accessions, further investigations, including disease assays, are required to corroborate the relationship between trichome density, RM277 and RM279 markers and a possible thrips resistance.

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Supplemental data

Supplemental Data 1: Description of seed morphological characters.
Supplemental Figure 1: Additional SSR marker profiles amplified from DNA bulks (RM201, R M220, RM241, RM259, RM270).
Supplemental Figure 2: PCR profile or heterogenic marker RM515 of DNA bulks.
Supplemental Figure 3: PCR profiles of expanded DNA bulks for SSR markers (RM84, RM207, RM224, RM480, RM518, RM536).
Supplemental Figure 4: PCR profiles of expanded DNA bulks for SSR markers (RM217, RM237, RM418, RM440, RM515, RM571).
Supplemental Figure 5: Map position of markers RM277 and RM279 on rice chromosomes 12 and 2.

Author contributions

All authors contributed to the study's conception and design. V. Koodalugodaarachchi prepared the material, the experiment, and the data collection, and wrote the first draft. D. S. Kekulandara and D. R. Gimhani provided advice on the experiment conduction and data analysis, and commented on the first and second drafts. The final manuscript was read and approved by all authors.

Conflict of interest statement

The authors declare no conflict of interest.

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