# Association of Commercial Rice Varieties with Weedy Rice Accessions (*Oryza sativa* complex) in Pulau Pinang's Rice Granary Area

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**Abstrak:** Varian padi angin merupakan rumpai yang paling ketara dalam petak sawah yang mengamalkan kaedah tabur terus. Kebanyakan varian padi angin seiras dengan variasi yang ditanam menyebabkan padi angin sukar dikawal. Persampelan telah dijalankan di empat lokasi jelapang padi Pulau Pinang. Sebanyak 36 varian padi angin yang berbeza telah dikenal pasti dari 4 lokasi tersebut: B, lokasi utara; P, lokasi tengah; A, lokasi selatan; dan N, lokasi barat daya. Padi liar (*Oryza rufipogon*), yang tumbuh di kawasan kajian, dikutip bersama dengan empat varieti yang ditanam secara meluas di sana (MR84, MR185, MR211 dan MR219). Objektif kajian adalah membuat perbandingan ciri morfologi antara padi angin dan varieti ditanam. Sebanyak 20 ciri dikenal pasti untuk perbandingan padi angin dan varieti ditanam. Analisa morpho metrik berjaya mengasingkan kesemua sampel kajian kepada dua kumpulan utama (A dan B) berasaskan 95% ketidaksamaan metrik. Kumpulan A terbahagi kepada 7 subkumpulan yang terdiri daripada beberapa varian padi angin, padi liar dan MR211 (varieti kawalan), sementara itu, kumpulan B terbahagi kepada 10 subkumpulan yang terdiri daripada baki varian padi angin dan 3 lagi varieti kawalan. Analisa dendogram menunjukkan bahawa varian padi angin dan varieti ditanam boleh dibezakan berasaskan ciri-ciri morfologi yang digunakan dalam kajian ini, kecuali MR211 (varieti yang ditanam) dan padi angin WR A8 yang terkumpul bersama dalam subkumpulan $A_2$. Analisa STRUCTURE menunjukkan kesemua individu kajian terbahagi kepada 18 kluster. Hasil kajian menunjukkan sebahagian gen di dalam varian padi angin telah dipengaruhi oleh varieti yang ditanam. Maklumat hasil kajian memberi manfaat dalam pembentukan protokol pengurusan rumpai dan amalan pertanian yang baik untuk pengawalan padi angin pada masa hadapan.

**Kata kunci:** Padi Angin, Ciri Morfologi, Sawah Padi Pulau Pinang, Struktur Tangkai, Rupa Bentuk, Pokok, Analisa Kluster

**Abstract:** Weedy rice (WR) is the most significant weed in direct-seeded fields. It has morphological characteristics similar to those of cultivated rice varieties. WR is more difficult to control than other weeds. We collected WR accessions from four sites within the Pulau Pinang rice growing areas. Thirty six different accessions were collected from each site: B, the northern site; P, the central site; A, the southern site; and N, the southwestern site. Wild rice (*Oryza rufipogon*), which grows in the sampled areas, was also collected together with four varieties (MR84, MR185, MR211 and MR219) that have been widely planted in these areas for a long period of time. The objective of this study was to compare the morphological characteristics of the WR accessions and cultivated rice. Twenty characteristics were observed for the comparison of WR accessions and rice cultivars.

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Morpho-matrix analyses allowed the specimens to be grouped to two main groups (A and B), based on a 95% dissimilarity matrix. Group A was subdivided into 7 subgroups consisting of a few WR accessions, wild rice and MR211 (control), and group B was subdivided to 10 subgroups consisting of other WR accessions and the 3 other control varieties. Dendrogram analysis indicated that the morphological traits used in this study were able to differentiate among the WR accessions and the cultivars, except for rice cultivar MR211 and WRA8, which grouped together in subgroup A2. STRUCTURE program analysis indicated that all individuals were distinguishable and were divided into 18 clusters. These results suggest that some genes of the WR accessions have been influenced by commercial varieties. The information gained from this study will be useful to develop rice weed management protocols and good agricultural practices to control WR in the future.

**Keywords:** Weedy Rice, Morphological Characteristics, Pulau Pinang Rice Fields, Panicle Structure, Plant Texture, Cluster Analysis

## INTRODUCTION

Weedy rice (WR) is the main competitor in rice growing areas, especially in South East Asia. WR and cultivated rice compete with respect to space, light and nutrients, and this competition affects the yield performance (Azmi *et al.* 2004). In Malaysia, 30%–50% yield losses have been reported in a direct-seeded rice field in which wild rice *(Oryza rufipogon)* was present at densities above 31 panicles/m$^2$, and under heavy infestation, lodging of WR plants may occur and cause total yield loss (Watanabe *et al.* 2000). There are many WR accessions that are found in direct-seeded fields, and the wide spread infestation with WR might be due to the use of machinery, contaminated seeds, irrigation and animal dispersal (Labrada 2007).

WR accessions possess morphological characteristics that are similar to those of cultivated rice varieties, such as leaves and stem nodes. Thus, WR is more difficult to control than other types of weeds. WR is taller than cultivated rice and has easy and early grain shattering characteristics. WR accessions can be classified based on panicle structure (open or compact panicle), grain colour (pigmented or not pigmented) and the presence or absence of awn. Morphological and topographical characteristics of the plants such as the shape and size of the seeds and the structure of incidental features have been useful criteria in the identification and classification of plant species (Noda *et al.* 1985).

A study was conducted by collecting a sample of WR accessions from four different rice-growing areas in Pulau Pinang in 2008. The purpose of this study was to identify the relationship between WR and cultivated rice using morphological characteristics and genomic DNA.

## MATERIALS AND METHODS

### Rice Specimens

Five hundred forty samples of WR (*Oryza sativa* complex) and 15 samples of wild rice (*O. rufipogon*) were collected from the 4 rice-field sites in Pulau Pinang. The WR accessions were collected from four sites within the Pulau Pinang rice-growing areas. Thirty six different accessions were collected from each site: B, the northern site; P, the central site; A, the southern site; and N, the southwestern site. Wild rice that was growing in the sampled areas was also collected. The seeds of each accession were identified and stored at 4°C. Sixty individuals of four rice cultivars (MR4, MR185, MR211 and MR219) that are widely planted in the selected area were used as control varieties for this study. Twenty plants from each accession were planted in the plant house with three replications. Crop establishment practice was based on the "High Yielding Rice" manual (Alias *et al.* 2002). A total of 615 samples were scored at maturity for 20 characteristics used in the description of the Standard Evaluation of Rice (SES) by the IRRI (1996).

### DNA Extraction and SSR Analysis

The upper parts (3–4 cm) of fresh young leaves from each accession were cut, cleaned with distilled water and then ground in liquid nitrogen. DNA was extracted by using cetyltrimenthylammonium bromide (CTAB) protocol (Doyle & Doyle 1987) with modifications. DNA samples were run on 0.8% agarose gels to assess the quality and quantity of DNA. The samples were then stored at −20°C. Eight pairs of Simple sequence repeat (SSR) primers were selected for the SSR analysis (Table 1).

Polymerase chain reactions (PCRs) were carried out using the protocol described by Othman (2004) with modifications. This reaction contained 4 µl of 5x buffer, 0.2 µl 5 mmol/l dNTPs, 2.0 µl of 25 mmol/l $MgCl_2$, 1.0 µl each of 10 µmol/l forward and reverse primers, 0.2 µl Taq polymerase (5 U/µl), 50 ng template DNA and distilled deionised water up to a final volume of 12.5 µl. The PCRs were conducted with following steps: pre-denaturation at 95°C for 5 min, followed by 34 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min and a final extension at 72°C for 5 min. The DNA amplification was performed in a PTC-100 Thermocycler (MJ Research Inc., USA).

The amplified products were electrophoresed on a 6% non-denaturing polyacrylamide gel and visualised by staining with ethidium bromide under ultraviolet (UV) light and photographed using an Alpha imaging system (Alpha Innotech Imaging Station, USA).

### Statistical Analyses

Morphological characteristics data were analysed using SPSS. Input for hierarchical clustering and relationships among the accessions were determined using the Euclidean distance as a similarity measure (SPSS 1985). Analysis was performed using an eigenvalues matrix as the variable to determine the relationships among the accessions with respect to the distance matrix.

Subsequently, the outputs were used in the cluster procedure. Between-groups linkage, also known as UPGMA (unweighted pair-group method using averages) linkage, was used as the default procedure. The distance between two clusters is the average distance between all inter-cluster pairs and the distances between clusters were used to construct a Tree Diagram.

**Table 1:** Eight pairs of SSR primers selected for the SSR analysis.

| No. | Primers | Motive | Primer sequences (5'–3') | Product size |
|---|---|---|---|---|
| 1 | RM157 | (CCT)6 | F: GTC GTC GAC CCA TCG GAG CCA C | 127–141 |
| | | | R: TGG TCG AGG TGG GGA TCG GGT C | |
| 2 | RM190 | (CT)11 | F: CTT TGT CTA TCT CAA GAC AC | 104–124 |
| | | | R: TTG CAG ATG TTC TTC CTG ATG | |
| 3 | RM303 | (GT)4 | F: GCA TGG CCA AAT ATT AAA GG | 143–205 |
| | | | R: GGT TGG AAA TAG AAG TTC GGT | |
| 4 | RM332 | (CTT)12 | F: GCG AAG GCG AAG GTG AAG | 162–180 |
| | | | R: CAT GAG TGA TCT CAC TCA CCC | |
| 5 | RM334 | (CCT)20 | F: GTT CAG TGT TCA GTG CCA CC | 144–200 |
| | | | R: GAC TTT GAT CTT TGG TGG ACG | |
| 6 | RM335 | (CCT)25 | F: GTA CAC ACC CAC ATC GAG AAG | 95–150 |
| | | | R: GCT CTA TGC GAG TAT CCA TGG | |
| 7 | RM544 | (TC)9 | F: TGT GAG CCT GAG CAA TAA CG | 180–248 |
| | | | R: GAA GCG TGT GAT ATC GCA TG | |
| 8 | RM418 | (ATT)21 | F: TCG  CGT ATC GTC ATG CAT AG | 259–311 |
| | | | R: GAG CAC ATA TGC CAC GTA CG | |
| 9 | RM144 | (ATT)11 | F: TGC CCT GGC GCA AAT TTG ATC C | 216–295 |
| | | | R: GCT AGA GGA GAT CAG ATG GTA GTG CAT G | |
| 10 | RM219 | (CT)17 | F: CGT CGG ATG ATG TAA AGC | 180–220 |
| | | | R: CAT ATC GGC ATT CGC CTG | |

In the genomic data analysis, each band was considered to be the result of one pair of SSR primers corresponding to one locus, with one band per allele. The results were evaluated using POPGENE32 v 1.31 (University of Calgary, Canada) (Yeh *et al.* 1999) to compute the deviation from the Hardy-Weinberg equilibrium at the marker and population levels. The STRUCTURE program (University of Chicago, USA) (Pritchard *et al.* 2000) was used to identify the most likely number of clusters present in the dataset. The program estimated the natural logarithm of the probability that an individual X is part of a population K [ln Pr(X/K)] using a Markov Chain Monte Carlo method (Falush *et al.* 2003; Gao *et al.* 2007). For this purpose, 25 different runs were applied from k=2 to k=25, with a burn-in period of 10000 iterations and an admixture model in which the allele frequencies were correlated.

## RESULTS

### Morphometric Analyses

Twenty morphological traits were used to compare the 615 individuals, which were then classified into 41 groups. These groups were 36 WR accessions, 4 rice cultivars and 1 wild rice species (Fig. 1). The dissimilarity matrix analysis suggested that the accessions were grouped into two main groups (A and B). Based on the 95% dissimilarity matrix, group A was subdivided into 7 subgroups ($A_1$, $A_2$, $A_3$, $A_4$, $A_5$, $A_6$ and $A_7$), and group B was subdivided to 10 subgroups ($B_1$, $B_2$, $B_3…B_{10}$).

There was one WR accession in each of the following four subgroups: $B_3$ (B7), $B_7$ (P8), $B_8$ (B4) and $B_{10}$ (B5). There were 3 rice cultivars, MR84, MR219 and MR185, which were grouped together into subgroup $B_4$. This dendrogram analysis indicated that the morphological traits used were able to differentiate among all of the WR accessions and the cultivars except for rice cultivar MR211 and WRA8, which were grouped together into subgroup $A_2$.

Dissimilarity matrix values varied among the WR accessions, rice cultivars and wild rice species. The results indicate that 60.7% of the accessions had values greater than 75. Among the accessions, 8 had values that ranged from 50 to 70, approximately 17% had dissimilarity matrix values that ranged from 25 to 50, and approximately 1% had values below 0.25. The smallest dissimilarity matrix values were identified between P5 and B1 (12.83) and between A9 and N3 (12.93). Meanwhile, the highest dissimilarity matrix values were found between wild rice and B4 and P8.

### Genomic Analyses

Among the 10 pairs of SSR primers used, 8 pairs revealed 95% of the observed polymorphisms in the 615 rice accessions. Two other primers, RM303 and RM332, only revealed a single allele for the corresponding locus. Therefore, the polymorphic primers were used in further analyses in this study. There were 653 to 675 alleles observed among the WR and rice cultivar specimens, with heterozygosity deficits ranging between 2.3% to 7.3%, with an average of 2.7%. Three primers, RM144, RM335 and RM190, did not show any heterozygosity deficit, and their FIS values ranged from −0.17 to −1.0. Five primers had FIS values ranging from −0.17 to 0.54. The FIS value is the inbreeding coefficient of an individual (I) relative to the subpopulation (S) values. A total of 57 alleles were identified among all accessions, with product sizes ranging from 95 to 304 bp. The average number of alleles was 6.3 per locus, with the number varying from 6 to 9 among the tested loci. The observed heterozygosity (Ho) for all accessions ranged from 0.586 to 1.0, while the expected heterozygosity (He) ranged from 0.503 to 0.778. The FIS values for the accessions ranged from −1.0 to 0.271.
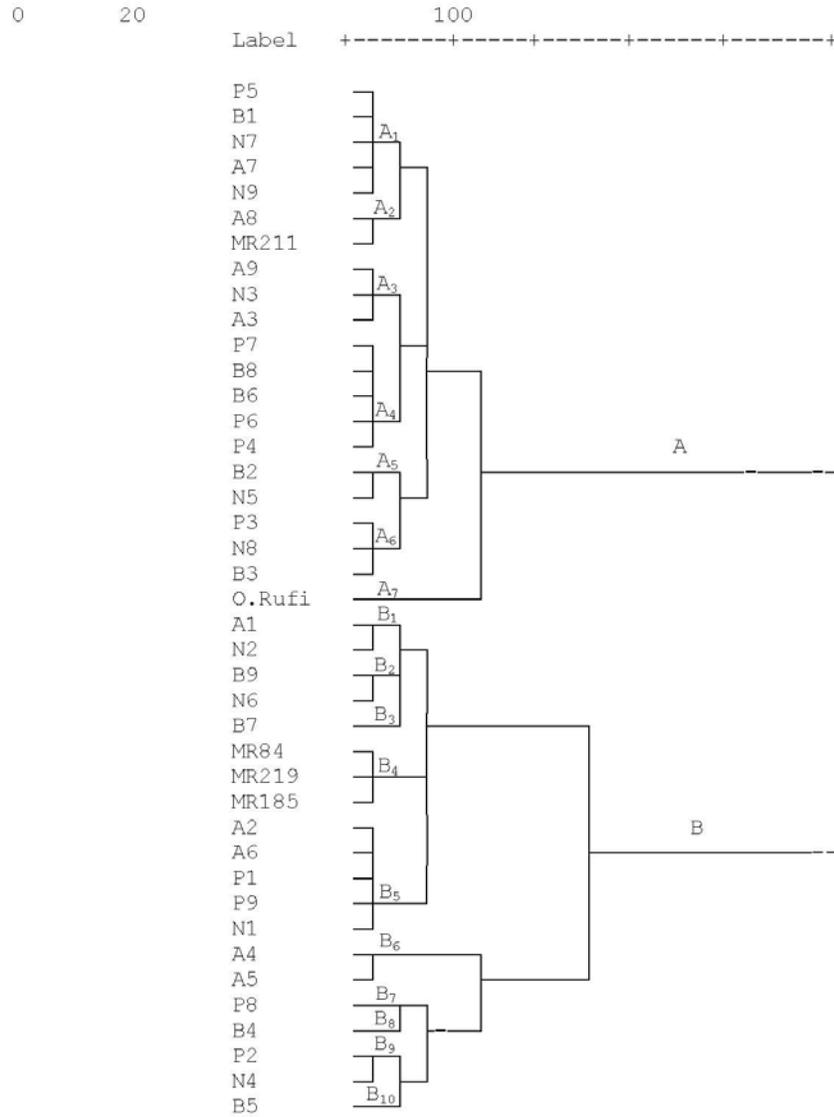
**Figure 1:** Tree diagram of hierarchical clustering using dissimilarity values from morphological characteristics of observed WR accession and rice cultivars.

The most likely number of clusters (K) present in the dataset was ascertained using 25 different runs of the STRUCTURE program, as shown in Figure 2. Ln 18 was the highest of average value of ln Pr(X/K) and was the

lowest mean ln (X/K). This result indicates that all individuals were distinguishable and could be divided into 18 clusters. The cluster memberships of several WR accessions, the wild rice species and the rice cultivars are shown in Table 2. The WR accessions A4, A7, A8, N3, N4 and N7 were identified to be dominant, with above 90% of the individuals in clusters 4, 5, 8, 10, 14 and 16. One rice cultivar (MR219) was predominantly located in cluster 1, with greater than 95% membership of the individuals, and nearly 94% of MR185 individuals belonged to cluster 3. 80% of rice cultivar MR211 individuals and 86% of WRPP4 individuals grouped in cluster 2. Rice cultivar MR84 and WRA7 were located in cluster 5, which contained 71% and 94% of their individuals, respectively. Wild rice was separated from the rice cultivars and several WR accessions; approximately 67% individuals of this accession were included in a cluster together with a few other rice accessions such as A2 (92%), P3 (56%), P6 (93%), B3 (94%) and B4 (94%). A few clusters, such as clusters 6, 7, 9, 12, 13, 15 and 17, had a large proportion (>60%) of memberships from at least 2 or 3 WR accessions.
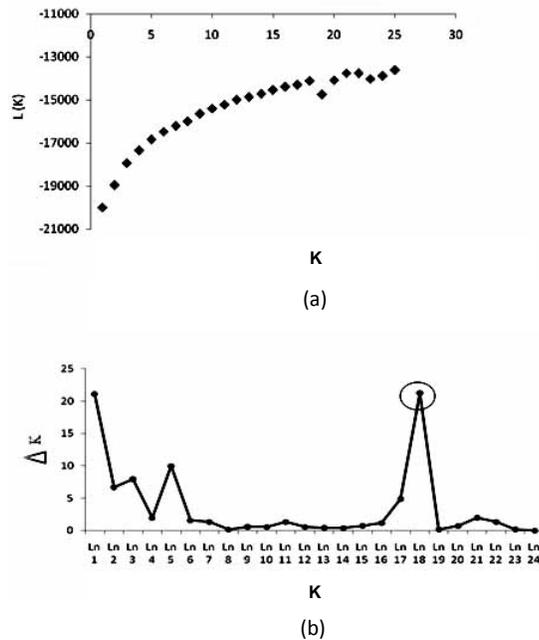


(a)



(b)

**Figure 2:** STRUCTURE analysis for forming genetic cluster for all observed variants; (a) shows the ln Pr(X/k) values of 25 different runs; (b) shows that the ln 18 was the maximum average ln Pr(X/k) value of 25 differents runs.

**Table 2:** Memberships of several WR, wild rice and rice cultivar.

| Inferred cluster | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| Membership (%) | 95.1 | 86.0 | 94.0 | 93.0 | 93.0 | 93.0 90.0 | 92.0 90.0 | 95.0 | 93.0 91.0 92.0 |
| Accessions | MR219 | P4 MR211 | MR185 | A8 N6 | A7 MR84 | N2 N9 | B1 B8 | N4 | N5 P8 B6 |
| Inferred cluster | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
| Membership (%) | 92.0 | 67.0 93.0 94.0 94.0 | 86.0 77.0 76.0 | 83.0 93.0 | 91.0 | 91.0 85.0 | 95.0 | 92.0 92.0 | 87.0 |
| Accessions | N7 | *O. rufipogon* A2 P6 B3 B4 | A6 P5 B7 | P9 B9 | A4 | A1 A5 | N3 | N1 A9 | A3 |

## DISCUSSION

### Morphometric Relationships

The results indicate that 76% of the WR accessions were different with respect to the morphological characters, resulting in higher dissimilarity matrix values. From this analysis, we were able to separate the WR accessions into several groups. Approximately 23% of the accessions have some similar characteristics and some dissimilar characteristics, which resulted in intermediate dissimilarity matrices that separated them into a few groups. At 95% dissimilarity, the matrix values showed that all accessions were separated into 17 groups due to differences in the culm characteristics, leaf characteristics, panicle structure, seed shattering, seed dimensions and weight, total spikelets, percentage of sterility and physiological characteristics.

This study showed that the arrangement of culm angles of WR, wild rice and cultivars differed from erect to semi-erect or procumbent growth. Most of the WR accessions had taller culm heights than the rice cultivars and wild rice species. The flag leaf length varied among all accessions, and the colour of the basal leaf sheath and leaf were either green, light green or dark green. Most of the WR accessions and rice cultivars produced compact panicle structures and were similar with respect to grain colour. WR matured 2 to 20 days earlier than the rice cultivars and the wild rice species. Greater numbers of seed shattered by a hand grip were counted for all of the WR accessions than for the rice cultivars (Zainudin *et al.* 2010).

The rice cultivars generally produced long-slender grains that were straw in colour and had lengths greater than 9.4 mm. WR accessions produced shorter

grains, intermediate in size between the medium and long grain types, which also varied in colour. The WR 1000-grain weight (TGW) ranged from 9.45 to 24.85 g compared to that of the rice cultivar that was greater than 24.7 g. WR accessions produced a total number of spikelets per panicle that ranged from 99 to 276, and rice cultivars produced 175 to 195 per panicle. The number of filled grains produced by the WR accessions was in the range of 94 to 217, compared to rice cultivars with numbers of filled grains ranging from approximately 118 to 137. It was observed that most of the WR accessions produced more primary and secondary branches with increased grain numbers per panicle (Zainudin *et al.* 2010). Compared to primary branch grains, secondary branch grains generally had a higher proportion of empty grains; the proportion of empty grains affects the seeds' setting ability and the seed distribution patterns (Xu *et al.* 2006; Tsnoda & Takahashi 1984). The similarity of morphological characteristics of WR and rice cultivars makes WR the most troublesome weed in direct-seeded rice fields in Malaysia (Wahab & Suhaimi 1991; Vaughan *et al.* 1995; Watanabe *et al.* 2000; Azmi *et al.* 2004, Azmi & Karim 2008).

**Cluster Analysis**
The average distance matrix of all the accessions and controlled varieties based on 15 characteristics ranged from 29.1% to 98.8%. The maximum average distance matrix derived for comparison between A7 and P2 was approximately 98.8%, closely followed by A8 and P2 at 91.40%; the minimum average recorded was 29.1% for the distance matrix between A2 and A3. Figure 1 shows that the WR accessions and control varieties were grouped into 18 clusters; 80% of the variance was accounted for by these clusters. These results confirmed that all of the control varieties were in the same cluster, which included one WR accession (A8). The WR accessions were divided into 18 groups; there were between one to five individuals in each group. The memberships of these clusters can be described either by the independent population histories of the groups that shaped the gene pools or the domestication history and breeding system of the population (Garris *et al.* 2005). There were a few accessions in each cluster due to similarities of the gene pools or allele frequencies associated with desirable plant traits. These phenomena occurred under the influence of adaptation to the flowering requirement and the evolution of day length neutrality, fostering temporal reproductive isolation and divergence. Their drought tolerance and early maturity are adaptive traits that could be usefully targeted in breeding applications (Glaszmann 1987; Parsons *et al.* 1999; Ni *et al.* 2002). Another factor is mixed population ancestry. The admixed individuals are likely to be the result of modern breeding or may be landraces belonging to groups. In particular, different subpopulations are likely to provide differing allele frequencies associated with desirable traits for plant improvement (Garris *et al.* 2003).

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## CONCLUSION

There are a few characteristics that can be used to distinguish WR accessions and rice cultivars. These characteristics were used to divide the WR accessions and rice cultivars into 18 groups. The results indicate that the plants had similarities in the gene pools and that allele frequencies associated with desirable plant traits can adjust themselves in the population. The admixed individuals, likely the result of modern breeding, contributed to the cluster sharing among the WR and commercial varieties. More in-depth studies on the morphological and physiological characters and on the genetic relationships of WR accessions should be performed to clearly define and compare all of the accessions. Information from this study will be useful in predicting the potential growth of a WR accession and its life cycle in the rice fields of Pulau Pinang, allowing the development of weed management protocols and good agriculture practices to control WR in the future.

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