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# Molecular and Agro-morphological Differences Among Traditional Rice Variety "*Pachchaperumal*" and Closely Related Weedy Rice Eco-types in Sri Lanka

**K.D.K. Karunarathna[1,2], S.R. Weerakoon[1*], S. Somaratne[1] and O.V.D.S.J. Weeresena[2]**

***ABSTRACT:*** *Weedy rice (*Oryza sativa *f.* Spontanea*) (WR) is one of the most nuisance weeds referred to as "red rice" which possesses higher morphological variability and has many similarities to wild and cultivated rice. Close morphological similarity between WR eco-types and cultivated rice varieties confuse identification of each other accurately. "*Pachchaperumal*", a traditionally cultivated rice variety indicates a close affinity to two WR eco-types found in Kurunegala and Matara Districts. Understanding genetic relationship among closely related WR eco-types and "*Pachchaperumal*" is important in preventing further mixing-up of grains. Present study focused on developing certain guidelines and protocols at morphological and molecular level to differentiate seeds of "Pachchapeumal" from two WR eco-types. Seeds of two WR eco-types (WR1 and WR2) and "Pachchapeumal" were planted in plastic pots with five replicates and arranged in a Complete Randomized Design. Morphological characterization was made using thirty characters according to Standard Characterization Catalogue. Collected data were subjected to cluster analysis, $\chi^2$ test and ANOVA. Molecular studies were conducted with eight SSR primers. On cluster analysis of morphological characters, WR1 grouped with "*Pachchapeumal*". Potential characters in differentiating rice varieties at field/farmer level included Leaf-Blade-Length, Leaf–Blade-Width, Panicle-Length, 100-grain-weight and days-of-heading. Seed-Coat-Color and Seed-Lemma-Color were similar across WR1, WR2 and "*Pachchaperumal*". Three SSR primer sets (RM44, RM11, RM84) clearly indicated its potential in differentiating closely similar WR eco-types from "*Pachchaperumal*".*

***Keywords:*** *Morphology;* Oryza sativa *f.* Spontanea*; "Pachchaperumal"; Weedy rice.*

## INTRODUCTION

Paddy cultivation in the country is facing many challenges and of those the emergence of rice weeds has become a serious threat. A number of studies have reported that yield loss due to the infestation of rice fields ranged from 30-40% (Labrada, 2007). However, the presence of "Weedy Rice" (*Oryza sativa* f. *Spontaneae*) became the most prominent weed problem in rice growing areas. Weedy rice (WR) was first reported in Sri Lanka in 1992 from the Ampara District and gradually spreaded in many areas of the country (Abeysekara *et al*., 2010).

WR currently occurs in varying population densities, in all agro-ecological zones of the country. The term "weedy rice" generally applies to all the species of the genus *Oryza* which behave as rice and grow in rotation with rice weeds. WR populations have been reported in many rice-growing areas in the world where the crop is directly seeded (Ferrero and Finassi, 1995). The origin of the weedy forms is suggested to be closely centered around cultivated rice. Many weedy plants share a greater number of the features of the two cultivated species *Oryza sativa* and *Oryza glaberrima* (Khush, 1997). However, at seedling stage, weedy plants are difficult to distinguish from the rice crop (Hoagland and Paul, 1978). The main problem faced by the farmers and the agronomists is the identification and differentiation of WR eco-types using morphological characters.

[1] Department of Botany, Open University of Sri Lanka, P.O. Box 21, Nawala, Sri Lanka
[2] Institute of Bio Chemistry, Molecular Biology and Biotechnology, University of Colombo, Sri Lanka.
* *Corresponding author,* Department of Botany, Open University of Sri Lanka, P.O. Box 21, Nawala, Sri Lanka.
*E-mail : shyamaweerakoon@gmail.com* Con no. 011 2881 383/0714933922

The varieties cultivated over a number of generations are in general referred to as traditional, indigenous or heirloom rice varieties. Cultivation of traditional rice varieties in Sri Lanka had been practiced under sanctity and well-planning. Thus, it was a sustainable process since the way of production and the production associated processes (Marambe and Amarasinghe, 2000). Under the foreign ruling of country during the 16th and 18th centuries, an emphasis was placed on plantation such as tea, rubber and the cultivation of traditional rice in Sri Lanka was neglected. However, cultivation of traditional rice varieties was given due emphasis back to the traditional rice cultivation during 20th century. A number of Sri Lankan traditional rice varieties contain higher amounts of Glutamic acid and vitamins, fiber and lower Glycemic index. Recent rice statistics of Sri Lanka indicated that 95% of the rice varieties grown are inbred and require higher inputs of agro-chemicals and chemical fertilizer for a higher productivity (Marambe, 2009). Though improved rice varieties produce comparatively higher yields, there is a considerable demand for traditional rice varieties because of grain quality, such as high ûber content (Wickramasinghe and Noda, 2008). Further, the interest of traditional rice varieties increase due to farmer perception (Eûsue *et al*., 2008), improvement of system sustainability (Abeyratne, 1956) and the higher adaptability to poor soils (Mandal *et al*., 1999). However, knowledge on the benefits of organic farming and health risks of chemical farming contributed significantly to the increase in the cultivation of traditional rice varieties such as *"Suwandel"*, *"Kaluheenati"*, *"Kuruluthuda"* and *"Pachchaperumal"* (Rathnasekara *et al*., 2010). *"Pachchaperumal"*, is one of the traditional varieties which comparatively rich in nutrients and proteins recommended for diabetes and also a considerable number of diseases associated cardiovascular system. The national and international demand make most

**Table 1**
**Morphological characters used for the characterization of WR eco-types and *"Pachchaperumal"* (PGRC, 1999)**

| *Number* | *Character* | *Descriptor status* |
|---|---|---|
| 1. | Seedling height (cm) | Recorded at the five leaf stage |
| 2. | Leaf blade length (cm) | Measured from top most leaf below the flag leaf on the main culm at late vegitative stage. |
| 3. | Leaf blade width (mm) | Measured at the widest portion of the leaf blade |
| 4. | Leaf blade pubescent | 1. Glabrous 2. Intermediate 3. Pubescent |
| 5. | Leaf blade color | 1. Pale green 2. Green 3. Dark green 4. purple tips 5. Purple margins 6. Purple blotch 7. Purple |
| 6. | Basal leaf sheath color | 1. Green 2. Purple lines 3. Light purple 4. Purple |
| 7. | Leaf angle | 1. Erect 2. Intermediate 3. Horizontal 4. Descending |
| 8. | Flag leaf angle | 1. Erect 2. Intermediate 3. Horizontal 4. Descending |
| 9. | Ligule length (mm) | Measured at late vegitative stage |
| 10. | Ligule color | 0. Absent 1. White 2. Purple lines 3. Purple |
| 11. | Collar colour | 1. Pale green 2. Green 3. Purple |
| 12. | Auricle colour | 0. Abscent 1. Pale green 2. Purple |
| 13. | Days of heading | No. of days from effective seeding to 50% heading |
| 14. | Culm length (cm) | From ground level to the base of the panicle |
| 15. | Culm number | Total no. Of grain bearing and non bearing tillers |
| 16. | Culm angle | 1. Erect 3. Intermediate 5. Open 7. Spreading 9. Procumbent |
| 17. | Inter node color After Full Heading | 1. Green 2. Light gold 3. Purple lines 4. Purple |
| 18. | Culm strength | 1. Strong 3. Moderately strong 5. Intermediate 7. Weak 9. Very week |
| 19. | Panicle length (cm) | From the base to the tip of the panicle |
| 20. | Panicle type | 0. Compact 5. Intermediate 9. Open |
| 21. | Secondary branching | 0. Abscent 1. Light 2. Heavy 3. Clustering |
| 22. | Panicle exsertion | 0. Well exsertion 3. Moderately 5. Justexterted 7. Partlyexserted 9. Enclosed |
| 23. | Awning after full heading | 0. Absent 1. Short and partly awned 5. Short and fully awned 7. Long and partly awned 9. Long and fully awned |
| 24. | Apicus color | 1. White 2. Straw 3. Brown 4. Red 5. Red apex 6. Purple 7. Purple apex |
| 25. | Lemma and palea color | 1. Straw 2. Gold 3. Brown spot on straw 4. Brown 5. Reddish to light purple 6. Purple spots on straw 7. Purple 8. Black 9. White |
| 26. | Lemma and palea pubescence | 1. Glabrous 2. Hairs on lemma keel 3. Hairs on upper portion 4. Short hairs 5. Long hairs |
| 27. | Sterile lemma color | 1. Straw 2. Gold 3. Red 4. Purple |
| 28. | Sterile lemma length | 1. Short 3. Medium 5. Long 7. Extra long 9. Asymmetrical |
| 29. | 100 grain weight | A random sample of 100 well developed grains dried 13% moisture content |
| 30. | Seed coat color | 1. White 2. Light brown 3. Speckled brown 4. Brown 5. Red 6. Variable purple 7. Purple |

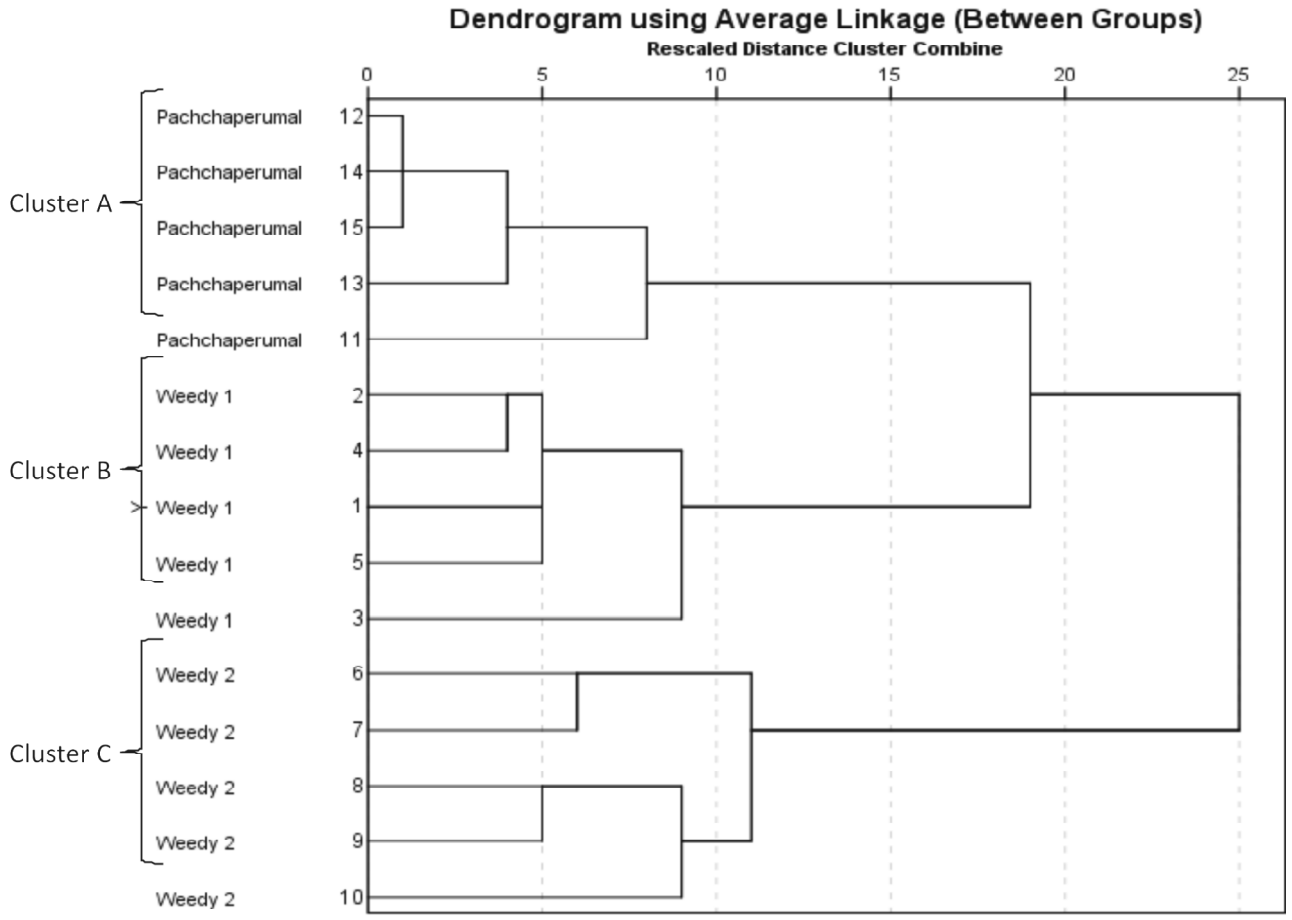


**Figure 1: Dendrogram derived from cluster analysis of morphological data of WR1, WR2 and "*Pachchaperumal*". (Cluster A-"*Pachchaperumal*", Cluster B- WR1 and Cluster C- WR2)**

of the farmers compelled to grow the traditional variety "*Pachchaperumal*".

The seeds of weedy rice (*O. sativa* f. *Spontea*) is characterize by red pericarp, thus it is commonly referred to as "red rice" (Cho *et al*., 1995). In general, the farmers in WR infested areas in Sri Lanka often encountered difficulties in differentiating WR eco-types and "*Pachchaperumal*" based on the morphological characters. The similar seed morphology of "*Pachchaperumal*" and WR seeds of certain eco-types preclude the separation of seed of "*Pachchaperumal*" and weedy rice make it even difficult at the consumer level. Thus, there is a growing concern on grains of certain WR eco-types are being marketed locally and internationally in place of "*Pachchaperumal*".

Molecular markers are useful and informative tool for estimating the genetic diversity and genetic relationships of closely related genotypes. Techniques for detecting genetic markers such as restriction fragment length polymorphisms (RFLP) (Sun *et al*., 2001), the random amplified polymorphic DNA (Qian *et al*., 2001), amplified fragment length polymorphisms (AFLP) (Saker *et al*., 2005), microsatellite or simple sequence repeats (SSRs) (Yan *et al*., 2007), and single nucleotide polymorphisms (Hayashi *et al*., 2004) have been widely in rice. SSR markers have been a powerful tool for this kind of research due to their abundance in eukaryotic genomes, co-dominance, and high polymorphisms (Gwag *et al*., 2010; Zhang *et al*., 2011). SSR markers have been used as allele-specific and co-dominant markers in population genetic and evolutionary studies of many plants (Mckhann *et al*., 2004; Upadhyaya *et al*., 2006) . Since, controlling of WR is crucial and study of agro-morphological and molecular relationship between WR and traditional rice varieties is an urgent needed in order to develop some guidelines to differentiate them at field and consumer levels. The objectives of the present study includes developing basic guidelines and protocols at morphological and molecular level to differentiate the traditional rice variety "*Pachchaperumal*" and WR eco-types.

**Table 2**
**Details of eight SSR primer pairs/ Description of SSR primer pairs used for detection of polymorphisms among *pachchaperumal* and weedy rice ecotypes**

| *Oligo name* | *Oligo sequence (5′- 3′)* | *Base pair size* |
|---|---|---|
| M13RM11F | TGTAAAACGACGGCCAGTTCTCCTCTTCCCCCGATC | 159 |
| PigtRM11R | GTTTCTTATAGCGGGCGAGGCTTAG | |
| M13RM14F | TGTAAAACGACGGCCAGTCCGAGGAGAGGAGTTCGAC | 210 |
| PigtRM14R | GTTTCTTGTGCCAATTTCCTCGAAAAA | |
| M13RM21F | TGTAAAACGACGGCCAGTACAGTATTCCGTAGGCACGG | 176 |
| PigtRM21R | GTTTCTTGCTCCATGAGGGTGGTAGAG | |
| M13RM 44F | TGTAAAACGACGGCCAGTACGGGCAATCCGAACAACC | 118 |
| PigtRM44R | GTTTCTTTCGGGAAAACCTACCCTACC | |
| M13RM84F | TGTAAAACGACGGCCAGTTAAGGGTCCATCCACAAGATG | 132 |
| PigtRM84R | GTTTCTTTTGCAAATGCAGCTAGAGTAC | |
| M13RM167F | TGTAAAACGACGGCCAGTGATCCAGCGTGAGGAACACGT | 147 |
| PigtRM167R | GTTTCTTAGTCCGACCACAAGGTGCGTTGTC | |
| M13RM205F | TGTAAAACGACGGCCAGTCTGGTTCTGTATGGGAGCAG | 141 |
| PigtRM205R | GTTTCTTCTGGCCCTTCACGTTTCAGTG | |
| M13RM280F | TGTAAAACGACGGCCAGTACACGATCCACTTTGCGC | 179 |
| PigtRM280R | GTTTCTTTGTGTCTTGAGCAGCCAGG | |

## METHODOLOGY

The seeds of two closely related WR eco-types of "*Pachchaperumal*" were collected from rice fields in Kurunegala (WR1) and Matara (WR2) Districts and seeds of "*Pachchaperumal*" was collected from RRDI (Rice Research and Development Institute), Batalagoda. Collected seeds were subjected to dormancy breaking treatments and sown in plastic trays in a plant house at the Open University of Sri Lanka, Nawala. Five replicates each with a single plant was planted in plastic pots with paddy soils. Replicates were arranged in Complete Randomized Design (CRD). Morphological characterization using thirty characters (Table 1) of two WR eco-types and "*Pachchaperumal*" was made using the Standard Characterization Catalogue (PGRC, 1999). Total genomic DNA was extracted from 7-day old seedlings of WR eco-types and "*Pachchaperumal*" using Ceygen Plant total DNA purification kit. Eight SSR primer pairs were used (Table 2).

The primer sequences and amplification conditions for primers were obtained from http://www.gramene.org/. A four-primer system (Schuelke, 2000) was used, which included a universal M13 oligo nucleotide (TGTAAAACGACGGCCAGT), labeled with one of four fluorescent dyes (6-FAM, NED, PET or VIC) (Table 3). The fluorescent dyes allow the products to be perplexed during electrophoresis; a special forward primer composed of a concentration of the M13 oligo nucleotide; and the pig tail reverse primer for SSR PCR amplification. All amplification reactions were carried out in 30 ml volume of which containing 1 × PCR buffer, 1mM dNTPs, 2mM SSR primers, 2mM $MgCl_2$, 50 ng of genomic DNA and 0.5 U of Taq polymerase.

The reaction conditions were : 95°C for 1min, followed by 30 cycles of 95°C (30 sec), 55°C (1 min), and 72°C (1 min), with 10 subsequent cycles of 95°C (30 sec), 53°C (45 sec), and 72°C (1 min), and a final extension at 72°C for 10 min. The SSR alleles were resolved on an ABI Prism 3100 DNA sequencer using GeneScan 4.1 software, and sized precisely using GeneScan 600LIZ ladder. Fragment analysis using capillary electrophoresis was performed using GENE MAPPER software and identified different peaks identified among WR1, WR2 eco-types and "*Pachchaperumal*". 30 morphological characters were analyzed using cluster analysis ANOVA and $\chi^2$ test on SPSS PC Ver. 20.

**Table 3**
**Four labeled primers used for the capillary electrophoresis/Descrition of primers labeled for fragment analysis using capillary electrophoresis to differentiate peaks among *pachchaperumal* and weedy rice ecotypes**

| *Oligo name* | *Oligo sequence (5′-3′)* | *Color of the Label Primer* |
|---|---|---|
| 5′- FAM- M13 (-21) | 5′(FAM) TGT AAA ACG ACG GCC AGT 3′ | Blue |
| 5′- NED- M13 (-21) | 5′(NED) TGT AAA ACG ACG GCC AGT 3′ | Yellow |
| 5′- PET- M13 (-21) | 5′(PET) TGT AAA ACG ACG GCC AGT 3′ | Red |
| 5′- VIC- M13 (-21) | 5′(VIC) TGT AAA ACG ACG GCC AGT 3′ | Green |

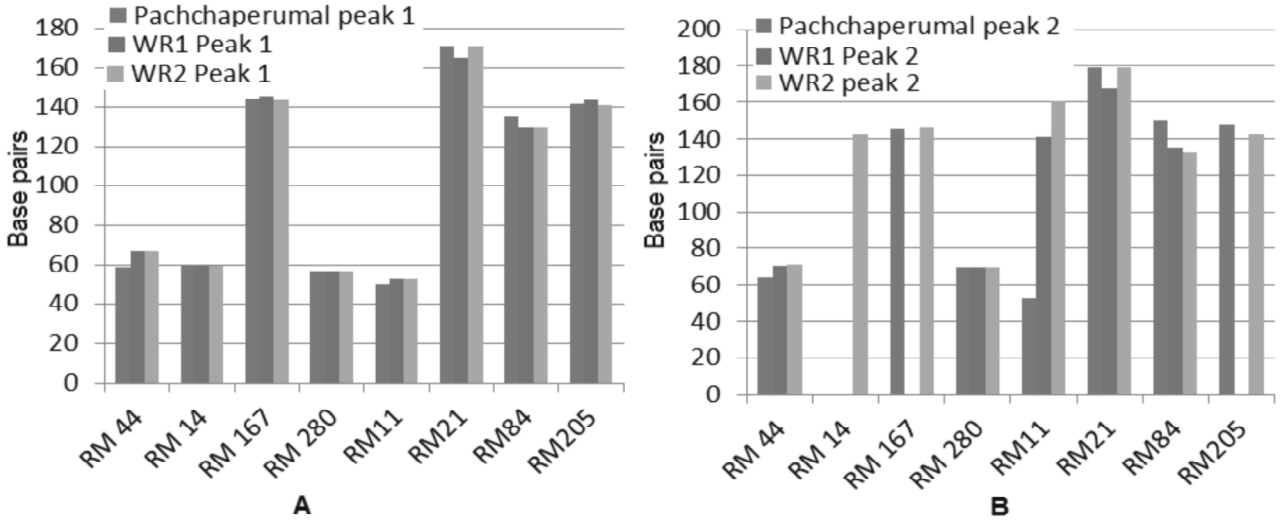


**Figure 2: Variation in Electrophorogram Peak 1 (A) and Peak 2 (B) with eight SSR primer sets along with size of base pairs in of Peak 1 for WR1, WR2 and "*Pachchaperumal*".**

**Table 4**
**Sepeation of means in clusters (A- "*Pachchaperumal*", B-WR1, C-WR2) based on six morphological characters. (The different letter across clusters indicate mean difference is significant at $p \leq 0.05$).**

| *Character* | *Cluster A* | *Cluster B* | *Cluster C* |
|---|---|---|---|
| Sdlh | 29.40 (0.37)a | 280 (0.37)a | 2.90 (0.78)a |
| LBL | 38.24 (1.18)a | 59.20 (0.82)b | 45.82 (3.67)b |
| LBW | 11.10 (0.33)a | 11.00 (0.35)a | 6.80 (0.37)c |
| PanicleL | 21.96 (0.84)a | 27.40 (0.97)b | 24.12 (2.35)a |
| Gw100 | 2.73 (0.08)a | 2.67 (0.03)b | 2.19 (0.09)c |
| DH84.80 | (2.44)a | 79.20 (1.66)a | 77.60 (1.54)b |

*Note:* Sdlh-Seedling height, LBL-Leaf Blade Length, LBW- Leaf Blade Width, Panicle L- Panicle Length, Gw100- 100 Grain Weigth, DH- Days of Heading.

## RESULTS AND DISCUSSION

### Morphological Characterization

According to a dendrogram depicted in Figure 1, there were two different groups of clusters identified at 80% simillarity phenone level. "*Pachchaperumal*" (Cluster A) and WR1 (Cluster B) fall into one group while WR2 (Cluster C) was separated into another group. The results of the mean separation test performed on six agro-morphological characters out of thirty of "*Pachchaperumal*", WR1 and WR2 eco-types are shown in Table 4. Out of the six agro-morphological characters, seedling height did not show a significant difference between the clusters. Other five characters; Leaf-Blade-Length, Leaf-Blade-Width, Panicle-Length, 100-grain weight and days of heading showed significant differences across the clusters, characterized by above mentioned morphological characters.

**Table 5**
**Chi-square test result for the 24 qualitative morphological characters**

| *Morphological character* | *Pearson Chi-Square Chi-Square value* | *df* | *Significance* |
|---|---|---|---|
| Leaf Blade Pubescence (LBP) | 16.667[a] | 4 | 0.002 |
| Leaf blade Color (LBC) | 11.667[a] | 2 | 0.003 |
| Basal leaf sheath color (BLSC) | 7.500[a] | 2 | 0.024 |
| Inter node color After Full Heading(INCAF) | 12.750[a] | 4 | 0.013 |
| Culm strength(CS) | 21.429[a] | 8 | 0.006 |
| Panicle type (PT) | 21.429[a] | 4 | 0 |
| Secondary branching (SB) | 10.750[a] | 4 | 0.03 |
| Panicle exsertion (PE) | 10.909[a] | 2 | 0.004 |
| Panicle angle (PA) | 10.909[a] | 2 | 0.004 |
| Awning after full heading (AWFH) | 30.000[a] | 6 | 0 |
| Apicus color (AC) | 14.229[a] | 4 | 0.007 |
| Seed coat color (SCC) | – | – | – |
| Lemma and palea color (LPC) | 11.667[a] | 2 | 0.003 |
| Lemma and palea pubescence (LPP) | 14.667[a] | 4 | 0.005 |
| Sterile lemma color (SLC) | – | – | – |
| Sterile lemma length (SLL) | 4.154[a] | 4 | 0.386 |
| Leaf angle (LA) | 6.500[a] | 6 | 0.37 |
| Flag leaf angle (FLA) | 30.000[a] | 6 | 0 |
| Ligule color (LC) | 15.000[a] | 2 | 0.001 |
| Ligule shape (LS) | 16.667[a] | 4 | 0.002 |
| Collar color (CC) | 11.400[a] | 4 | 0.022 |
| Auricle color (AC) | 15.000[a] | 2 | 0.001 |
| Culm number (Cno.) | 13.600[a] | 10 | 0.192 |
| Culm angle (CA) | 15.214[a] | 6 | 0.019 |

At the field level, farmers can identify WR from "*Pachchperumal*" plants by observing some morphological characters such as Leaf-Blade -Length,

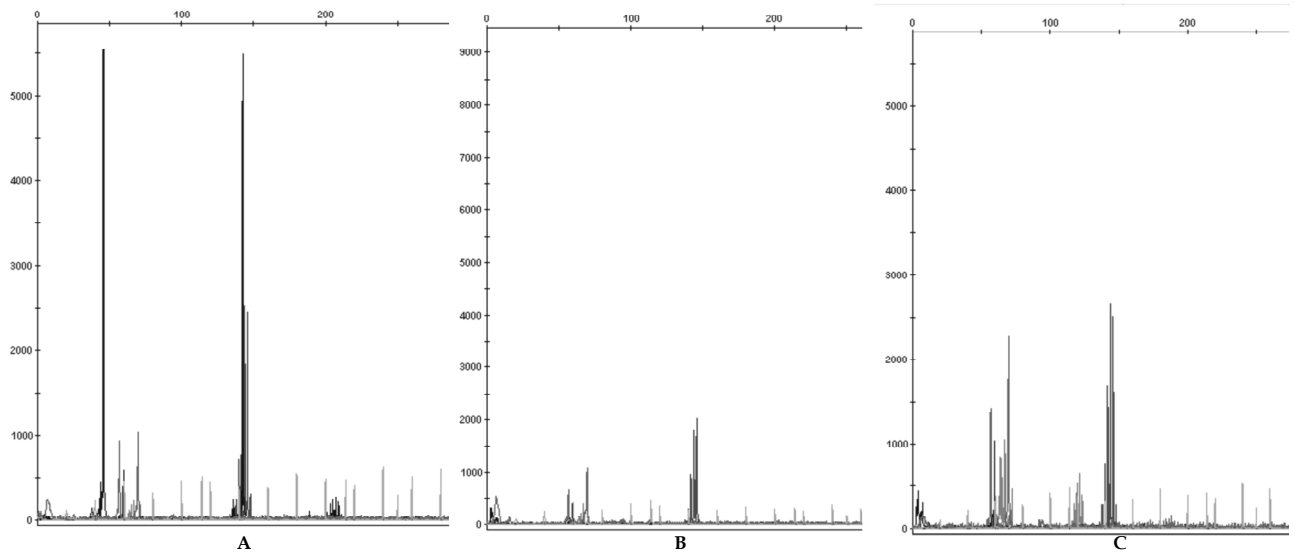


**Figure 3: Electrophorogram derived from the capillary electrophoresis for WR1 (A), WR2 (B) and "*Pachchaperumal*" (C) with four Primer Pairs (RM 44, RM 280, RM 14, RM 167)**

Leaf–Blade-Width, Panicle-Length, 100-grain-weight and Days of heading. The results of $\chi^2$ test shown in Table 5 indicated that seed-coat-color and seed-lemma-color are more or less similar across WR1, WR2 and "*Pachchaperumal*" exemplifying the complexity of the problem in identifying rice grains at consumer level.

**Molecular Characterization**

Of the eight SSR primer pairs, three sets (RM44, RM11, RM84) clearly differentiated WR eco-types from "*Pachchaperumal*" with reference to the Electrophorogram Peak 1 and Peak 2. (Figure 2A and 2B).

Electrophorogram derived from the capillary electrophoresis for WR1 (Figure 3A), WR2 (Figure 3B) and "*Pachchapermal*" (Figure 3C) ) had given different peaks at different base pair size using 1st four Primer pair sets ( RM 44, RM 280, RM 14, RM 167).

Electrophorogram derived from the capillary electrophoresis for WR1 (Figure 4A), WR2 (Figure 4B) and "*Pachchapermal*" (Figure 4C) respectively, gave different peaks at different base pair size using 2nd four primer pair sets ( RM 11, RM 21, RM 84, RM 205).

Out of the eight SSR primer pairs (RM44, RM280, RM14, RM167, RM11, RM21, RM84, RM205), three primer sets (RM44, RM11, RM84) demonstrated that they can be used to differentiate WR eco-types from "*Pachchaperumal*".

The study showed that it is difficult to identify the seeds of "*Pachchperumal*" and weedy rice eco-types at consumer/market level using only the morphological characters. However, with molecular analysis using SSR primers (RM44, RM11, RM84), it was possible to identify the seeds of "*Pachchperumal*" and weedy rice eco-types (Figure 5).

## CONCLUSIONS

There is an uncertainty associated with differentiation of the traditionally cultivated rice variety, "*Pachchaperumal*" from weedy rice eco-types which show close morphological similarities. At consumer/market level, this confusion creates problems in exporting and marketing the pure product of "*Pachchaperumal*". However, SSR molecular markers RM44, RM11 and RM84 have shown their potential in differentiating WR eco-types from "*Pachchaperumal*".

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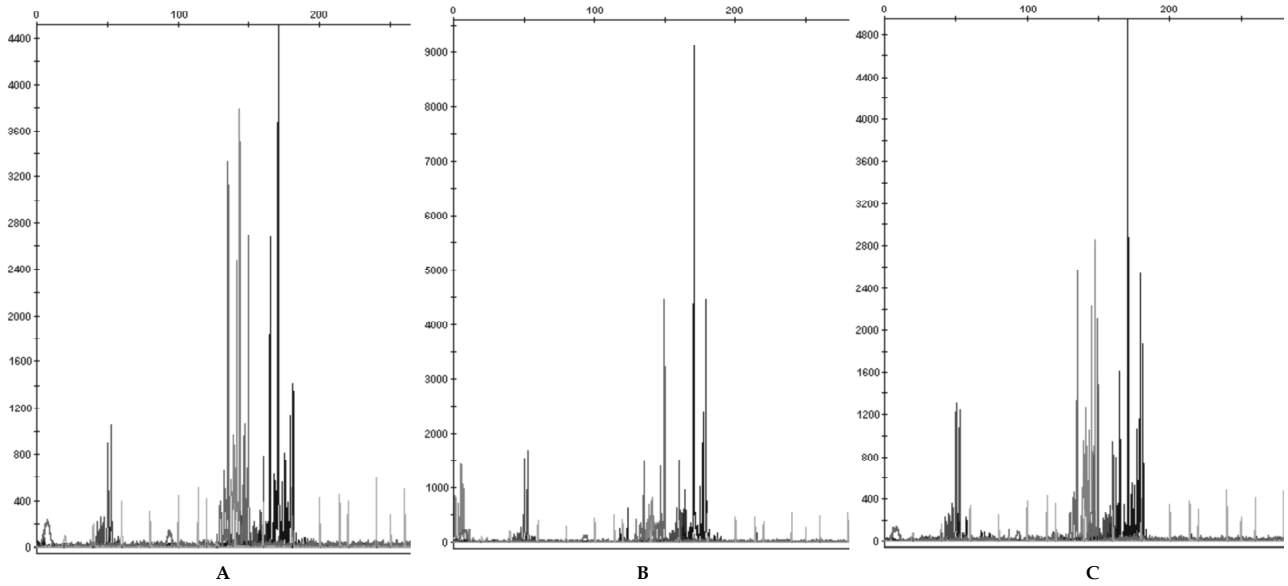
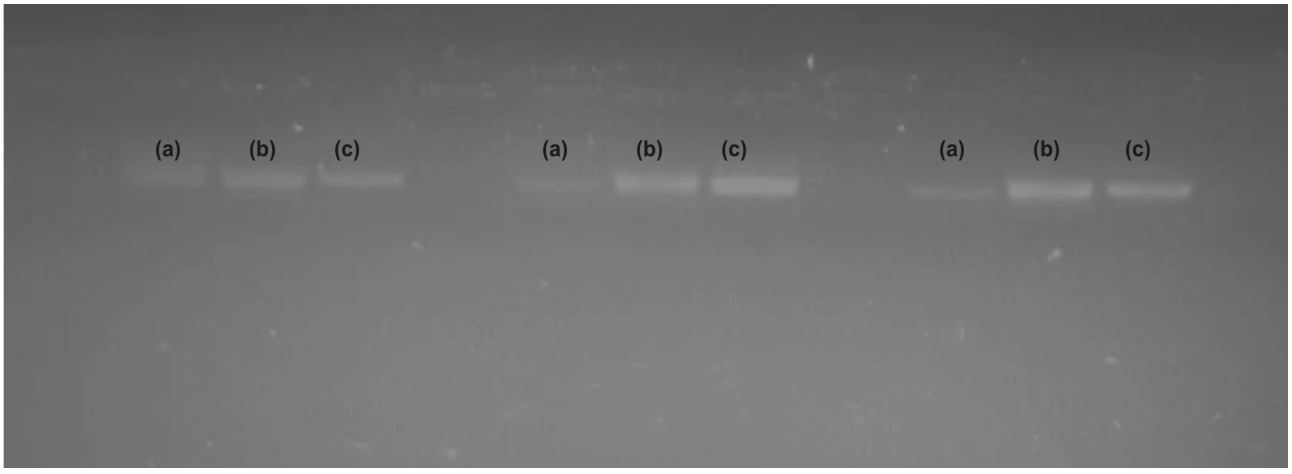



**Figure 4: Electrophorogram derived from the capillary electrophoresis for WR1 (A), WR2 (B) and "*Pachchaperumal*" (C) with four Primer Pairs (RM 11, RM 21, RM 84, RM 205)**




**(a) "Pachchaperumal", (b) Weedy rice 1, (c) Weedy rice 2**

**Figure 5: SSR gel picture for 3 primer sets (RM 11,RM44, RM84) for "*Pachchaperumal*", Weedy rice eco-type 1, Weedy rice eco-type 2**

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