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Tropical Agricultural Research Vol. 17: 48 - 57 (2005)

Molecular Screening of Selected Rice Varieties with Specific Markers for Gm2 and Gm4(t) Gall Midge Resistant Genes

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ABSTRACT. *Molecular screening for gall midge resistance was done using 47 selected rice varieties including 26 improved and 17 traditional varieties and four wild species. Specific primers were used to detect presence/absence of resistant genes. F10 primer was used to screen test varieties for Gm2 resistance. Phalguna and ARC6650 were used as the resistant and susceptible references, respectively. An amplified fragment of 600 bp was obtained with F10 primer with respect to all resistant and susceptible species/varieties except traditional varieties Perillanel and Podiwee, indicating that the Gm2 marker cannot be used to identify the presence of Gm2 rice gall midge resistant gene in tested (46) rice samples. Perillanel and Podiwee varieties behave similar to ARC6650 with F10 primer. Primer for Gm4(t) gene gave two different amplified fragments. Resistant varieties gave an amplified fragment of 570 bp while susceptible varieties amplified a fragment of 583 bp. Selected rice varieties were screened with respect to the resistant variety Ob 677 and susceptible varieties Tulsi and TN-1. It was found that, out of 46 tested 26 varieties including improved and traditional varieties amplified a fragment of resistance (570 bp). The rest of the species/varieties amplified a fragment of susceptibility (583 bp). Molecular screening results obtained for Gm4(t) to be confirmed with screening the varieties in the green house.*

INTRODUCTION

The Rice Gall Midge (RGM) is a serious pest in South and Southeast Asia and some parts of West Africa. There are two species of RGM, the Asian Rice Gall Midge, *Orseolia oryzae* (Wood-Mason) and the African Rice Gall Midge, *Orseolia oryzivora* (Diptera: Cecidomyiidae) (Hill, 1987). The Asian gall midge has been reported as the pest of rice *(Oryza sativa* L.) from several Asian countries like Bangladesh, China, Cambodia, India, Indonesia, Lao PDR, Myanmar, Nepal, Sri Lanka, Thailand and Vietnam (Bentur *et al.*, 2003).

Gall midge attack has been an increasing problem over the last two decades because of the emergence of new biotypes (Kalode and Bentur, 1989). New biotypes of RGM have emerged in India, China and Sri Lanka (Katiyar *et al.*, 2001). Biotypes emerge

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due to selection pressure on native population through resistant genes making resistant rice verities susceptible. In Sri Lanka, the first serious damage by RGM was reported in 1934 (Hutson, 1934) and since then the degree of damage has been increasing. The estimated yield loss in Sri Lanka as a percentage of the potential yield in affected areas is about 10 - 20% (Nugaliyadde and Dissanayake, 2000).

Controlling the pest with systemic insecticides is not economically and environmentally friendly. Hence, emphasis has been given to breed rice varieties with resistance to RGM. Development of resistant rice varieties against RGM was initiated in Sri Lanka in 1969 using conventional breeding methods (Nugaliyadde *et al.*, 1996). However, conventional breeding methods are time consuming, laborious and expensive. In this regard, DNA Marker-Assisted Selection (MAS) serves as a time effective means of improving gall midge resistance. Molecular markers can be used to identify genes. Use of molecular markers, facilitate in assessing resistance even at the seedling stage, without waiting until the end of the season. It increases the efficiency of breeding for resistance by allowing selection to be conducted even when the insect pressure is low. It also permits selection in locations where the gall midge is absent or where the prevalent biotype differs from that in the target environment. In addition, MAS is ideal for pyramiding genes to achieve more durable resistance against a specific biotype or broad spectrum of resistance against several biotypes (Katiyar *et al.*, 2001).

Nine 'major' Gall midge resistant genes (Gm1, Gm2, gm3, Gm4(t), Gm5, Gm6(t), Gm7, Gm8 and Gm9) have been identified (Jain *et al.*, 2004). Molecular markers have been developed for five of the above genes in India and China (Gm2, Gm4(t), Gm6(t), Gm7 and Gm8) (Mohan *et al.*, 1997; Nair *et al.*, 1995, 1996; Katiyar *et al.*, 2001; Sardesai *et al.*, 2002; Jain *et al.*, 2004). However, molecular markers for RGM resistance in Sri Lanka have not yet been developed.

The objective of this study is to test and optimize specific molecular markers available for gall midge resistant genes Gm2 and Gm4(t) and molecular screening of selected rice varieties in Sri Lanka for resistant genes. These results could be used for the development of RGM resistant rice varieties by gene pyramiding.

MATERIALS AND METHODS

DNA extraction

Forty-seven seed samples of rice were obtained from the gene bank at the Plant Genetic Resources Center (Table 1). Twenty to 30 plants of each sample were grown in the green house in plastic trays and leaf samples for DNA extraction were taken from 10 day-old seedlings. DNA was extracted using CTAB method (ICGEB, 2003) with some modifications. An aliquot of 1.5 g of leaf tissue per sample were ground to a fine powder in liquid nitrogen using pre-cooled mortar and pestle. The leaf powder was transferred into 10 ml of pre- warmed (56 °C) isolation buffer [1.5% CTAB, 0.075 M Tris-Cl (pH8.0), 0.01 M EDTA (pH 8.0), 1 M NaCl] in polypropylene tubes (50 ml). The tubes were incubated at 56 °C for 20 minutes in a shaking water bath. Then 10 ml of Chloroform:Isoamyl alcohol (24:1) was added and the samples were centrifuged at 3000 rpm for 25 minutes using a bench top centrifuge. The aqueous phase was transferred into a new tube and the Chloroform:Isoamyl alcohol step was repeated. The supernatant of each sample was transferred into a new tube and 2/3 volume of chilled iso-propanol was added. Resulted DNA was removed out and

washed with 70% ethanol 2-3 times, followed by air-drying for 2-3 hours to remove all ethanol. Then DNA was dissolved in 1 ml of TE buffer [10 mM Tris.Cl (pH8.0), 1mM EDTA (pH 8.0)] and stored at 4 °C.

DNA was treated with RNase (50 µg/ml). It was incubated for 30 min at 37 °C. The samples were allowed to cool and equal volume of Choloroform: Isoamyl alcohol (24:1) was added and mixed for 5 min. Then the samples were centrifuged at 4 °C for 25 min at 3000 rpm, and the supernatant was collected, 1/10th volume of 3 M Sodium Acetate (pH 5.2) was added and mixed well. After that, 2/3 volume of chilled iso-propanol was added and mixed gently to precipitate the DNA. The samples were centrifuged at 3000 rpm for 15 min at 4 °C. Resulted DNA was washed with 70% ethanol, dried, dissolved in 100 µl of TE buffer (pH 8.0) and stored at -20 °C.

The DNA samples were quantified using a spectrophotometer. Samples were then diluted to obtain final concentration of 20 ng/µl in TE buffer (pH 8.0) and stored at 4 °C.

DNA amplification

PCR was performed using Eppendorf DNA thermocycler to check and optimize the primers of molecular markers Gm2 and Gm4(t) as described by Nair *et al.* (1995) and (1996) respectively with a modification in the volume as described below.

Optimization of PCR conditions

PCR reactions were done in reaction volumes of 50 µl, 25 µl, 12.5 µl and 10 µl reducing the PCR component ratio.
Primers for Gm2 gene amplification;

PF10 (Forward) 5'- GGAAGCTTGGCTTATAGTAACTAG-3'
PF10 (Reverse) 5'-GGAAGCTTGGAAATGCAAGATCTT-3'

Primers for Gm4(t) gene amplification;

43 (Forward) 5'-TTATTGATGAGGACTTAGGG-3'
43 (Reverse) 5'-TGGATAGGTTAGCAGAGCTG-3'

Finally, PCR was carried out for all the samples in 10 µl reaction volumes that consisted, 35-40 ng of template DNA, 200 µM each primer, 0.1 units of Taq polymerase and 10 X PCR buffer [10 mM Tris-Cl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin] per sample.

Table 1. Selected rice species/varities for the study

Exotic/Improved Rice varieties for GM	Traditional Rice varieties	Wild Rice species
1. CR 95 JR 721-3	27. Perillanel	44. *Oryza nivara*
2. CR 94 – 13	28. Malagu samba	45. *Oryza rufipogen*
3. Leuang pratew	29. Dewaredderi 26081	46. *Oryza.eichingeri*
4. Ob 2552	30. Dahanala	47. *Oryza rhizomatis*
5. MR 1523	31. Sulai 27614	
6. 3837 Siam	32. Oddavalan 2449-20	
7. Bg 300	33. Pokkali 8558	
8. Bg 352	34. Murunga 307	
9. Bg 357	35. Suduheenati	
10. Bg 358	36. Kohumawi	
11. Bg 360	37. Kaluheenati 39 MY 3254	
12. Bg 359	38. Vellai illankalayan 28061	
13. Bg 304	39. Podiwee A 8	
14. Bg 276-5	40. Kuruluthudu wi B 13	
15. Bg 94-1	41. Pachchaperumal 2462/11	
16. BW 361	42. Vellaiperunel 28724	
17. BW 363	43. Madael 39 MY 137	
18. At 362		
19. Ptb 18		
20. Ptb 21		
21. Ob 678		
22. TN-1		
23. IR 36		
24. Phalguna		
25. Tulsi		
26. ARC 6650		

Observation of amplified products

Amplified DNA samples were subjected to electrophoresis on 1.4% agarose gel in 0.5 X TBE (0.045 M Tris-borate, 0.001 M EDTA) electrophoresis buffer at 5 V/cm for 100-120 minutes for Gm2 PCR products while for 360 minutes for Gm4(t) PCR products. Two micro liters of loading buffer (Xylene cyanol, bromophenol blue and glycerol) and the PCR product (8 μl) were mixed and loaded into each well. Ten kilo base ladder was used to track the fragment size. Developed gels were stained with ethidium bromide (0.5 μg/ml) and observed under UV light.

RESULTS

The procedure used to extract DNA from rice samples gave high yields of good quality DNA (200 - 300 μg/ml from 1.5g of 10-day old rice leaves). Spectrophotometer reading (OD_{260}/OD_{280}) was in between 1.5 to 1.8.

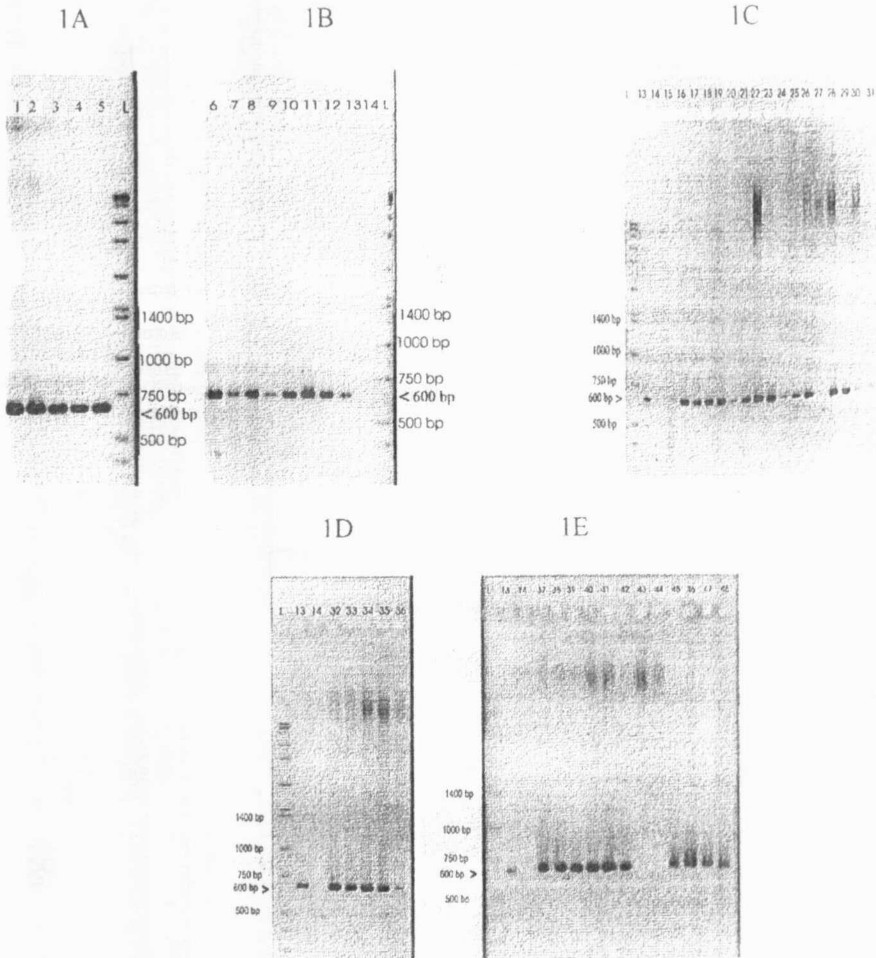

Fig. 1. **PCR based screening for Gall Midge resistant and susceptible lines in rice with specific primers (F10) for Gm2 gene**

1A: 1) Bg357, 2) Bg300, 3) Bg358, 4) Bg352, 5) Bg360.

1B: 6) CR95 JR 721-3, 7) Ob 2552, 8) MR1523, 9) Bg94-1, 10) TN-1, 11) 3837 Siam, 12) Leuang pratew, 13) Phalguna [R], 14) ARC 6650[S].

1C: 13) Phalguna [R], 14) ARC 6650 [S], 15) CR 94-13, 16) Ptb 21, 17) Ptb 18, 18) IR 36, 19) Ob 678, 20) BW 361, 21) At 362, 22) Bg359, 23) Bg276-5, 24) BW 363, 25) Bg304, 26) Oddavalan 2449-20, 27) Perillanel, 28) Pokkali 8558, 29) Suduheenati, 30) Murunga 307, 31) Phalguna [R].

1D: 13) Phalguna [R], 14) ARC 6650 [S], 32) Dahanala, 33) Vellaiperunel 28724, 34) Pachchaperumal 2462/11, 35) Kaluheenati 39 MY 3254, 36) Vellai illankalayan 28061.

1E: 13) Phalguna [R], 14) ARC 6650 [S], 37) Sulai 27614, 38) Kohumawi, 39) Malagu samba, 40) Kuruluthudu wi B 13, 41) Dewaredderi 26081, 42) Madael 39 MY 137, 43) Podiwee 8, 44) Perillanel, 45) *Oryza eichingeri*, 46) *Oryza rhizomatis*, 47) *Oryza nivara*, 48) *Oryza rufipogen*

Optimization of PCR conditions

Initial experiments carried out to optimize PCR conditions showed that there is no difference in amplification when the volume of the reaction mixture was reduced to 10 µl. Therefore, 10 µl reaction volume was used in all the experiments.

Molecular screening for Gm2 gene

Amplified DNA band generated by F10 was found at 0.6 kb level after separation by agarose gel electrophoresis as previously described by Nair *et al.* (1995). As evident in Figure 1 [1A, 1B, 1C, 1D, 1E], the resistant variety Phalguna amplified DNA while susceptible variety ARC6650 did not show any amplification as reported by Nair *et al.* (1995). All the other varieties used for the study were screened with respect to these two known varieties as resistant and susceptible, respectively. According to the results obtained, it is clear that all the rice species/ varieties contain Gm2 gene except Perillanel (Figure 1 Sample Nos. 27 and 44) and Podiwee (Figure 1 Sample No. 43) varieties. These two varieties behave similar to ARC6650.

Molecular screening for Gm4(t) gene

Marker for Gm4(t) gene amplified two different regions with 570 bp size in resistant varieties and 583 bp size in susceptible varieties (Nair *et al.*, 1996). Ob 677 variety has been identified as resistant variety with Gm4(t) while Tulsi and TN-1 has been identified as susceptible lines (Huang *et al.*, 1998; Nair *et al.*, 1996; Mohan *et al.*, 1997). According to the results, Bg357, Bg358, Bg300, Bg359, Bg360, MR 1523, CR95 JR 721-3, Ob 2552, Ptb18, IR36, Bg276-5, Bg304 and the traditional varieties, Perillanel, Pokkali 8558, Suduheenati, Murunga 307, Dahanala, Vellaiperunel 28724, Pachchaperumal 2462/11, Podiwee A8, Kaluheenati 39 MY 3254, Vellai Illankalayan 28061, Sulai 27614, Kohumawi, Dewaredderi 26081, Madael 39 MY 137 contain Gm4(t) gene while Bg352, CR 94-13, Leuang pratew, Ptb 21, BW 361, At 362, BW 363, Oddavalan, Malagu samba, Kuruluthudu wi and the wild species, *Oryza nivara, Oryza rufipogen, Oryza eichingeri, Oryza rhizomatis* did not carry Gm4(t). In Figure 2A the line CR 94-13 did not show any amplification and subsequently it was confirmed that it was due to an error in the PCR mixture as it produced the band at 583 bp levels when rechecked (Figure 2 B lane 18).

DISCUSSION

Specific marker for Gm2 has been developed in India using parents, Phalguna [Resistant (R) line] and ARC 6650 [Susceptible (S) line]. According to Nair *et al.* (1995), testing of Gm2 marker was done using recombinant inbred (RI) lines (F5-F6 generation) derived from a cross between ARC 6650 (S) and Phalguna (R). In this experiment, Phalguna has used as the checked variety for resistance and ARC 6650 as the check variety for susceptibility for gall midge. In the present study, Phalguna and ARC 6650 gave results similar to Nair *et al.* (1995). However, results showed that the varieties tested in this experiment had resistant gene except Perillanel and Podiwee as evident in Figures 1A, 1B, 1C, 1D and 1E. Since Bg94-1 and TN 1 were found to be highly susceptible varieties in the field, presence of Gm2 according to the specific primer F10 is misleading. It may be due to the reason that F10 has developed for crosses between Phalguna and ARC 6650 and it is for a specific cross.

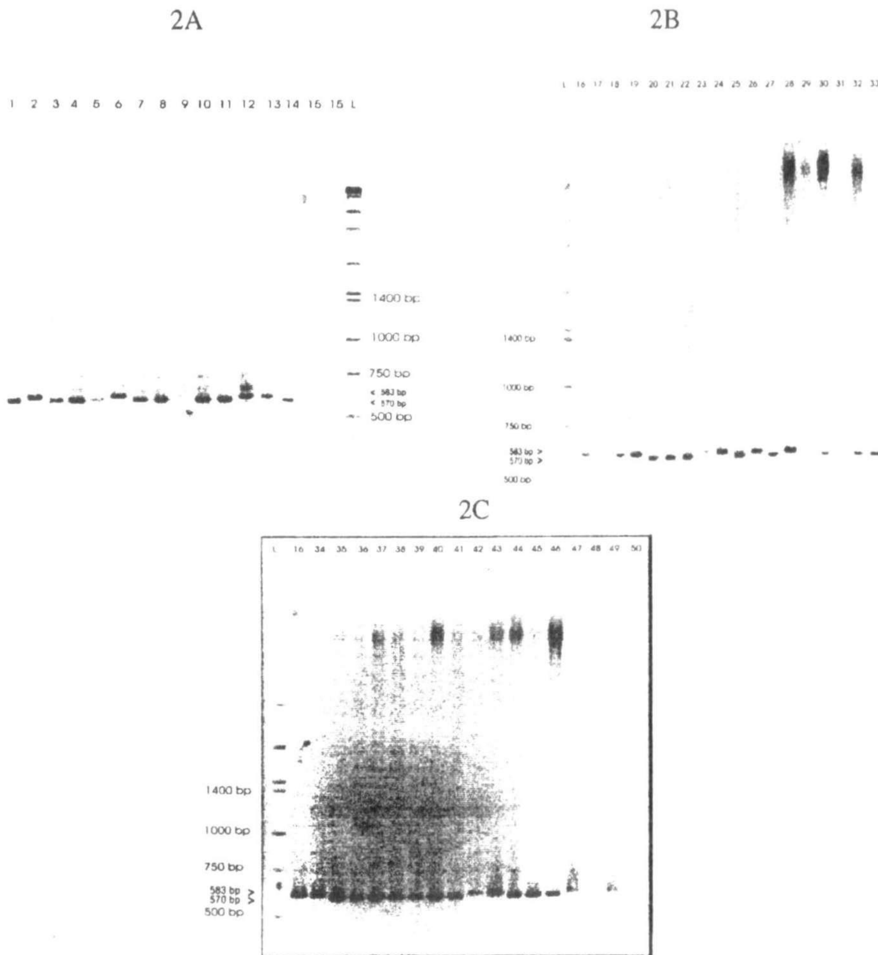

Fig. 2. PCR based screening for Gall midge resistant and susceptible lines in rice with specific primers for Gm4(t) gene

2A: 1) Ob 678, 2) TN-1, 3) Bg358, 4) Bg357, 5) Bg360, 6) Bg352, 7) Bg300, 8) MR1523, 9) CR94-13, 10) CR 95JR 721-3, 11) Ob 2552, 12) Leuang pratew,13) TN-1, 14) Ob 677, 15) Control.

2B: 16) TN-1, 17) Tulsi, 18) CR 94-13, 19) Ptb21, 20) Ptb18, 21) IR36, 22) Ob 678, 23) BW 361, 24) At 362, 25) Bg276-5, 26) BW 363, 27) Bg304, 28) Oddavalan 2449-20, 29) Perillanel, 30) Pokkali 8558, 31) Suduheenati, 32) Murunga 307, 33) Dahanala

2C: 16) TN-1, 34) Tulsi, 35) Vellaiperunel 28724, 36) Pachchaperumal 2462/11, 37) Podiwee A 8, 38) Kaluheenati 39 MY 3254, 39) Vellai illankalayan 28061, 40) Sulai 27614,41) Kohumawi, 42) Malagu samba, 43) Kuruluthudu wi B 13, 44) Dewaredderi 26081, 45) Madael 39 MY 137, 46) Bg359, 47) *Oryza eichingeri* 48) *Oryza rhizomatis,* 49) *Oryza nivara,* 50) *Oryza rufipogen*

Traditional varieties Perillanel and Podiwee did not give any amplified product with F10 similar to ARC 6650, which is the susceptible variety used in the study. Therefore, it could be sugested that these two varieties may contain characters that are very similar to Indian variety ARC 6650 with respect to F10 specific primer. Specific marker for Gm4(t) gene has been developed in India using F3 population derived from a cross between rice varieties Abhaya (resistance to RGM) and Tulsi (susceptible to RGM). In our experiment, Ob 677 which contain Gm4(t) (Huang *et al.*, 1998) used as the check variety for resistance and Tulsi and TN-1 as the check variety for susceptibility for RGM. Ob 677 gave same band as Abhaya in Nair *et al.* (1996) and totally susceptible varieties Bg 94-1 and TN-1 gave same band corresponding to Tulsi. However, some resistant varieties as CR 94-13 and Leuang gave amplification of 570 bp size fragment while some RGM susceptible rice varieties in the field (traditional rice varieties) amplified resistant fragment of 570 bp. Therefore, there is a difference between molecular screening results and the field screening data. The results obtained have to be confirmed with screen house experiments. It is also noticed that the two fragments of 570 bp and 583 bp (13 bp) were able to separate in a 1.4% agarose gel when electrophoresis was carried out at 5 V/cm for 360 minutes. Work is currently in progress towards the screening in green house.

CONCLUSIONS

The DNA protocol used in this study yielded a large amount of good quality DNA (200-300 µg /ml from 1.5 g of 10day-old rice leaves). One fifth of PCR components used by Nair *et al.* (1995, 1996) gave good amplification reducing the cost per reaction. PCR conditions used were perfect to amplify Gm2 and Gm4(t) with specific markers. Agarose 1.4% gel electrophoresis at 5 V/cm is good to separate fragments with 13 bp differences.

Although, Gm2 marker could be used for identification of Phalguna and ARC6650 and their progenies, F10 primer cannot be used to identify presence of Gm2 RGM resistant gene in tested (46) rice samples. Traditional varieties Perilland and Podiwee which are susceptible to RGM in Sri Lanka behaved similar to ARC6650 the susceptible parent used in India in developing Gm2 specific primer. Results of molecular screening using Gm4(t) specific primer has to be further confirmed with green house screening.

ACKNOWLEDGEMENTS

. Authors would like to thank Dr. R.M.T. Rajapakse, Head of Seed Conservation and Gene Bank, Plant Genetic Resources Center, Gannoruwa and Dr. L. Nugaliyadde, Senior Lecturer, University of Ruhuna for their guidance and support given to us throughout this study. Dr. D.H. Muthukuda Arachchi, Senior Deputy Director and former Senior Deputy Director Dr. A.H.M. Jayasooriya, Plant Genetic Resources Center, Gannoruwa for granting permission to carry out this study. Authors wish to extend their sincere thanks to Mr. W.L.G. Samarasinghe, Mr. J.H.B.H. Bandara, Ms. R. Ariyadasa and Ms. S.K. Hewakapuge for their assistance. Financial assistance given by SL-USDA germplasm development project No. 01 is gratefully acknowledged.

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