Received: 15 Oct 2020 | Revised: 01 Feb 2021 | Accepted: 09 Feb 2021

Electronic Journal of Plant Breeding

Research Article

Morphological and molecular diversity of thermosensitive genic male sterile lines in rice (*Oryza sativa* L.)

K. Sai Rekha[1*], R. Saraswathi[1], M. Kumar[1], S. Manonmani[1], M. Raveendran[2] and S. Robin[1]

[1]Centre for Plant Breeding and Genetics, TNAU, Coimbatore, Tamil Nadu, India.
[2]Centre for Plant Molecular Biology & Biotechnology, TNAU, Coimbatore, Tamil Nadu, India.
***E-Mail:**ssaishine@gmail.com

Abstract
An investigation was taken up to study the genetic relationship of thermosensitive genic male sterile lines developed at Tamil Nadu Agricultural University, Coimbatore using morphological traits and SSR markers. Morphological and floral traits revealed a significant and wide genetic variation among TGMS lines. SSR markers survey using 100 SSR markers revealed that 27 were polymorphic, amplifying a total of 71 alleles with an average of 2.67 alleles. Based on the morphological characters, TNAU 18S exhibited better performance for a number of tillers per plant, angle of glume opening and panicle length and TNAU 45S expressed good floral characters. Cluster analysis differentiated six TGMS lines into four clusters. There is a difference in the dissemination of TGMS lines into different groups based on molecular and morphological data may be due to selection pressure, sampling error and genetic drift. The diverse TGMS lines obtained from cluster analysis could be used directly in the crossing program for the production of high heterotic two-line rice hybrids.

Key words
Rice, Thermosensitive Genic Male Sterility, Jaccard's Similarity Coefficient, Euclidean Distance, Cluster analysis, SSR

INTRODUCTION

Rice (*Oryza sativa* L.) is the important cereal crop and most consuming food source in Asia. Rice is cultivating in more than 100 countries and nearly 18,000 varieties of rice occupied 25% of the food grain production in the World. However, an increase in the population in the World demands 880 million tonnes by 2025, as compared to the present production of 560 million tonnes (Latha *et al.,* 2003). The green revolution created a pathway to increase global rice production and also to congregate the demands of the increasing population in the World (Yuan and Fu 1995). Recent progress in different plant breeding techniques shaped the possibility of increasing the yield and one of the sources is through hybrid production.

Currently, the cytoplasmic genic male sterility (CMS) system is the most commonly used male sterility system for hybrid production in rice crop. Even though effective, a CMS system of hybrid rice breeding is cumbersome and expensive. In three-line system, the maintaining of restorer lines for the fertile restoration of "A" line is one of the major drawbacks. The hybrid breeding of a two-line system utilizing thermosensitive genic male sterility (TGMS) is a better choice to overcome problems associated with three-line breeding. In a country like India, where TGMS is considered more useful than the photo-period sensitive genic male sterility (PGMS) system because of marginal temperature fluctuations (Virmani, 1996). To increase the demand for TGMS in heterosis breeding, the characterization of new TGMS lines and analysing diversity among TGMS lines are very essential. Very less information is available on the level of diversity among various TGMS lines both at the morphological

and molecular level. Therefore, the present investigation was taken up to characterize the six TGMS lines using morphological and molecular data and also to enumerate the level of genetic diversity based on cluster analysis.

MATERIALS AND METHODS

Six TGMS lines developed by the Department of Rice, Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore were used in this study. A total of 14 morphological and floral characters viz., days to 50% flowering, plant height, the number of productive tillers, panicle exertion, angle of glume opening, 100 seed weight, pollen sterility, spikelet sterility, panicle length, stigma length, stigma breadth, anther length, anther breadth and pollen volume were measured in accordance with the (SES, IRRI, 1996).

The DNA was extracted from fresh young leaves using the CTAB method proposed by Murray and Thompson (1980). The quality of DNA was measured through O.D. at 260/280 nm and 260/230 nm in a Nanodrop Spectrophotometer (Jenway Genova Nano, UK). The purity of genomic DNA was also checked on 0.8 per cent agarose gel before amplification. A set of 100 randomly selected markers comprising of 70 random markers and 30 Hypervariable SSR markers were used in this study. The DNA from each sample was diluted to 25 ng/µl for further amplification. Amplification was done using 10 µl reaction mixture composed of 1 µl of template DNA, 4.6 µl of Milli-Q-water, 0.5 µl of forward primer (20 picomoles/ µl), 0.5 µl of reverse primer (20 picomoles/ µl), 1 µl 10X Taq buffer, 1 µl 2 mM dNTPs, 0.4 µl 25 mM $Mgcl_2$ and followed by 0.03 µl Taq polymerase enzyme (5 U/µl) (Fermentas, Thermo Fisher, USA). Amplification conditions were followed as initial denaturation (94˚C for 5 min) and 35 cycles of amplification (94˚C for 45 sec), primer specific temperature of 30 sec and extension at 72˚C for 45 sec followed by a final extension for 10 min at 72˚C. Amplified products were resolved on 3 % agarose gels stained with ethidium bromide and visualized under UV gel documentation system (BIO-RAD, Gel Doc™ XR+, USA). The size of the amplified fragments was estimated with the help of the software of the gel documentation system using a 100 bp DNA ladder (Gene DireX, USA) as the size standard.

The morphological data were standardized by subtracting the mean value and dividing by the standard deviation to calculate Euclidean distances (*mdij*). The matrix of these values denoted as MD. Morphological similarities (*msij)* was calculated as (1 – *mdij*) and the matrix of these values was denoted as MS. Using the matrix MS = (1 – MD), Unweighted Pair Group Method with Arithmetic averages (UPGMA) cluster analysis was performed using the statistical package NTSYS-PC 2.0 (Rohlf *et al.,* 1993) producing a dendrogram map depicting the relationship among the lines relative to the morphological characteristics.

The amplified products of each primer were scored based on the presence as “1” and absence as “0” of the band for SSR analysis. Each band was considered as one unit and only clear and unambiguous bands were used for further analysis. The data from expressed SSR bands was used to generate Jaccard`s coefficients. The Jaccard`s coefficients were used to construct a dendrogram using the UPGMA using statistical package NTSYS-PC 2.0 (Rohlf *et al.,* 1993).

RESULTS AND DISCUSSION

Six TGMS lines were analysed using nine morphological characters and the mean data was presented in **Table 1**. All the six TGMS lines were also studied for floral traits and pollen sterility and spikelet sterility during the sterile phase during the summer season. Wide variation was observed in days to first flowering among six TGMS

Table 1. Morphological and floral characters of six TGMS lines

Character	TNAU14S	TNAU18S	TNAU39S	TNAU45S	TNAU60S	TNAU95S	Mean	S.D.
Days to first flowering	105	90	85	90	90	108	94.67	9.42
Plant height (cm)	87.6	90	69.5	89	86.3	94.3	86.12	8.59
Number of tillers	16	24	22	20	14	12	18	4.73
Panicle exsertion (%)	83	92.8	93	90	79	85	87.13	5.7
Angle of glume opening	20	23	20	21	20	20	20.67	1.21
Pollen sterility (%)	97	98	98	98	100	100	98.5	1.22
Spikelet sterility (%)	97	98	98	98	100	100	98.5	1.22
100- seed weight (g)	2.31	2.32	2.44	2.04	2.31	1.97	2.23	0.19
Panicle length (cm)	12	20.25	11	14.5	13	18	14.79	3.62
Stigma length (mm)	0.21	0.24	0.23	0.27	0.24	0.24	0.24	0.02
Stigma breadth (mm)	0.05	0.05	0.04	0.08	0.06	0.06	0.06	0.01
Anther length (mm)	0.27	0.19	0.22	0.31	0.24	0.28	0.25	0.04
Anther breadth (mm)	0.05	0.04	0.05	0.06	0.04	0.05	0.05	0.01
Pollen volume (mm^2)	0.1	0.09	0.08	0.09	0.08	0.13	0.1	0.02

lines. TNAU 39S (85 days) had a shorter duration, TNAU 14S (104 days) and TNAU 95S (108 days) were long in duration and the remaining three lines *viz*., TNAU 18S (90 days), TNAU 45S (90 days) and TNAU 60S (90 days) were medium in duration. TNAU 95S (94.3 cm) was recorded as the tallest among all the lines and TNAU 39S (69.5 cm) was a dwarf type. Numbers of tillers were observed more in TNAU 18S (24) and were less in TNAU 95S (12). Panicle exertion ranged from 83 to 93 per cent. Maximum panicle exertion was seen in TNAU 39S (93°) followed by TNAU 18S (92.8°) and the minimum was observed in TNAU 14S (83°). Two TGMS lines (TNAU 60S and TNAU 95S) showed 100 per cent pollen and spikelet sterility and the remaining lines are in the range of 97-98 per cent pollen and spikelet sterility. The stable pollen sterility showed by TNAU 95S was also reported by Srimathi *et al.* (2019) and Kanimozhi *et al.* (2018). There is a narrow variation in the angle of glume opening which ranged from 20-23˚ among the lines. Panicle length was observed to be more in TNAU 18S (20.25 cm) and while less in TNAU 39S (11 cm). There is a narrow variation in 100 seed weight ranged from 1.97 to 2.44 g. TNAU 45S had the highest stigma length (0.27 mm), stigma breadth (0.08 mm), anther length (0.31 mm) and anther breadth (0.06 mm) among all TGMS lines and TNAU 95S (0.13) had the highest pollen volume compared to all TGMS lines.

Euclidean distance (ED) was calculated to establish the genetic relationship among six TGMS lines using fourteen morphological traits and presented in **Table 2**. Euclidean distance values ranged from 4.464 to 6.558 indicating the presence of a wide range of genetic diversity among the six TGMS lines. The ED value was maximum (6.558) between the genotypes TNAU 39S and TNAU 95S, indicating that these genotypes are diversely related to each other. Meanwhile, the minimum ED value was observed between genotypes TNAU 95S and TNAU 60S (4.464) and followed by genotypes TNAU 14S and TNAU 39S (4.507) indicating that these genotypes were closely related to each other.

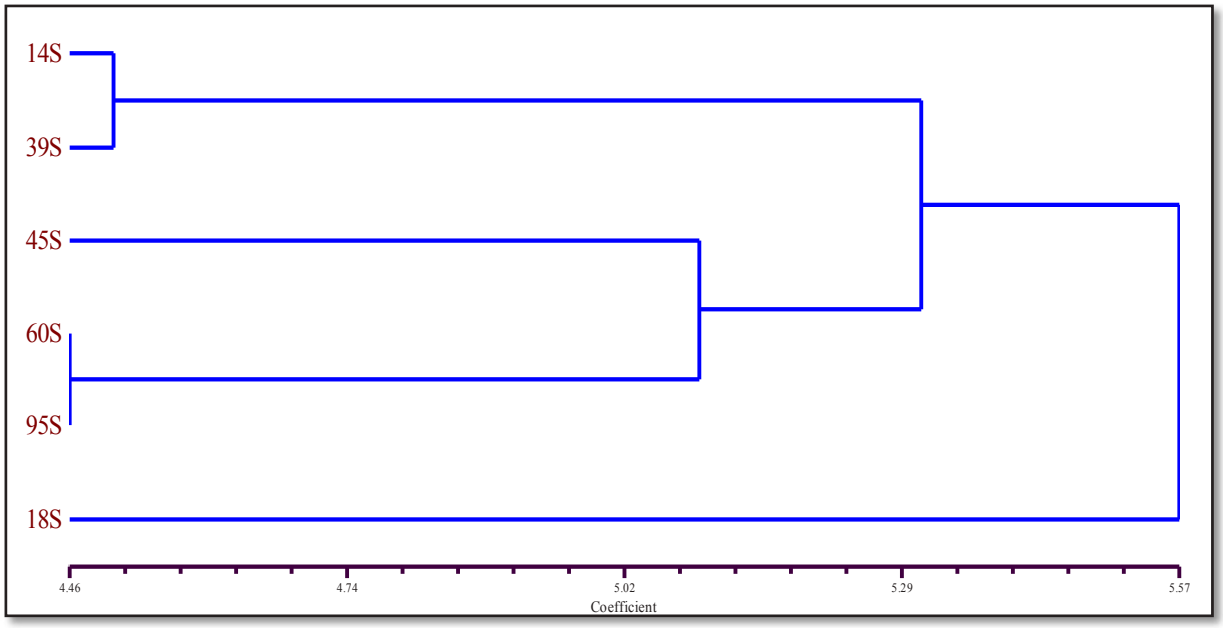

Fig. 1 Dendrogram using Euclidean distance based on morphological data among the six TGMS lines

Table 2. Euclidean distance values among six TGMS lines based on the morphological data

	TNAU14S	TNAU18S	TNAU39S	TNAU45S	TNAU60S	TNAU95S
TNAU14S	0.000					
TNAU18S	5.806	0.000				
TNAU39S	4.507	4.846	0.000			
TNAU45S	5.316	5.661	5.608	0.000		
TNAU60S	4.929	5.328	4.542	5.160	0.000	
TNAU95S	4.914	6.200	6.558	5.022	4.464	0.000

The Euclidean distance values were used to construct the UPGMA dendrogram (**Fig. 1**). Cluster analysis grouped the six TGMS lines into four clusters (**Table 2**) of which cluster I had two TGMS lines TNAU 14S and TNAU 39S, cluster II had one TGMS line (TNAU 45S), cluster III had two TGMS lines (TNAU 60S and TNAU 95S) and cluster IV had only one TGMS line (TNAU 18S). TGMS lines *viz.,* TNAU 14S and TNAU 39S of cluster I and TNAU 60S and TNAU 95S of cluster II were fallen in the same cluster and representing close relation between them. The two TGMS lines TNAU 45S and TNAU 18S were grouped in different clusters, indicating that they are more diverse from the remaining TGMS lines.

Analysis of molecular diversity among six TGMS lines with 27 SSR markers out of 100 SSR markers gave clear, consistent and scorable amplification profiles with all the six TGMS lines used in this study. The amplified 27 primers generated a total of 71 fragments, out of the 49 were reported as polymorphic and recorded 69.01% polymorphism. Nearly 2-5 polymorphic alleles were amplified per primer and the average number of alleles recorded was 2.67. The primer RM16643 amplified six alleles, whereas RM297 generated five alleles followed by RGNMS 3805a and RM536, each of which scored four alleles and markers RGNMS 2997, RM7075, RM3870, RM594 and RM31 generated three alleles. The remaining eighteen SSR markers amplify two alleles each.

Jaccard`s similarity coefficient was portrayed to predict the genetic relatedness among six TGMS lines using 27 polymorphic markers and presented in **Table 3**. Jaccard`s similarity coefficient values were in the range of 0.344 to 0.653, which indicates the presence of a wider range of genetic diversity at the molecular level among the six TGMS lines. The similarity coefficient value was maximum (0.653) between the genotypes TNAU 39S and TNAU45S indicating that there is a maximum degree of similarity expressed in their genetic background for these genotypes. However, the minimum similarity coefficient value was observed between genotypes TNAU14S and TNAU 45S (0.344) and followed by genotypes TNAU 14S and TNAU 39S (0.355) represents that these genotypes are far related.

Table 3. Jaccard's similarity coefficient values among six TGMS lines

	TNAU14S	TNAU18S	TNAU39S	TNAU45S	TNAU60S	TNAU95S
TNAU14S	1.000					
TNAU18S	0.417	1.000				
TNAU39S	0.355	0.553	1.000			
TNAU45S	0.344	0.517	0.653	1.000		
TNAU60S	0.520	0.379	0.446	0.580	1.000	
TNAU95S	0.357	0.338	0.448	0.547	0.604	1.000

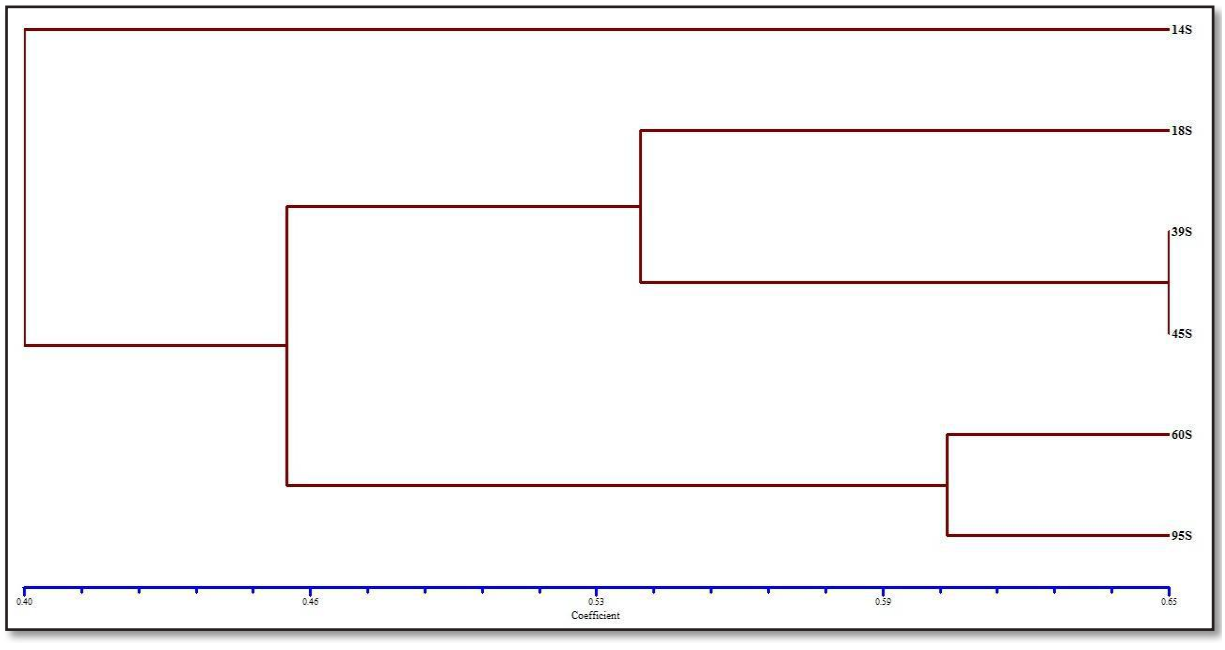

Fig. 2. Dendrogram using Jaccard's similarity coefficient based on SSR marker data among six TGMS lines

Jaccard's similarity coefficient values were used to construct the UPGMA dendrogram (**Fig. 2**). Cluster analysis grouped the six TGMS lines into three clusters of which cluster I had a single TGMS line TNAU 14S, cluster II had three TGMS lines (TNAU 18S, TNAU 39S and TNAU 45S) and cluster III had two TGMS lines (TNAU 60S and TNAU 95S). TGMS lines *viz.,* TNAU 18S, TNAU 39S and TNAU 45S of cluster II were closely related to each other which represent very low variation between them. Similarly, two TGMS lines TNAU 60S and TNAU 95S of cluster III were also found to be closely related.

Molecular markers create a pathway to measure variation at the genetic level and emerged as a valuable tool in plant breeding (Gebhardt *et al*., 1991). For assessing the genetic diversity microsatellite markers have been used in rice (Subudhi *et al.,* 1997; Aggarwal *et al.,* 1999; Garland *et al.,* 1999; Virk *et al.,* 2000; Ravi *et al.,* 2003; Yu *et al.,* 2003) and also to predict genetic purity of genotypes SSR markers are used in rice (Olufowote *et al.,* 1997; Yashitola *et al.,* 2002). In the present study, 100 random SSR markers were employed for the characterization of six TGMS lines from Tamil Nadu Agricultural University. Among 100 SSR primer pairs used in this study, 27 were polymorphic and produced a total of 71 alleles with an average allele per pair as 2.67.

There is some degree of similarity between dendrograms generated from morphological and molecular data (**Table 4**), wherein TNAU 60S and TNAU 95S have been included in the same cluster in both dendrograms which revealed the high degree of similarity between these genotypes. Whereas, TNAU 18S formed an independent cluster in both the dendrograms which showed a diversity of this line from other lines. There is also some degree of correspondence between dendrograms generated by morphological traits and SSR marker data, wherein the TNAU 45S is included with TNAU 39S in SSR data but it formed an independent cluster in morphological data analysis and similarly TNAU 14S formed an independent cluster in SSR based dendrogram but it was included in a cluster along with TNAU 39S in morphological data.

Table 4. Clusters grouped between six TGMS lines based on morphological traits and molecular data

Clusters	Based on morphological traits	Based on SSR markers
Cluster I	TNAU18S	TNAU18S
Cluster II	TNAU60S & TNAU95S	TNAU60S & TNAU95S
Cluster III	TNAU14S	TNAU45S
Cluster IV	TNAU39S & TNAU45S	TNAU14S & TNAU39S

Molecular markers differentiated six TGMS lines into four clusters and morphological characters discriminated six TGMS lines into four different clusters. Comparing the dendrograms formed based on molecular data and morphological traits, two similar clusters were formed in both situations. The lines TNAU 60S and TNAU 95S fit in one cluster while TNAU 18S formed an independent cluster in both the data analysis. There is also a deviation in the cluster formation in dendrograms formed based on molecular and morphological traits related to TGMS lines TNAU 39S, TNAU 45S and TNAU 14S. Poor genome coverage leads to a sampling effect which might be the reason for an increase in standard error in molecular marker analysis for estimating similarity and so resulted in the least correlation between molecular data compared with morphological data (Bohn *et al.,* 1999).

The two-line hybrid rice production is the best alternative to the three-line hybrid breeding system because the absence of restoration of fertility as in the case of three-line breeding system. Hence, in order to get better heterosis in two-line hybrid production, there is a need to cross the diverse parents to develop superior hybrids (Xiao *et al.,* 1996). This investigation of predicted genetic diversity of parental lines and its role in framing the clusters into the same heterotic pools was also reported by Xangsayasane *et al.* (2010). The morphological characterization in combination with marker-based genetic distance along with forms a valuable tool in determining genetic relationships among TGMS lines. The traits such as anther length and breadth, stigma length and breadth contribute more to self-pollination (Savitha *et al.,* 2017) and it is not desirable for male sterility. For successful two-line hybrid production in rice, lines having a wide genetic relationship are prominent to produce more heterotic and superior hybrids.

ACKNOWLEDGEMENT

This study was financially supported by the Department of Rice, Tamil Nadu Agricultural University and ICAR scheme "Consortium Research Platform on Hybrid Crops- Rice".

REFERENCES

Aggarwal, R., Brar, D., Nandi, S., Huang, N. and Khush, G.S. 1999. Phylogenetic relationships among *Oryza* species revealed by AFLP markers. *Theoretical and Applied Genetics.,* **98**:1320–1328. [Cross Ref]

Bohn, M., Utz, H.F. and Melchinger, A.E. 1999. Genetic similarities among winter wheat cultivars determined on the basis of RFLPs, AFLPs, and SSRs and their use for predicting progeny variance. *Crop science.,* **39**:228–237. [Cross Ref]

Garland, S.H., Lewin, L., Abedinia, M., Henr,y R. and Blakeney, A. 1999. The use of microsatellite polymorphisms for the identification of Australian breeding lines of rice (*Oryza sativa* L.). *Euphytica*., **108**:53. [Cross Ref]

Gebhardt, C., Ritter, E., Barone, A., Debener, T., Walkemeire, B., Schachtschabel, U., Kaufmann, H., Thompson, R.D., Bonierbale, M.W., Ganal, M.W., Tanksley, S.D. and Salamini, F. 1991. RFLP maps of potato and their alignment with the homoeologous tomato genome. *Theoretical and Applied Genetics.,* **83**:49–57. [Cross Ref]

IRRI (International Rice Research Institute). 1996. Standard evaluation system for rice.

Kanimozhi, P., Pushpam, R., Binodh, A.K., Kannan, R. and Pillai, M.A. 2018. Evaluation of TGMS lines for good floral and outcrossing related traits in rice. *Electronic Journal of Plant Breeding*, **4:** 1497-1502. [Cross Ref]

Latha, R., Thiyagarajan, K. and Senthilvel, S. 2003. Inheritance of thermo-sensitive genic male sterility in rice. *Journal of Genetics & Breeding,* **57**:89–91.

Murray, M. and Thompson, W.F. 1980. Rapid isolation of high molecular weight plant DNA. *Nucleic acids research*., **8:** 4321–4326. [Cross Ref]

Olufowote, J.O., Xu, Y., Chen, X., Goto, M., Susan, R.M., Park, W.D., Henry, M.B. and Robert, H.D. 1997. Comparative evaluation of within-cultivar variation of rice (*Oryza sativa* L.) using microsatellite and RFLP markers. *Genome*, **40**:370–378. [Cross Ref]

Ravi, M., Geethanjali, S., Sameeyafarheen, F. and Maheswaran, M. 2003. Molecular marker-based genetic diversity analysis in rice (*Oryza sativa* L.) using RAPD and SSR markers. *Euphytica.,* **133**:243–252. [Cross Ref]

Rohlf, J., Rohlf, F., Rohlf, E. and Rohlf, J. 1993. NTSYS-pc. Numerical taxonomy and multivariate analysis system. Version 1.80

Savitha, P., Usha Kumari, R. and Vanniarajan, C. 2017. Correlation between selected morphological floral characters and yield components related traits in *Oryza sativa* L. *Indian Journal of Experimental Biology.,* **55:** 642-647.

Srimathi, K., Pillai, M.A., Aananthi, N. and Rajababu, C. 2019. Genetic studies on TGMS lines for development of superior two-line rice hybrids. *Electronic Journal of Plant Breeding*, **10:** 620-626. [Cross Ref]

Subudhi, P., Borkakati, R., Virmani, S. and Huang, N. 1997. Molecular mapping of a thermosensitive genetic male sterility gene in rice using bulked segregant analysis. *Genome*., **40:**188–194. [Cross Ref]

Virk, P., Zhu, J., Newbury, H., Bryan, G.J., Jackson, M.T. and Ford-Lloyd, B.V. 2000. Effectiveness of different classes of molecular marker for classifying and revealing variation in rice (*Oryza sativa*) germplasm. *Euphytica*., **112**:275–284. [Cross Ref]

Virmani, S.S. 1996. Hybrid rice. *In: Advances in agronomy. Elsevier.*, pp 377–462. [Cross Ref]

Xangsayasane, P., Xie, F., Hernandez, J.E. and Borromeo, T.H. 2010. Hybrid rice heterosis and genetic diversity of IRRI and Lao rice. *Field crops research*., **117**:18–23. [Cross Ref]

Xiao, J., Li, J., Yuan, L., McCouch, S.R. and Tanksley, S.D. 1996. Genetic diversity and its relationship to hybrid performance and heterosis in rice as revealed by PCR-based markers. *Theoretical and Applied Genetics.,* **92**:637–643. [Cross Ref]

Yashitola, J., Thirumurugan, T., Sundaram, R., Naseerullah, M.K., Ramesha, M.S., Sarma, N.P. and Ramesh, V.S. 2002. Assessment of purity of rice hybrids using microsatellite and STS markers. *Crop Science.,* **42:**1369–1373. [Cross Ref]

Yu, S., Xu, W., Vijayakumar, C., Ali, J., Fu, B.Y., Xu, J.L., Jiang, Y.Z., Marghirang, R., Domingo, J., Aquino, C., Virmani, S.S. and Li, Z.K. 2003. Molecular diversity and multilocus organization of the parental lines used in the International Rice Molecular Breeding Program. *Theoretical and Applied Genetics.,* **108:**131–140. [Cross Ref]

Yuan, L. and Fu, X.1995. Technology of hybrid rice production. Food and Agriculture Organization (FAO).