GENETIC DISTANCE ANALYSIS OF HYBRID RICE PARENTAL LINES BASED ON MORPHOLOGICAL TRAITS AND RAPD MARKERS

P T NGHIA, J P S MALIK[1], M P PANDEY[2] AND N K SINGH[2]

ABTRACT

The genetic similarities of 19 hybrid rice parental lines were estimated using morphological traits and RAPD markers. Mahalanobis distance and standard taxonomic distance were computed from ten quantitative morphological traits. Molecular genetic distances derived from Dice's similarity coefficients were based on 133 RAPD markers. Cluster dendrograms were generated for both the standard taxonomic distances and molecular genetic distances. Genetic variability existing among thermo-sensitive genic male sterile (TGMS) lines was much lower than that among cytoplasmic genic male sterile (CMS) lines. Standard taxonomic distance method was found to be a more accurate method in clustering genotypes as compared to Mahalanobis distance method. PCR analysis based on 10 RAPD primers could detect sufficient polymorphisms for the germplasm characterization and genetic distance study. The cluster analysis based on RAPD markers was able to reveal close genetic relationships between different rice genotypes used in the hybrid rice breeding program.

Key words: hybrid rice, diversity, RAPD, marker, PCR

INTRODUCTION

The exploitation of hybrid vigor (heterosis) seems to be one of the options apart from modified new plant type for making further breakthrough in rice yield potential. Rice F_1 hybrids utilizing cytoplasmic male sterility (CMS) system obtained 15 to 20 per cent higher yield as compared to the best semi-dwarf inbred cultivars (Yuan *et al.* 1994). In recent times, thermo-sensitive genic male sterility (TGMS) system is being considered as a more effective alternative to the CMS system for hybrid seed production (Lu *et al.* 1994). To promote the hybrid-rice breeding program more effectively, an accurate classification of parental lines into heterotic groups is essential to help plant breeders in choosing parents and predicting of the performance of F_1 hybrids.

Traditionally, genetic distance analysis of rice cultivars was based on agronomic-morphological traits (Mahapatra *et al.* 1995; Kaw 1995). Recent studies have addressed molecular genetic markers such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) (Wang & Tanksley 1989; Mackill *et al.* 1996; Cao & Oard 1997). A moderate or strong

[1] Department of Genetics & Plant Breeding, G.B.Pant Univ. of Agri. & Tech., Pantnagar-263 145, India.
[2] Department of Molecular Biology & Genetic Engg., G.B.Pant Univ. of Agri. & Tech., Pantnagar-263 145, India.

association between heterosis of F_1 hybrids and genetic divergence of parents has been analyzed (Arunachalam & Bandyopadhyay 1984; Smith *et al*., 1990; Zang *et al.*, 1995; Xiao *et al*., 1996), the effectiveness of genetic distance analysis of parents in predicting F_1 hybrid performance, however, is not always as good as expected (Dudley *et al.* 1991; Jian Ying Peng *et al.* 1991). The level of correlation between molecular markers based distances and hybrid performance is dependent on the germplasm used (Melchinger *et al.* 1990 and Saghai Maroof *et al.* 1997).

For the past several years, intensive hybrid rice breeding program at Pantnagar (India) has led to the development of many promising CMS, TGMS and restorer lines. These lines are under evaluation or being used for producing F_1 hybrids.

OBJECTIVES

- Investigate genetic divergence among hybrid rice parental lines and to compare different methods of genetic distance analysis based on morphological traits and RAPD markers. This will aid the long- term objective of identifying an effective way for predicting the level of heterosis in F_1 hybrids.
- Attentions should be paid identify an effective approach how to predict the level of heterosis among F1's hybrids.

MATERIALS AND METHODS

Plant materials

Nineteen rice genotypes including eight CMS seven TGMS and four restorer lines were evaluated in this study (Table 1). They were produced at Pantnagar, India and the International Rice Research Institute (IRRI), Manila, Philippines. These genotypes possessed some essential features required for hybrid breeding program like complete and stable male sterility (except restorer lines), higher out-crossing rate, better grain quality, good plant type and adaptability.

Methods of distance analysis

Morphological traits of these parental lines were measured on plants raised in the field at the Crop Research Center, G.B.Pant University of Agriculture and Technology, Pantnagar in 1997. Based on ten selected quantitative traits i.e. days to 50 per cent flowering, plant height, number of panicles per plant, panicle length, length of flag leaf, width of flag leaf, number of spikelets per panicle, 1000-grain weight, length of brown grain, width of brown grain, Mahalanobis distances (Rao, 1952) and standard taxonomic distances (Sneath & Sokal, 1973) were computed. Mahalanobis distances were then used to group genotypes into clusters following the method described by Tocher (Rao, 1952).

For standard taxonomic distance analysis, the means of genotypes were normalized prior to cluster analysis by dividing with the standard deviation and subtracting the mean of each trait. The matrix of standard taxonomic distances (D_{ij}) for individuals i and j and N morphological traits were computed as below.

$$Dij = [\Sigma (Xki - Xkj)^2 / N]^{1/2}$$

Cluster analysis was then conducted on taxonomic distance matrix using unweighted pair-group method with arithmetic mean (UPGMA) (Nei & Li, 1979).

Table 1. List of different male sterile and CMS restorer lines of rice evaluated

S. No.	Accession	Parentage Origin	Remarks	Origin
1.	IR58025A	IR48483A/8*Pusa167-120// Pusa167-120	CMS (WA)	IRRI
2.	IR62829A	IR46828A/7*IR29744//IR29744	CMS (WA)	IRRI
3.	PMS 2A		CMS (WA)	PAU
4.	IR54755A	IR1529-680/O. Officinalis// IR1529-680	CMS (ARC)	IRRI
5.	Pant CMS2A	V97A/IET6223	CMS (WA)	PANT
6.	IR68897A	IR62829A/6* IR62856//IR62856	CMS (WC)	IRRI
7.	IR68281A	IR58025A/6* IR54718-C3//IR54718-C3	CMS (WC)	IRRI
8.	IR69617A	IR58025A/8*54718-C2// IR54718-C2	CMS (WC)	IRRI
9.	UPRI92-133	IR8/IR127-2	Restorer	IRRI
10.	IR31802	IR13168-143/IR12340-10// IR9129-209	Restorer	IRRI
11.	UPRI89-20	C681030/IR13429-57	Restorer	PANT
12.	UPRI89-43	IET4141/CR98-7216	Restorer	PANT
13.	UPRI95-140	Spontaneous Mutant	TGMS	PANT
14.	UPRI95-167	UPRI95-140/UPRI95-117	TGMS	PANT
15.	UPRI97-58	UPRI95-140/IR36	TGMS	PANT
16.	UPRI97-59	UPRI95-140/UPRI95-141	TGMS	PANT
17.	UPRI97-60	UPRI95-140/UPRI95-141	TGMS	PANT
18.	UPRI97-61	UPRI95-140/UPRI95-141	TGMS	PANT
19.	UPRI97-62	UPRI95-140/UPRI95-141	TGMS	PANT

CMS (WA) = Cytoplasmic male sterile (wild abortive)
IRRI = International Rice Research Institute
PANT = G.B.Pant University of Agriculture & Technology
PAU = Punjab Agricultural University, Ludhiana
TGMS = *Thermo-sensitive genic male sterile*

DNA extraction and RAPD analysis

For each genotype, 1 g of young etiolated leaves from seedlings were taken for DNA extraction using the method described by Dellaporta *et al.* (1983). Purified DNA was dissolved in TE buffer and quantified with Hoefer Fluorometer DyNA quant 200 (Hoefer Scientific Instruments, San Francisco). The DNA solutions were then diluted in TE buffer to a working concentration of 1 ng/µl and stored at –20°C until PCR amplification. The 25 µl polymerase chain reaction mixture contained 1 ng of template DNA, 200 µM of each dNTP (Genei, Bangalore, India), 0.2 µM of decamer primer (Operon Technologies, See Table 2), 0.5 U Taq polymerase and

1 X reaction buffer containing 1.5 mM $MgCl_2$ (Genei, Bangalore, India). The thermocycler (Perkin-Elmer, model) was operated for one cycle at 94°C for 5 min. and then programmed for 45 cycles of 94°C (30 sec.), 35°C (1 min.) and 72°C (2 min.). It was followed by a final amplification step of 5 min. at 72°C. Amplified DNA samples were electrophoresed on polyacrylamide gel with 7.5% acrylamide, in 1 X TAE buffer at 80 V for 4 hrs, stained with ethidium bromide and photographed under UV light. The RAPD profiles were scored visually for the presence (1) and absence (0) of bands. The combined table of scored bands obtained from 19 rice genotypes using ten RAPD decamer primers was used for computing genetic distances (Nei & Li 1979).

$$GD = 1 - [2N_{ij} / (N_i+N_j)]$$

Where N_{ij} is the number of shared bands between two genotypes and N_i and N_j are the total number of bands for genotypes i and j, respectively. Genetic distances were then used to construct a cluster dendrogram by the UPGMA method.

Matrix comparisons

To compare the RAPD genetic distance matrix with the morphological distance matrices based on Mahalanobis distance and standard taxonomic distance methods, it was assumed that genetic distance values obtained by these methods were normally distributed. The Pearson product moment correlation coefficient (r) was then computed for estimating a linear relationship between any two genetic distance matrices. High absolute value of correlation coefficient indicates that one matrix is a good predictor of the other. This product moment correlation (r) is equivalent to the value obtained from the normalized Mantel Statistic (Smouse *et al.*, 1986).

RESULTS AND DISCUSSION

RAPD analysis

Ten decamer primers selected on the basis of their effectiveness in previous studies (Ko *et al.* 1994; Mackill 1995; Cao & Oard 1997; Raghunathachari 1977) were used to detect polymorphisms among the 19 hybrid rice parental lines (Table 2). One hundred thirty three RAPD loci were generated by all ten primers. This resulted an average of 13.3 bands per primer. Out of 133 bands, 27 bands (20.3%) were monomorphic for all the genotypes while 106 bands (79.7%) were found to be polymorphic for one or more genotypes. The number of bands per primer ranged from 4 to 23. No single primer was found to produce distinct banding patterns for all genotypes. However, the primer OPD08 was somewhat unique as it could distinguish 17 out of 19 genotypes tested of (Fig. 1). Primer OPD08 was also selected for genotype identification in previous studies (MacKill 1995, Cao & Oard 1997). Primer OPJ13 was found particularly useful in discriminating among seven TGMS genotypes that were similar in phenotypic appearance (Fig. 2). It is interesting to note that, but using only two primers i.e. OPD08 and OPJ13, all the 19 rice genotypes could be differentiated. Thus, these results show that RAPD technique being technically simpler, quicker, relatively inexpensive and non-radioactivity, can generate sufficient polymorphisms for germplasm

characterization and genetic distance studies.

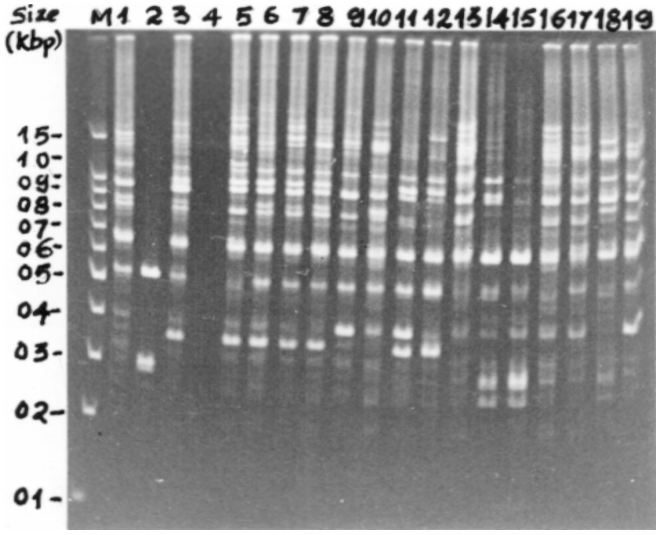

Fig 1: RAPD profile of 19 hybrid rice parental genotypes obtained with primer OPD08. Lane M: DNA size marker "100 DNA ladder", L1: IR58025A, L2: IR 62829A, L3: PMS2A, L4: IR 54744A, L5: Pant 2A, L6: IR68897A, L7: IR68281A, L8: IR 69617A, L9: UPRI 92-133, L10: IR31802, L11: UPRI 89-20, L12: UPRI 89-43, L13: UPRI 95-140, L14: UPRI 95-167, L15: UPRI 97-58, L16: UPRI 97-59, L17: UPRI 97-60, L18: UPRI 97-61, L19: UPRI 97-62.

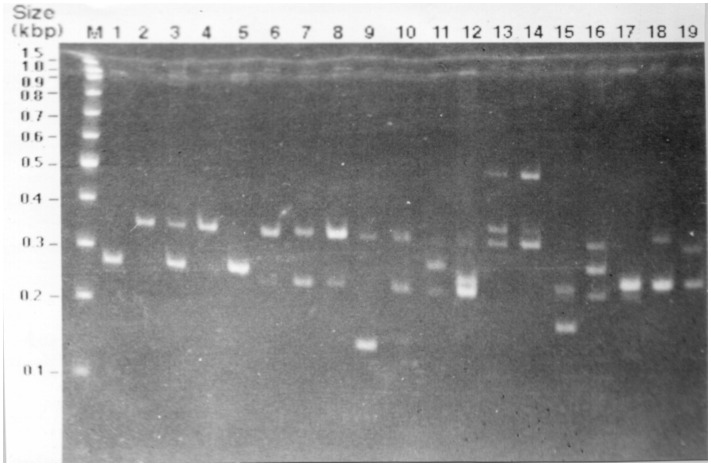

Fig 2. RARD profile of 19 hybrid rice parental genotypes obtained with primer OPJ13. Lane M: DNA size marker "100 DNA ladder", L1: IR 58025A, L2: IR62829A, L3: PMS2A, L4: IR 54744A, L5: Pant2A, L6: IR 68897A, L7: IR 68281A, L8: IR69617A, L9: UPRI 92-133, L10: IR31802, L11 UPRI 89-20, L12: UPRI 89-43, L13: UPRI 95-140, L14: UPRI 95-167, L15: UPRI 97-58, L16: UPRI 97-59, L17: UPRI 97-60, L18: UPRI97-61, L19: UPRI97-62.

Table 2. Ten decamer primers and the number of RAPD loci detected on acrylamide gels

Primers	Sequence (5' to 3')	Monomorphic loci		Polymorphic loci		Total no. of RAPD loci
		No.	(%)	No.	(%)	
OPC07	GTCCCGACGA	1	7.14	13	92.86	14
OPC15	GACGGATCAG	8	50.00	8	50.00	16
OPD08	GTGTGCCCCA	0	0.00	23	100.00	23
OPJ08	CATACCGTGG	3	37.50	5	62.50	8
OPJ13	CCACACTACC	0	0.00	8	100.00	8
OPF06	GGGAATTCGG	5	35.70	9	64.30	14
OPF13	GGCTGCAGAA	9	52.94	8	47.06	17
OPF14	TGCTGCAGGT	0	0.00	11	100.00	11
OPF17	AACCCGGGAA	1	5.55	17	94.45	18
OPK11	AATGCCCCAG	0	0.00	4	100.00	4
Total		27	20.30	106	79.70	133

Cluster analysis based on different methods

Analysis of the relationship based on 133 RAPD loci revealed that the genetic distances among 19 genotypes ranged from 0.094 (90.6% similarity) to 0.344 (65.6% similarity) (data not shown). Genetic divergence among restorers from 0.094 to 0.269, while that among restorers from 0.143 to 0.197. CMS group showed the most diversity that varied from 0.134 to 0.344. The RAPD cluster pattern is presented in Fig. 3. It showed five clusters at the cut off 0.20 genetic distance level and eight clusters at the cut off 0.17 genetic distance level. All the TGMS lines were grouped in one cluster at 0.20 level, but they were divided into two sub-clusters according to their parentage relationship at 0.17 level. Two CMS lines, IR68281A and IR69617A, which were developed through genome substitution of IR58025A by repeated back crossing to male parents IR54718-C_3 and IR54718-C_2, respectively, were closely clustered together with the genetic distance of 0.134. These two CMS lines might be considered as sister lines derived from the same ancestral origin. Four leading CMS lines viz. IR62829A, IR54755A, IR58025A and PMS2A formed two clusters. The cluster of IR54755 A and IR62829A was genetically much more closer to TGMS cluster as compared to the cluster of IR58025A and PMS2A. However, despite being in the same cluster, the genetic similarity between IR54755A and IR62829A is only 76.6%. Pant CMS2A was grouped into the same cluster with seven other genotypes at 0.20 level but it was separated into distinct sub-cluster at 0.17 level.

Table 3. Clustering of rice genotypes based on Mahalanobis distances (Tocher's Method).

Cluster	Genotypes	No. of genotypes
I	IR58025A, PMS2A, UPRI95-140, UPRI95-167, UPRI97-58, UPRI97-59, UPRI97-60, UPRI97-61, UPRI97-62	9
II	IR62829A, IR54755A, IR68897A	3
III	IR68281A, UPRI92-133R	2
IV	IR69617A, IR31802R	2
V	UPRI89-20R	1
VI	UPRI89-43R	1
VII	Pant CMS2A	1

Another measures of dissimilarity were based on morphological traits. By using Mahalanobis distance, all genotypes were grouped into seven clusters (Table 3). Cluster I grouped all TGMS lines plus IR58025A and PMS2A. Morphologically, these two CMS lines were different from TGMS lines in terms of grain size, duration and length of flag leaf (data not shown). Two CMS genotypes IR62829A and IR54755A were grouped in cluster II. Pant CMS2A that was revealed to be the most diverse genotype and was grouped separately into cluster VII. In general, Pant CMS2A showed maximum genetic distances from other genotypes (Table 4). When the same morphological data was subjected to standard taxonomic distance analysis, five clusters were formed at the cut off genetic distance level of 1.233 (Fig. 3). Standard genetic distances of 19 genotypes varied from 0.453 to 2.313 (data not show). The cluster pattern based on standard distance method could separate two CMS lines IR58025A and PMS2A from TGMS cluster. Furthermore, the distance value between TGMS cluster and the cluster containing IR58025A and PMS2A was higher than that between TGMS cluster and the cluster of IR62829A and IR54755A. This observation was consistent with the cluster analysis obtained from RAPD markers. The main agreement between Mahalanobis distance and standard distance methods was that Pant CMS2A was revealed as the most diverse genotype from all the other genotypes. Analysis of the degree of relationship between three distance matrices (Mahalanobis distance, standard taxonomic distance and molecular genetic distance) revealed that the genetic distance matrix estimated from standard distance method was in better correlation with that estimated from RAPD markers ($r=0.34$, $P<0.005$) as compared to that obtained from Mahalanobis distance method ($r=0.18$, $P>0.05$). It is generally accepted that molecular markers such as RFLP and RAPD represent genetic variation at DNA level, providing more accurate measures of relationships between individuals without the influence of

environmental variation (Miller & Tanksley, 1990). Eventually, the RAPD and other molecular markers (RFLP & AFLP) based on estimates should become the reference for assessing the quality of estimates based on morphological information (Van Beuninger & Busch 1997). Therefore, our findings suggest that standard taxonomic distance method is more accurate than Mahalanobis distance method in assigning genotypes into clusters for taxonomic classification. Similar results also reported by Beer *et al*. (1993) and Mahapatra *et al*. (1995).

Table 4. Average intra- and inter-cluster distances [(D)=$\sqrt{D^2}$] among seven clusters.

Cluster	I	II	III	IV	V	VI	VII
I	3.268	3.970	3.971	4.170	4.078	4.122	4.363
II		3.657	4.227	4.609	4.480	4.731	4.640
III			3.545	4.459	4.252	4.695	4.803
IV				3.899	4.441	4.891	5.203
V					0.000	4.229	4.656
VI						0.000	4.963
VII							0.000

Comparison of cluster dendrograms obtained with standard distances and RAPD markers offered an explanation for the difference between two methods. The typical difference between two methods was the inclusion of Pant CMS2A into a cluster. Standard distance method grouped Pant CMS2A into separate cluster that had the largest distances from all other genotypes. Moreover, two CMS sister lines IR68281A and IR69617A were separately grouped into two diverse clusters. Therefore, clustering of genotypes by standard distance method largely reflects the differences in morphological traits (Table 5). For instance, Cluster II grouped three CMS lines viz. IR54755A, IR62829A and IR69617A which were characterized by short plant height, early duration, lesser number of spikelets per panicle, long-erect flag leaf and small grain size. Cluster III grouped four CMS lines IR58025A, PMS2A, IR68897A and IR68281A. These lines were relatively taller, later duration, higher number of spikelets per panicle and with long-slender grain size. Cluster V grouped Pant CMS2A characterized by early duration, semi-dwarf, short flag leaf, bold and large grain size. In contrast, RAPD method could faithfully reveal and group genotypes into clusters according to their pedigree relationships. A possible explanation for the failure of morphological method which did not reflect genetic relationship between genotypes might be based on genotype environmental interaction effects and the different combination of alleles/genes resulting in morphological similarities or differences that are not proportional to the underlying genetic difference (Cao & Oard 1997). Eventually, the difference

between two cluster patterns as well as a low correlation between them (r=0.34, P<0.005) suggests the need of further study to test whether morphological trait or RAPD analysis can be used to predict heterotic performance of F_1 hybrids.

Regarding a low genetic variability within TGMS group obtained by both morphological and RAPD marker methods, we then asked whether RAPD markers could be used as a means to broaden genetic diversity within parental stock. Because RAPD method could faithfully reveal genetic relationship between genotypes, all the elite lines might be subjected to RAPD analysis before using them for the development of new CMS or TGMS lines. In this way, only those elite lines that are diverse from each other will be hybridized to develop a new parental stock. This approach could maximize opportunities to obtain superior hybrids because unrelated parents would be expected to contribute unique desirable alleles at different loci (Tatineni *et al.* 1996).

Table 5. Cluster means and variation for morphological traits based on standard taxonomic distance method at the genetic distance level of 1.233.

Characteristic	Cluster				
	I	II	III	IV	V
Days to 50% flowering	78.4±3.1	76.6±2.3	91.0±6.1	90.5±5.3	77.0
Plant Height (cm)	76.9±6.3	68.4±0.7	81.9±7.1	116.2±16.8	86.0
Panicles/Plant	122±1.0	128±1.1	14.9±0.6	11.4±0.8	12.5
Panicle length (cm)	23.9±0.8	24.0±1.1	26.8±1.9	26.3±0.5	26.1
Length of flag leaf (cm)	39.6±4.2	36.5±3.1	32.6±6.1	33.8±6.4	33.7
Width of flag leaf (cm)	1.50±0.15	1.28±0.35	1.38±0.15	1.63±0.15	1.51
Spikelets/panicle	166.5±15.2	125.6±20.6	179.8±18.8	164.9±15.4	150.0
1000 grain wt. (g)	20.2±1.1	19.6±2.5	19.5±1.0	23.1±1.4	24.4
Length of brown grain (mm)	6.29±0.29	6.95±0.39	7.08±0.19	6.99±0.25	5.43
Width of brown grain (mm)	2.14±0.15	1.97±0.28	1.88±0.11	2.17±0.21	2.63

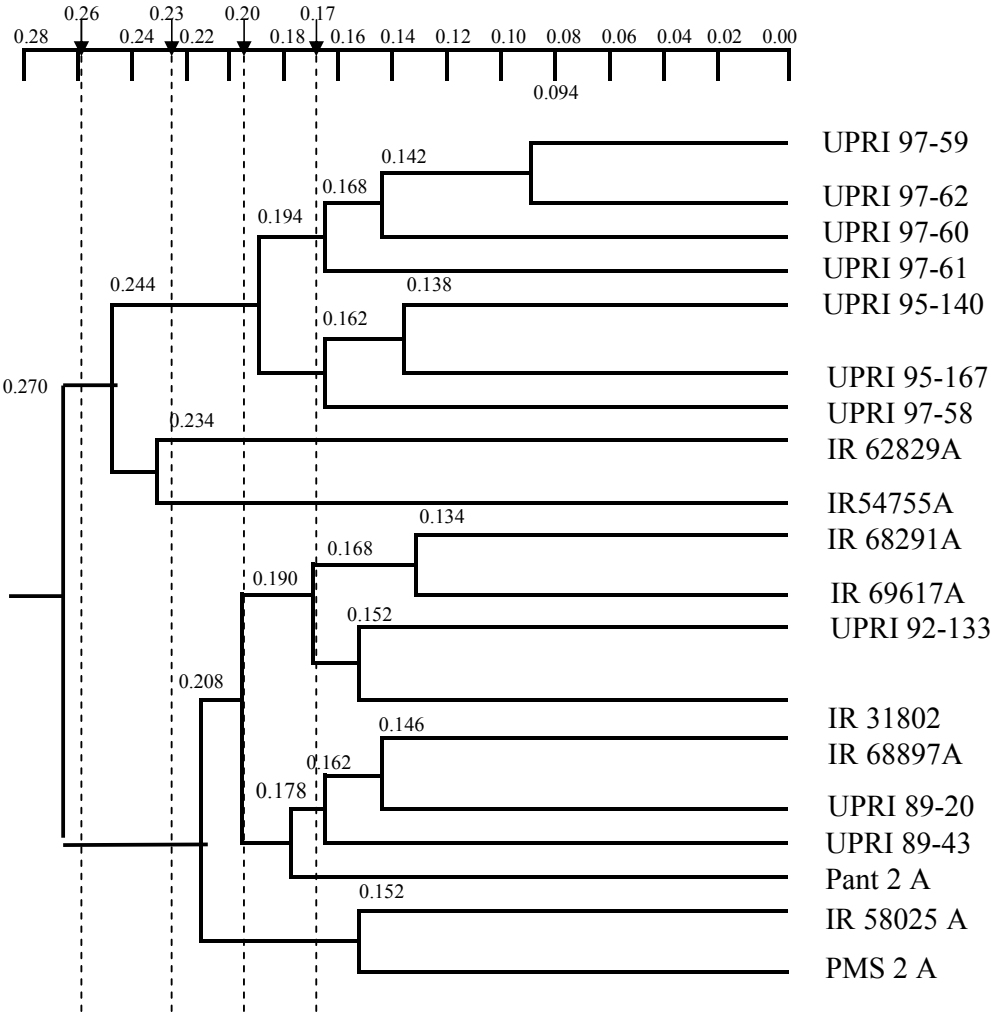

Fig. 3 Dendrogram of genetic distances of 19 parental rice genotypes constructed from 133 RAPD loci. Scale on the top is genetic distance derived from Dice's Coefficient of similarity.

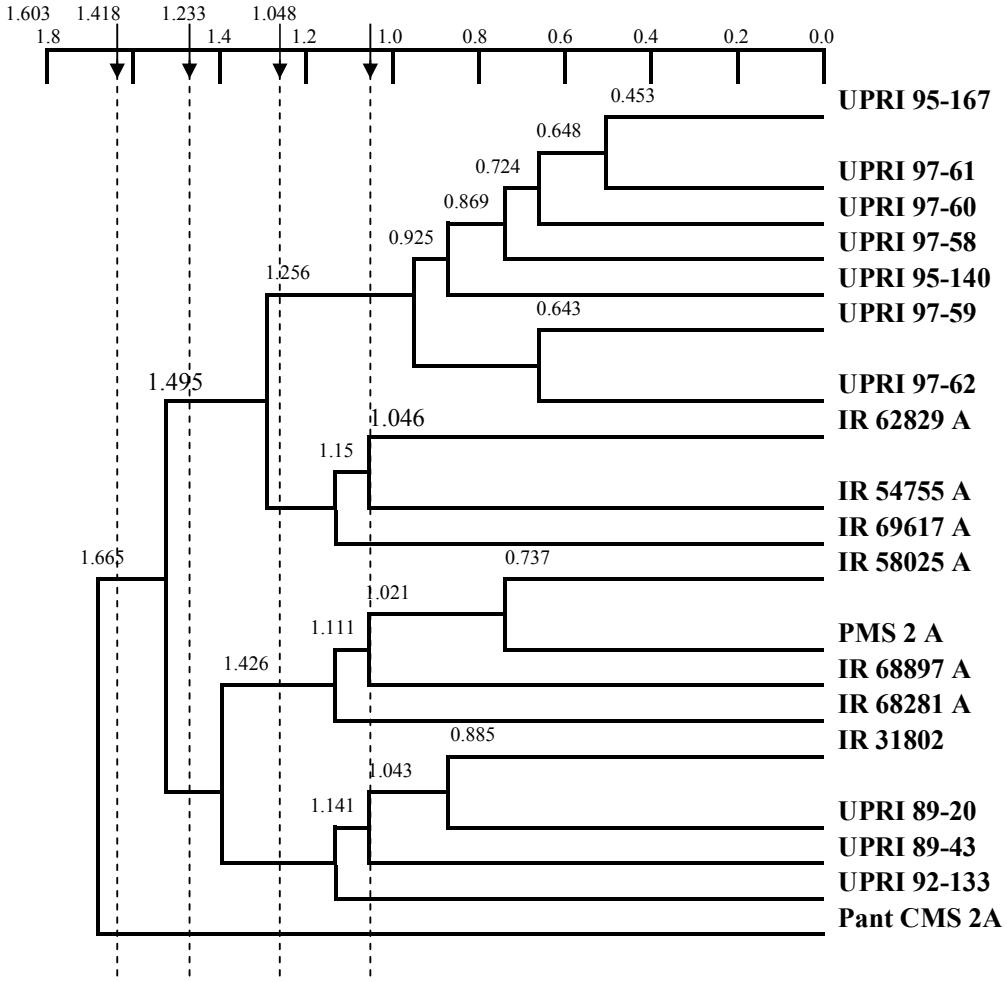

Fig. 4 Dendrogram of standard taxonomic distance of 19 parental rice genotypes constructed from 10 morphological characters. Scale on the top is taxonomic distance.

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TÓM TẮT

Phân tích khoảng cách di truyền của các vật liệu lúa lai bằng đánh dấu RAPD

Sự gần gũi về kiểu di truyền của 19 giống bố mẹ dùng trong chương trình lúa Ưu Thế Lai được đánh giá dựa trên các đặc tính hình thái và đánh dấu phân tử RAPD. Khoảng cách Mahalanobis (D^2) và khoảng cách hình thái chuẩn được tính từ 10 đặc tính nông học. khoảng cách di truyền phân tử được tính từ 133 băng RAPD dựa trên phương pháp " Hệ số giống nhau " của Dice. Biểu đồ phân nhánh của khoảng cách hình thái chuẩn và khoảng cách di truyền phân tử cho thấy sự khác biệt về kiểu gene giữa các dòng bất dục đực mẫn cảm với nhiệt độ (TGMS) nhỏ hơn nhiều so với sự khác biệt về kiểu gene giữa các dòng đực bất dục tế bào chất (CMS). Khoảng cách hình thái chuẩn được xác định như là một phương pháp phân nhóm chính xác hơn khoảng cách Mahalanobis. Sử dụng 10 đoạn mồi phân tử RAPD đủ để phân biệt ở mức phân tử và tính toán khoảng cách di truyền giữa các giống lúa. Phân tích nhóm bằng phương pháp RAPD phù hợp với các mối quan hệ huyết thống giữa các bố mẹ khác nhau dùng trong nghiên cứu lúa Ưu Thế Lai.