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EVALUATING DIFFERENCE OF YIELD TRAIT AMONG RICE GENOTYPES *(Oryza sativa* L.*)* UNDER LOW MOISTURE CONDITION USING CANDIDATE GENE MARKERS

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ABSTRACT

The difference of yield parameters among eighty five diverse upland rice genotypes was evaluated under low moisture condition. Ten candidate gene primers were used to investigate correlation with yield parameters. The results revealed that difference between genotypes with all morphological traits was significant at the 5% probability level. By single marker analysis (SMA) and stepwise multiple regression analysis (SMRA), the candidate gene markers showed significant correlations with such morphological traits at the 5% and 1% probability levels. The genetic diversity of rice genotypes was identified by the Dice coefficient of similarity from 64% to 100% and many groups were established by clustering based on candidate gene marker data. The dendrogram obtained from the candidate gene marker was more discriminatory than the one obtained from morphological traits.

Key words: Candidate gene, Dendrogram, SMA and SMRA.

INTRODUCTION

Rice *(Oryza sativa* L.*)* is central to the lives of billions of people around the world. It is one of the world's main staple crops, with nearly 2.5 billion people depending on it as their main food (International Year of Rice, 2004). Rice is cultivated in at least 114, mostly developing countries and is the primary source of income and employment for more than 100 million households in Asia and Africa (FAO, 2004). Of the 840 million people suffering from chronic hunger, over 50% live in areas dependent on rice production. About 80% of the world's rice is produced on small farms, primarily to meet family needs, and poor rural farmers account for 80% of all rice producers (FAO, 2004).

One of the major challenges in agriculture is to produce more food with limited water. Rice is mainly grown in the submerged conditions but there is a need to develop strategy for growing under aerobic conditions to decrease water use in rice production. Because of a large portion of the world's poor farm in rain fed systems where the water supply is unpredictable and droughts are common. In Asia, about 50% of all the rice land is rain fed, and although rice yields in irrigated systems have doubled and tripled over the past 30 years, only modest gains have occurred in rain fed rice systems (Fischer *et al.*, 2003b). The rice is sensitive to drought at different developmental stages, particularly during the tillering and reproductive stage when varied degrees of sterility can arise under drought stress (Widawsky and O'Toole, 1990). Moisture stress is the single most important factor limiting rice productivity in the rain fed habitats (Widawsky and O'Toole, 1990; Fischer *et al.*, 2003a).

Candidate genes are the genes involved in the variation of a trait based on its biological function or map localization. These candidate genes can be used for identifying favorable alleles as well as following their inheritance in segregating populations. Linked markers will always have the problem of recombination, often demands the use of flanking markers for selection. Furthermore,

identification of the sequence change that imparts a desirable phenotype will allow the development of a marker, specific for the favorable allele. In addition to avoiding problems with recombination, this would also allow the use of the marker in nearly any population, and also as a general screen of germplasm or elite breeding lines for genes of interest (Mackill and McNally, 2004). In our study, candidate genes have been used as molecular markers to establish their relation with certain traits under aerobic conditions and evaluate diversity among diverse rice genotypes.

MATERIALS AND METHODS

Materials

10 primers designed base on sequence of candidate genes were screened by PCR analysis and were selected for analyzing the DNA sample of diverse rice genotypes (Table 1).

The plant material includes 85 diverse rice genotypes obtained from International Rice Research Institute, Philippines.

Experimental design and layout

The diverse rice genotypes were evaluated for yield parameters under aerobic conditions of cultivation (Low moisture stress conditions).This experiment was under taken in Shattigeri. The genotypes were raised during summer (dry) season. The experiment was laid out in randomized complete block design (RCBD). The genotypes were grown under aerobic conditions without puddling and water stagnation, and the irrigation was given at an interval of 5 days. Morphological traits consisting of Plant height (cm), Number of panicles, Number of tillers, Number of spikelets per panicle, Grain yield (g) and straw weight (g) were observed for three plant of each genotype to record data.

Extraction of genomic DNA

The genotypes were grown in an experimental field at University of Agricultural Sciences, GKVK, Bangalore. The leaves were collected at maturity and dried properly in an oven at 55^0C. Dried leaves were then powdered in a mixer, and the fine powder was used for the DNA extraction. DNA was extracted by following the Porebski *et al* (1997) method with certain modifications. The DNA pellet was dissolved in 300 µl of TE buffer and stored at -40^0C. DNA quantification was done at OD_{260nm} and was diluted to a final concentration of 12.5ηg μl^{-1} and 2µl of this DNA was used for the PCR amplification.

Candidate gene amplification

Amplification was carried out in a 20 µl reaction mixture containing 1X PCR buffer, 4mM dNTPs, 5µmol primers, 1 unit *Taq* DNA polymerase and 25 ng of DNA template. The first stage was denaturation of template DNA at 95^0C for 5 min to ensure that template DNA was denatured. The next stage, repeated cycles were carried out with 45 times including denaturation of template DNA at 95^0C for 1 min, primer annealing at 47^0C- 55^0C (varying according to the primer) for 1 min and primer extension at 72^0C for 2 min followed by a final extension at 72^0C for 7 min.

PCR products were separated on a 1.5 % agarose gel containing ethidium bromide. 1X TBE buffer was used for preparation of gel and for running the gel. It was run for 2.5 hr at 85V. the size of the fragments were determined by using 500 bp DNA ladder marker and the gel was visualized under ultraviolet light and documented using gel documentation system (Herolab GmbH Laborgerate).

Table 1: The candidate gene primers with their sequences use for analysis

SL. No.	Sequence of candidate gene primers (5'-3')	Candidate genes	Functions of candidate genes	References
1	5'AAATCCCTTAGGCACAACA3' 5'TTTGCGAAACAAACTCGAAA3'	ACC synthase	Catalyzing the formation of ethylene from AdoMet (S-adenosyl-L-methionine)	Boller *et al.*, 1979; Dong *et al.*, 1992
2	5'ATATCGCCCTCGCTCTTCGACCA3' 5'GTCATCCAGAACCAGAACGCCGT3'	Chitinase	Hydrolysis of chitin, defense response against pathogenic microbes, might be involved in normal plant growth and development.	Collinge *et al.*, 1993; de Jong *et al.*, 1992
3	5'CTCAGCGCCCTCATATCCAG3' 5'GATGCGGTCTTGAACCCTGC3'	Chitinase basic	Involved in protecting nodule tissues, especially the root nodule meristem, against external pathogenic bacteria or incompatible symbionts rather than a defense response against internal pathogenic symbionts	Kim and An, 2002
4	5'TCATTTGCTACTCTCCGCCGCT3' 5'CATTTTTCGCCACGGTCTTTAG3'	LEAFY	Transcription factor involved in the regulation of flower development	Sliwinski, 2006
5	5'GATCGGGGTGTGCTACGGATGA3' 5'GCTGATGGGGTAGACGTGCTGCA3'	Glucanase	Hydrolyzing the β-1,3-glucans found in cell walls of many phytopathogenic fungi that attack plants	Anuratha *et al.*, 1996
6	5'TCCGTGAATTCAATACTAACACTC3' 5'ACTCAATTCTCGACCAAACTTGGC3'	Heat shock protein	Function as chaperones and play important role in normal growth and stress tolerance	Hayes and Dice, 1996
7	5'TTTAAGCGACCAGGAAGAGTATG3' 5'AGTAATTCTTTGACCACTGGGAA3'	Maltose binding regulatory protein	Factor involved increasing maltose under cold-stress condition	Lu and Sharkey, 2006
8	5'CTTAGCTTAATGCTGCTGGTGGC3'	Peroxidase	Contribute to resistance, including	Gross, 1980;

SL. No.	Sequence of candidate gene primers (5'-3')	Candidate genes	Functions of candidate genes	References
	5'GAGTCCACCTTGGAGCAGCTGAG3'		oxidation of hydroxy-cinnamyl alcohols into free radical intermediates, phenol oxidation polysaccharide cross-linking, cross-linking of extension monomers and lignification	Schmid and Feucht, 1980; Fry, 1986; Everdeen *et al.*, 1988; Grisebach, 1981; Walter, 1992
9	5'TGTGGAAGGCGTTCATGGA3' 5'TGAGCGAGGTAGTCCTC3'	Pathogenesis related protein 10	A gene family of pathogenesis related protein	Osmark *et al.*, 1998
10	5'GTGGTGCTAAGAAGAGGAAGA3' 5'TCAAGCTTCAACTCCTTCTTT3'	Ubiquitin 5	Factor involved proteolytic pathway	Smalle and Vierstra, 2004

Single Marker Analysis (SMA) and Stepwise multiple regression analysis (SMRA)

The data was analyzed using SAS (Statistical Analysis Software) v6.12 program (SAS, 1989) and ANOVA was performed by Fisher's method using the General Linear Model (GLM). The regression values (R^2) were calculated by correlation between each trait and each marker by SMA. The correlation between each trait and all markers was also evaluated by SMRA.

Clustering analysis

NTSYS-pc (Rohlf, 2004) was used to know the genetic similarity among the diverse rice genotypes. The similarity coefficient was computed using Dice coefficient (Dice, 1945; Nei and Li, 1979) to create similarity matrix using molecular marker data. Genotypes were clustered using SAHN method (Sequential, Agglomerative, Hierarchical, and Nested clustering) base on similarity matrix.

Genotypes were also clustered base on Correlation coefficient (CORR) using morphological traits data.

RESULTS AND DISCUSSION

Evaluation of diverse rice genotypes for morphological traits

The diversity among genotypes was identified from coefficient of variation (CV) and clustered base on traits (Figure 1). Variation among genotypes was the highest with number of panicles (13.32%) and was the lowest with plant height trait (4.5%).

The performance of 128 genotypes was examined under irrigation and four different types of drought stress. It is suggested that variation in flowering time, potential yield and drought patterns need to be considered for development of drought resistant cultivars using specific physiological traits (Pantuwan, 2002).

Evaluation of plant height, number of tillers, number of panicles, panicle length, total plant length, maximum root length, root number, root volume, stem weight, shoot dry weight, silicon content in stem and total silicon content in stem was carried out under low-moisture stress conditions. Wide variability of ten varieties and 148 doubled haploid lines was observed for all the traits (Vinod et al., 2006).

Correlation between various morphological traits and candidate gene markers in rice

A minimum of one and maximum of three alleles per locus were detected for all the 10 primer pairs. However, polymorphism between genotypes was

found with 9 primer pairs (ACCsyn, Chitinase, Chitinase basic, LFY, Gluc, Hsp, MBRL, PR10 and UBQ5). Good bands were obtained from the 10 primer pairs and varied in size between 200bp and 1700bp. Some examples of these were showed on Figure 2 and 3.

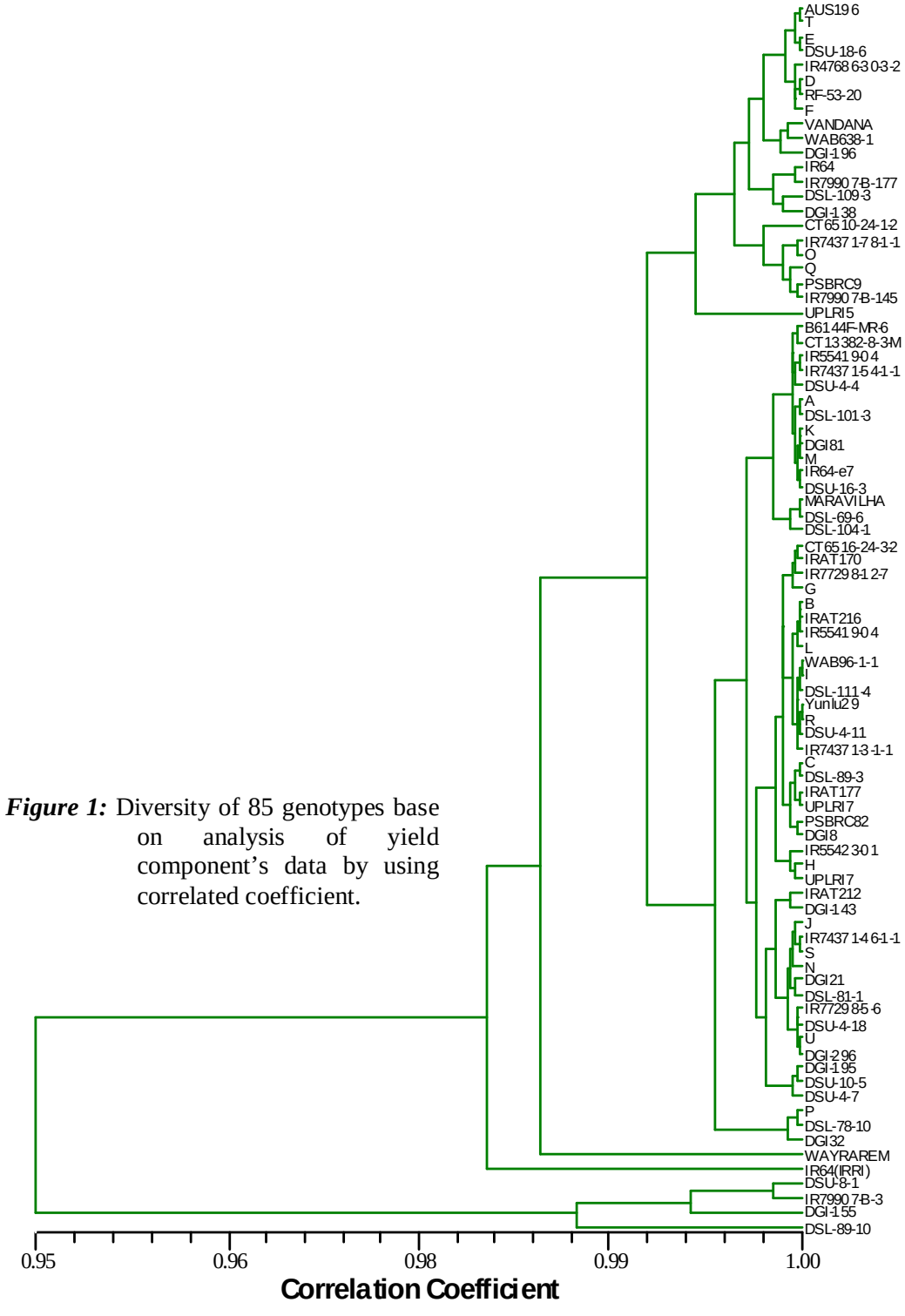

Figure 1: Diversity of 85 genotypes base on analysis of yield component's data by using correlated coefficient.

The results of SMA and SMRA showed that eight of ten primers (ACCsyn, Chitinase, Chitinase basic, Gluc, LFY, MBRL, PR10 and UBQ5) used in study show significant correlation with morphological traits. $Gluc_{1000}$ marker was correlated with the largest number of traits (Grain yield, number of tillers, number of panicles and straw weight), followed by LFY_{300} (number of tillers, number of panicles and plant height) and $UBQ5_{350}$ (number of panicles, number of spikelets and plant height), $Chitinase_{350}$, $Chitinase\ basic_{1500}$, $Gluc_{700}$, and $MBRL_{400}$ markers with two traits (Grain yield and number of panicles, grain yield and straw weight, grain yield and number of panicles, and number of tillers and number of panicles respectively) and remaining markers ($ACCsyn_{1100}$, $Chitinase_{500}$, LFY_{1200}, LFY_{700}, LFY_{600}, $PR10_{900}$, $PR10_{700}$, $PR10_{600}$, and $UBQ5_{250}$) with one trait each (Table 2).

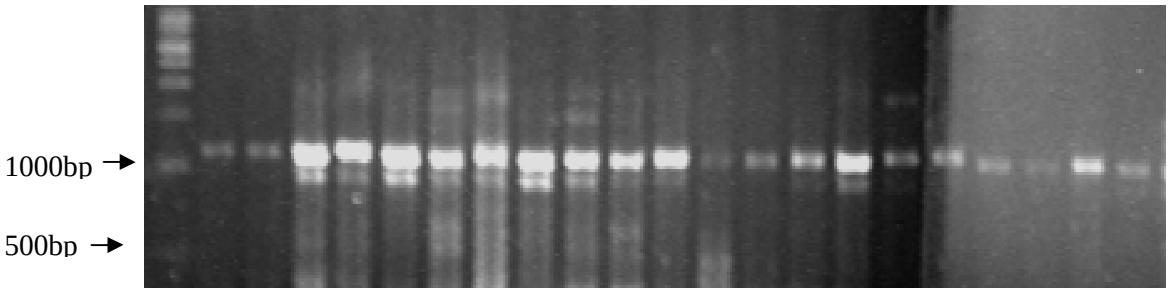

Figure 2: PCR products with MBRL primer
M: 1kbp ladder, 1: PCBRC 9, 2: UPL RI 7, 3: Yunlu 29, 4: IR 76569-259-1-1-3, 5: IR 76569-243-2-1-4, 6: DGI-196, 7: DSL-89-3, 8: DSL-104-1, 9: DGI-195, 10: IR64, 11: DSU-16-3, 12: DSU-4-18, 13: DSL-109-3, 14: DGI-296, 15: DSL-101-3, 16: DSL-89-10, 17: DGI 21, 18: DSU-4-11, 19: DGI-138, 20: DGI 32, 21: DSU-4-7

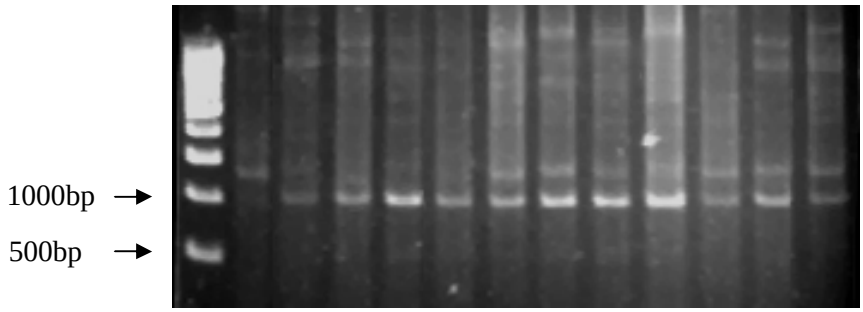

Figure 3: PCR products with Gluc primer
M: 1kbp ladder, 1: Yunlu 29, 2: IR 76558-156-4-1-3, 3: IR 76569-259-1-1-3, 4: IR 76569-243-2-1-4, 5: IR 76569-166-4-2-2, 6: DGI-196, 7: DSL-89-3, 8: DSL-104-1, 9: DGI-195, 10: IR64, 11: DSU-16-3, 12: RF-53-20

Table 2. Candidate gene markers correlated with morphological traits based on analysis of 85 rice genotypes

Markers	Grain Yield		No. of tillers		No. of panicles	
	SMA	SMRA	SMA	SMRA	SMA	SMRA
ACC syn_{1100}	-	-	-	-	-	-
$Chitinase_{350}$	0.057*	0.052*	-	-	0.073*	0.073*
$Chitinase_{500}$	-	-	-	-	0.045*	0.045*
Chitinase $Basic_{1500}$	-	0.059*	-	-	-	-
$Gluc_{1000}$	0.059*	0.059*	0.094**	0.048*	0.098**	0.047*
$Gluc_{700}$	0.048*	0.044*	-	-	-	0.037*
LFY_{1200}	-	0.043*	-	-	-	-
LFY_{300}	-	-	0.057*	-	0.066*	-
LFY_{600}	-	-	-	-	-	-
LFY_{700}	-	0.050*	-	-	-	-
$MBRL_{400}$	-	-	0.070*	0.070*	0.053*	-
$PR10_{600}$	-	-	-	0.044*	-	-
$PR10_{700}$	-	-	-	-	-	-
$PR10_{900}$	-	-	-	-	-	0.058*
$UBQ5_{250}$	-	-	-	-	-	0.050*
$UBQ5_{350}$	-	-	-	-	0.055*	-
ACC syn_{1100}	-	-	-	-	0.055*	0.063*
$Chitinase_{350}$	-	-	-	-	-	-
$Chitinase_{500}$	-	-	-	-	-	-
Chitinase $Basic_{1500}$	-	-	-	-	0.093**	0.093**
$Gluc_{1000}$	-	-	-	-	0.051*	-
$Gluc_{700}$	-	-	-	-	-	-
LFY_{1200}	-	-	-	-	-	-
LFY_{300}	-	-	0.071*	0.071*	-	-
LFY_{600}	0.056*	0.0562*	-	-	-	-
LFY_{700}	-	-	-	-	-	-
$MBRL_{400}$	-	-	-	-	-	-
$PR10_{600}$	-	-	-	-	-	-
$PR10_{700}$	-	-	-	-	0.041*	-
$PR10_{900}$	-	-	-	-	-	-
$UBQ5_{250}$	-	-	-	-	-	-
$UBQ5_{350}$	0.055*	-	0.047*	-	-	-

*= significant at 5% probability level; ** = significant at 1% probability level.

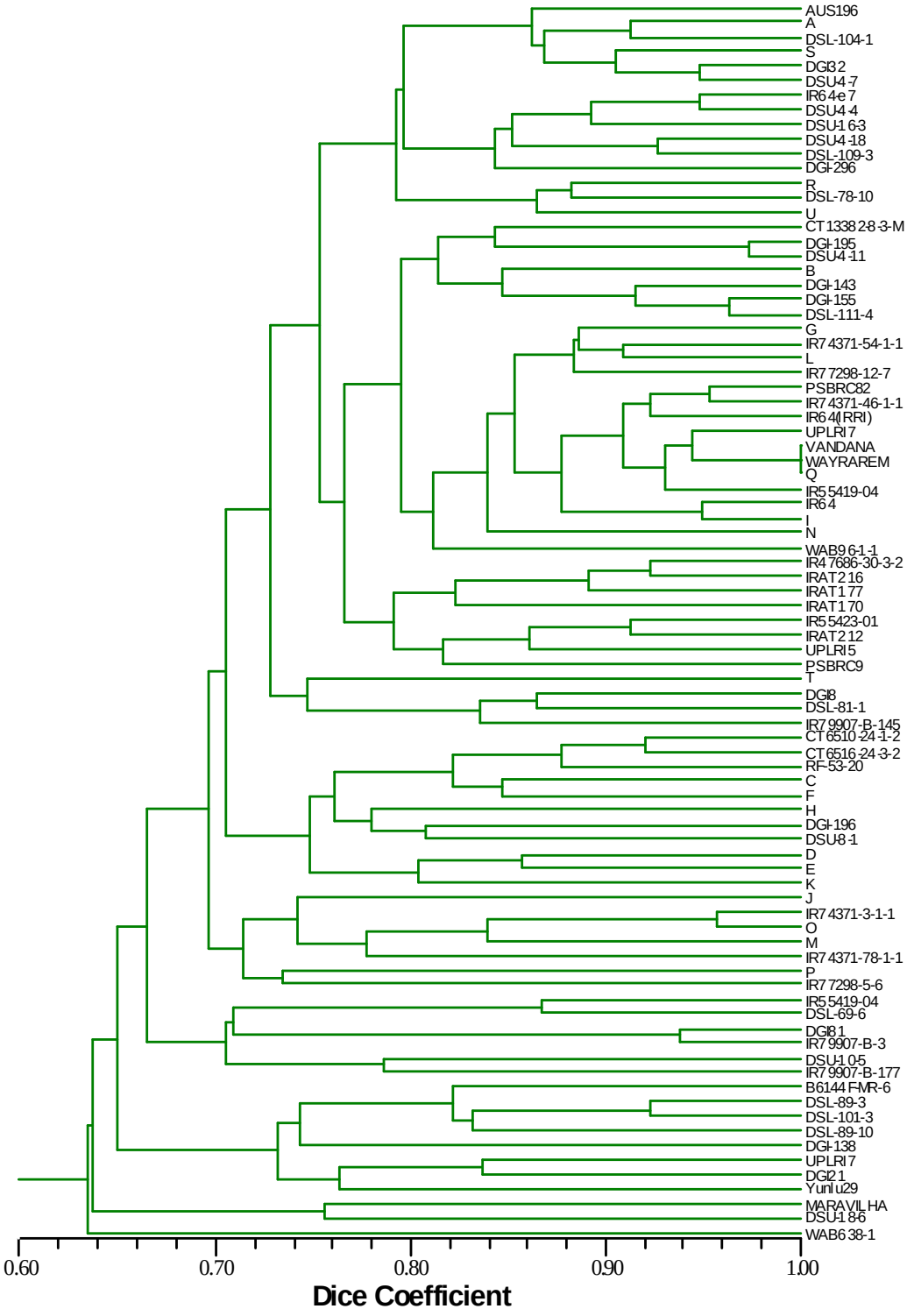

Figure 4: Diversity of 85 genotypes base on analysis of candidate gene data by using Dice coefficient.

In earlier study, single-marker analysis performed on the phenotypic and genotypic data generated revealed strong association (< 0.01) of four candidate genes, *LTP, KCDL, EXP13* and *EXP15* with certain shoot characters viz., total plant length, plant height, number of productive tillers, number of tillers, stem weight, shoot dry weight, silicon content in stem and total silicon content in stem (Vinod et al., 2006). Under low moisture stress conditions, four candidate genes (*RAB21, EXP15, CIS* and *EXP13*) were associated (< 0.01) with shoot traits such as plant height, panicles length, silicon content in stem and root characters such as maximum root length, root number and root volume

Evaluation of diversity among diverse rice genotypes using candidate gene markers analysis

Eighty-five diverse rice genotypes were clustered by the Dice coefficient of similarity from 64% to 100% (Figure 4). The clustering was started at the Dice similar coefficient of 64%, in that WAB638-1 genotype differentiated with other genotypes. The diverse genotypes were divided into many subgroups with increasing of the Dice similar coefficient. The VANDANA, WAY RAREM and PR 27699-B-D808-4-4 genotypes were similar at the Dice similar coefficient of 100% with ten candidate gene primers used in study.

Ogunbayo *et al.* (2005) evaluated both morphological and genetic variations exist between the 40 rice accessions. The dendrogram obtained from the molecular marker was more discriminatory than the one obtained from morphological marker. In our study, we observed a similarity coefficient ranging from 64% to 100% for marker data (Figure 4) while from 95% to 99.98% for morphological data (Figure 1), suggesting that diversity of eighty-five diverse rice genotypes was higher by using marker analysis. Matrix comparison plot module of NTSYS-pc program was also used to compare between the clustering base on morphological data and the clustering base on marker data. Similarity was evaluated by correlated coefficient of them, which was significant at 1% probability level, but correlated coefficient was low (3.8%).

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Đánh giá tính trạng năng suất của các giống lúa (*O. sativa* L.) trong điều kiện độ ẩm thấp, sử dụng gene dự tuyển

Sự khác biệt về các chỉ tiêu năng suất của 85 mẫu giống lúa cạn (*O. sativa* L.) được đánh giá trong điều kiện độ ẩm thấp. Sự tương quan giữa các chỉ tiêu đó với một số gen dự tuyển cũng được đánh giá thông qua phân tích tương quan. Kết quả thu được cho thấy sự khác biệt giữa các genotype là có ý nghĩa ở mức 5%. Nhiều gen dự tuyển biểu hiện tương quan có ý nghĩa với các chỉ tiêu năng suất ở cả hai mức 5% và 1%. Trên cơ sở phân tích các gene dự tuyển, sự đa dạng di truyền của các kiểu gen cũng được xác định thông qua hệ số tương đồng Dice từ 64% tới 100% và nhiều nhóm khác nhau được hình thành. Sự đa dạng di truyền trên cơ sở phân tích các gen dự tuyển là lớn hơn so với phân tích các chỉ tiêu năng suất.