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EFFICIENCY OF SSR MARKERS SYSTEM IN ACCESSING GENETIC VARIATION FOR RICE *(ORYZA SATIVA* L.) GENOTYPES

Balqees H. AL- Musawi , Nidhal Abd H. AL-Badeiry[1] and Mohammed A.AL-Anbari[2]
Department of Biology, College of Science, Karbala , Karbala province, Iraq
[1]Department of Biology, College of Education for girls, Kufa University, Kufa province, Iraq
[2]Department of Agronomy, College of Agricultural, Karbala , Karbala province, Iraq
* Corresponding author : E-mail balkeasbio@gmail.com

Abstract

To estimate the genetic diversity in fifteen rice (*Oryza sativa* L.) genotypes differed in drought and salinity tolerance by using two primers RM585 and RM8085 (SSR) markers related to above traits and used to fingerprint among genotypes. The results indicated that among SSR markers used, 6 SSR loci were polymorphic and produced a total of 10 alleles. The number of alleles per locus created by each marker varied from 4 to 6 alleles with an average of 5 alleles per locus. The overall size of amplified fragments ranged from 142.946 to 216.064 bp. The polymorphic information content (PIC) values obtained from the microsatellite primer panels ranged from 0.635 to 0.788 with an average of 0.712. This Unweighted Pair Group Method (UPGMA) cluster analysis separated the 15 genotype into two major groups : The first cluster A contained 7 genotypes cluster A and a large cluster B. The major group A comprised 7 genotypes, while the second cluster B comprised 8 depending on their geographic origin, their ancestor. The results specified the ability of SSR markers to identify and to detect the allelic diversity and genetic variation among the studied rice genotypes and findings the prospect role for further improvement of drought and salinity tolerance rice genotypes through marker helpful breeding.
***Key words*:** SSR, Genetic Diversity, Salinity, (UPGMA), Drought Tolerance.

Introduction

Rice (*Oryza sativa* L.) is one of the most significant cereal crops in the world and the main food for more than three billion people in the World (Ma *et al.,* 2007). The tremendous growth of human population worldwide has increased the demand for rice production (Liang *et al.*, 2010) requiring an improvement of 50% by the year 2025 (Kush, 2001). More than 90% of rice is produced and consumed in Asia (FAO, 2004). Rice is the seed of the monocot plants, belonging to the family Graminae and subfamily Orazoidea. which is a model plant for genomic research (Sasaki and Burr, 2000), which have (2n=24) AA-genome and highly polymorphic with wide geographical and genetic differentiation (Sarla and Swamy, 2005). The transformation of some highly productive rice lands for residential and industrial purposes has pushed rice cultivation to less productive areas such as drought, saline and flood prone areas. The major environmental factor that constrains the productivity and stability of plants (Araus *et al.,* 2002). Rice (*Oryza sativa* L.) farming is drought (water stress).Which is the most important limiting factor for plant agriculture worldwide, can cause earnest losses of yield and productivity in ultimate crop plants in regions (Massonnet *et al*., 2007). Salinity (Salt stress) is the second utmost widespread soil problem in a major constraint to cereal production worldwide (Tuteja *et al*., 2012), respectively rice growing countries after drought.

Molecular markers are powerful tools for evaluation of genetic diversity, relationships and have always been a dominant tool in the estimation of genetic variation among the PCR based molecular markers to drought and salinity tolerance. Microsatellite or SSR marker analysis is favorable to identify major gene locus for salt and drought tolerance that can be beneficial for plant breeders to develop new genotypes. This markers have been widely applied in rice genetic studies as they are able to reveal significantly high levels of allelic diversity and higher degree of polymorphism in rice (Nei, 1973). It is being successfully applied in fingerprinting and variety identification, which proved to be ideal for making gene and QTL mapping in plants (Nandakumar *et al.,* 2004). Genetic variation of rice genotypes in Iraq and assessment of their salinity and drought tolerance were evaluated using SSR markers.

Water stress tolerance characters are quantitative traits, and so postmortem of these complex traits into component genetic factors is a necessary to tamper the traits. Genome mapping using molecular genetic markers offers an stellar chance to locate genes or QTLs controlling quantitative characters (Tanksley, 1993). Markers related with genes controlling a trait of benefit

could be used in the chosen of genotypes in a breeding program. Hence the present study was strived to know and understand the genetic diversity of the drought and salt tolerant of rice genotypes for the region and develop unique fingerprint for each genotype under molecular level.

The main objective of this study is to this microsatellite database will be useful for rice breeders in identifying desirable traits and selection of appropriate parents in rice breeding programs. This baseline data also helps in generating a suitable practical method in salt tolerant genotypes management (Kanawapee *et al*., 2011). Also to research the genetic diversity among fifteen rice genotypes varying in their tolerance to salinity and drought using SSR markers for developing individual fingerprint for each genotype, and to recognize SSR markers related with some drought tolerance traits or QTLs, and to recognize the better genotype to be used as donors for drought tolerance in breeding program in the future for expansion the suitability of this unique rice molecular marker characterizing among rice genotypes and gave equally helpful for the scientific community and farmers.

Materials and Methods

Plant Materials

Fifteen different genotypes of *Oryza sativa* seeds were collected from seed bank of AL-Mashkhab Rice Research Station (AMRRS). Najaf-Iraq. Illustrated listed in Table (1).

Table 1 : Rice genotypes used in the study

Genotypes' Name	Pedigree	Breeding Institute
1. Amber 33	Local (Iraqi)	AMRRS
2. Amber al-Baraka	Introduced from India	AMRRS
3. Amber Furat	Technology& Science Ministry/ Baghdad	AMRRS
4. Amber Baghdad	Technology& Science Ministry/ Baghdad	AMRRS
5. Amber Menathera	Technology& Science Ministry/ Baghdad	AMRRS
6. Sumar	Technology& Science Ministry/ Baghdad	AMRRS
7. Dijlah	Introduced from China	AMRRS
8. Ghadeer	Introduced from IRRI (Philippines).	AMRRS
9. Brnamge -4	Introduced from IRRI(Philippines).	AMRRS
10. Dorfak	Sepidrood/Salari- Iran	AMRRS
11. Gohar	Pusa1238-1/Pusa1238-81-6-Iran	AMRRS
12. Khazar	IR2071-625-1-52/TANU7456-Iran	AMRRS
13. Shiroudi	Khazar / Deylamani – Iran	AMRRS
14. Neda	Amol3/Hassansarayee/sangetarom-Iran	AMRRS
15. Nemat	Amol3/sangetarom - Iran	AMRRS

* AMRRS = AL-Mashkhab Rice Research Station

METHODS

Genomic DNA Extraction; Rice Germplasm

Isolation of genomic DNA method was carried according to the protocol by Plant DNA Extraction Kit (Cat. No: GP100) provided by (Geneaid Biotech. Ltd.; Taiwan Company). The Genomic DNA Mini Kit supplies a quick and easy method for purgation total DNA (including genomic DNA, mitochondrial and chloroplast DNA) according to the isolation of genomic DNA from plant tissue protocol with some modification. Young leaves of 15-20 days old seedlings from fifteen rice genotypes were done DNA extracted.

Gel Electrophoresis

Electrophoresis was conducted at 80 V, 400 mA for 130 min at genomic DNA for PCR amplifications. The gels were spotted with ethidium bromide and visualized under UV Transilluminator and images were captured using Vilber Lourmat, Taiwan gel documentation system. The (100bp) DNA ladder supply from Bioneer / Korea (Sambrook and Russel, 2001).

Quantification and Optimization of DNA Concentration

The segregation DNA samples were quantified by UV visible spectrophotometer (Thermo Scientific, Germany). Absorbance at 260-280 nm and DN concentration was determined. DNA concentration = OD260 × 50 µg/ml× dilution factor. The original DNA concentrations were determined and adjusted to 30 ng/µl. then stored at - 20°C until use.

Primer Selection of Microsatellite /SSR Marker:

The selected Primers were amplified the SSR marker RM585 and RM8085, which screened against 15 genotypes at a time. These primers were used in the study for molecular characterization of rice genotypes. Details in the Table 2.

Table 2 : The size and sequence of the microsatellite markers (SSRs)

SSR Marker			Primer sequence (5'-3')	No. Ch.	Repeat Motif	bp	Reference
1	RM585	F	CAGTCTTGCTCCGTTTGTTG	Ch.6	(TC)45	233	Md *et al*. (2015)
		R	CTGTGACTGACTTGGTCATAGG				
2	RM8085	F	TGCGTTTCGATTTCTTTTTA	Ch.1	(AG)26	119-128	Salumkhe *et al*. (2011)
		R	GGAAAGTTGTGTTCTTTGGC				

F = Forward R = Reverse

Detection of SSR Marker by PCR Amplification

PCR Master Mix Reaction Preparation

PCR reactions was performed with final volume of 20 µl mixture and PCR master mix reaction was prepared by using (Maxime PCR PreMix (i-Taq). Provided from iNtRON Company in Korea .This reaction mixture containing 1 µl each primer (10 pmol), 2 µl template DNA (30 ng/µ1), Thereafter, sample volume was adjusted to 20 µl with nuclease-free water. After that, putted in standard PCR tubes containing the PCR PreMix as lyophilized materials containing all other components necessary to PCR reaction. Then the tube placed in vortex centrifuge and transferred in (Techne PCR thermocycler).

PCR Master mix	Volume
DNA template	2µL
F. primer 10pmol	1µL
R. primer 10pmol	1µL
PCR water	16µL
Total volume	20µL

Polymerase Chain Reaction (PCR) Program

The thermal cycling (Techne TC-5000 Thermal Cycler, UK) was programmed as follows: initial denaturation of 5 min at 94°C and then 35 cycles of the following three steps: 94°C for 30 Sec , 55°C for 30 Sec and 72°C for 1min and ultimate incubation or expansion at 72°C for 5 min .

Data Analysis

This resulting microsatellite data was analysed using Power Marker V 3.25 software (Liu and Muse, 2005) to calculate the number of alleles, heterozygosity and polymorphic information content (PIC). Analyzed by the binary procedure. Individual SSR patterns were contrast with samples. The increasing well-marked amplified fragments or band profiles were scored appeared graph data, numerous bands as present (+) or absent (-) for each genotype. Genetic distance was computed using (Nei, 1972), standard genetic distance (Ds). The distance method of (Nei, 1972), was used with program Power Marker V3.25 for the construction of phylogenic tree was generated using (UPGMA) which means Un weighted pair-group method with arithmetic.

Results and Discussions

The present realization, isolated genomic DNA from high yield rice genotypes were determined in electrophoresis and separated band for the presence of genomic DNA of *Oryza sativa* (Figure-1). In SSR-PCR reactions, this fifteen rice genotypes have been executed using with two SSR primers. Genomic DNA was segregated in 25 days fresh leaves for SSR analyses.

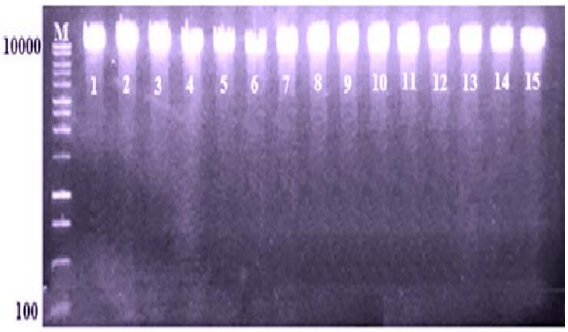

Fig. 1 : Electrophoresis of total genomic DNA of rice genotypes run on 1.5% agarose, Lanes: 1-Amber 33 , 2-Amber Al-Baraka, 3- Amber Furat, 4- Amber Baghdad , 5- Amber Munatherha, 6-Sumar, 7- Dijlah, 8- Ghadeer, 9- Brnamge-4-, 10- Dorfak, 11- Gohar, 12- Khazar, 13- Shiroudi, 14-Neda, 15-Nemat. M= 100-10000 bp DNA ladder marker.

SSR Analysis

The results obtained in these experiments revealed that two primers were initially screened against the fifteen rice genotypes cultivated in Iraq. Which have amplified the specific regions and produced specific bands (SSR primers were RM585, RM8085). For scoring and data analysis, the amplified loci were run on agarose gel with high concentration. Microsatellites as they are co-dominant marker thus heterozygote produces two bands revealing the amplification of the two loci and could be readily identified (Wu *et al.,* 2010).

Each primer produced different amplification products (SSR profiles), the results of these two SSR primers will be illustrated individually in details below:

1. Primer RM585

Using this primer for detected positions of QTLs traits related to salinity tolerance for rice genome of which located on the long arm of chromosome 6 Table 2. This primer gave 18 amplified bands distributed among six alleles, that 6 of which were polymorphic, 12 monomorphic and 1 unique bands at molecular size of 224.99 bp in Sumar. Presence of unique alleles are significant because they may be diagnostic for specially regions with a genome specific to genotype, a particular of rice. This ability primer to recognize a unique annealing site on genome increase its ability to produce a unique DNA fingerprint for a particular genotype (Al-Ghufaili and Al-Tamimi, 2017). Amplified of bands had been produced among fifteen rice genotypes ranging in their molecular size from 162.138 bp to 224.99 bp. The higher number of amplified bands was 18 due to high percentage for primer efficiency was 54.54%. The total number of alleles was 6 this reverse on high value of Gene diversity and PIC were 0.8156, 0.7888 respectively. The total number of alleles Major Allele was 182.287 while score 0.2333 in Major Allele frequency, this may due to that an insertions or deletions or substiutions mutations could cause a change in primer annealing sites consequently change size of amplified fragment product, because it could change distance between two annealing sites of primer on target DNA (Fadoul *et al.,* 2013). The polymorphic bands was six. The presence of polymorphic band in primers resulted in giving value for efficiency, discriminatory value and polymorphism, because these criteria are highly dependent upon number of polymorphic band produced by particular primer (Graham and McNicol, 1995). The value of heterozygosity was varied 0.2000 for locus with a mean of 0.1000 (Table 4). The higher the heterozygosity values, the broader the genetic diversity. Gene diversity, often indicated to as expected heterozygosity and is defined as the eventuality that two randomly selection alleles from the population are various (Liu and Muse, 2005). This PIC values for the SSR loci ranged from 0.7888, with an average of 0.7123. The highest PIC values provides an estimate of the discriminating power of the marker (Nagy *et al.*, 2012). The average polymorphic information content value specified in this study agreed with earlier findings announced based on SSR marker in rice inbred lines (Moniruzzaman *et al.,* 2012) Figure 2. This primer differed in their capacity in producing polymorphic amplified profiles among studied rice genotypes which gave was DNA profiles (fingerprints) for (6) Sumar. This consistency of a major gene locus for salt tolerance near a microsatellite marker can be applied by plant breeders to choose further efficiently to best understand salt tolerance at the reproductive period. Selection programs that will assist in improving salt tolerant rice genotypes. This marker was also announced as highly polymorphic in rice genotype for labeling salt tolerant genes (Islam, 2004; Niones, 2004) also (El-Refaee *et al.,* 2006) announced that 80% of the all tested SSR primers presented polymorphic pattern in rice.

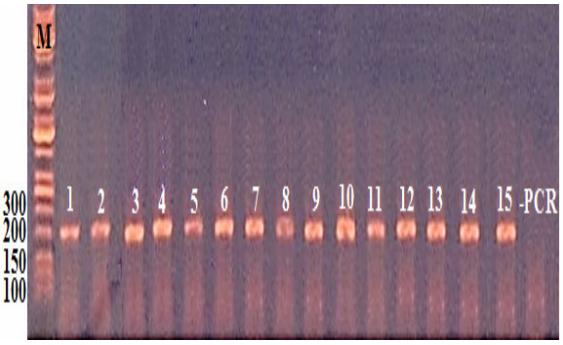

Fig. 2 : SSR amplification product with primer RM585 run on 1.5% agarose, Lanes: 1-Amber 33 , 2-Amber Al-Baraka, 3-Amber Furat, 4- Amber Baghdad , 5- Amber Munatherha, 6-Sumar, 7- Dijlah, 8- Ghadeer, 9- Brnamge-4-, 10- Dorfak, 11-Gohar, 12- Khazar, 13- Shiroudi, 14-Neda, 15-Nemat represent rice genotypes. M= 100-10000 bp DNA ladder marker.

2. Primer RM8085

Using this primer for detected of positions QTLs traits related to drought tolerance for rice genome of which located on the long arm of chromosome 1. Table 2. This primer gave 15 amplified bands distributed among four alleles, these no gave any unique fingerprint and polymorphic band, but gave 15 monomorphic band. Presence of unique alleles are significant because they may be diagnostic for specific regions with a genome specific to a genotype of rice, using SSR primers. Amplified of bands had been produced among fifteen rice genotypes ranging in their molecular size from 142.947 bp to 167.08 bp. The number of amplified bands was 15 due to high percentage for primer efficiency was 45.45%. Increase and decrease of number amplified bands related directly to increase and decrease number of annealing sites recognized by primer which dependent mainly on template DNA sequence and changes resulted of diverse reasons including mutation that cause changing in annealing sites of primer and affect number of main and amplified bands (Tahir, 2014). The total number of alleles was 4 this reverse on value of Gene diversity and PIC were 0.6844, 0.6358 respectively. The total number of alleles Major Allele was 158.037 while score 0.4667 in Major Allele frequency, due to An insertions or deletions or substiutions mutations could cause a change in primer annealing sites consequently change size of amplified fragment (product), because it could change distance

between two annealing sites of primer on target DNA (Fadoul *et al.,* 2013). Thus dissimilarity between results using the same primer on rice genome with diverse genotypes could occur as resulted in RM8085 by (Anandan *et al*., 2016) compared with results in current study. Monomorphic bands were 15 bands. Monomorphic band refer to constant, identical or conserved sequences on genome that ensure belonging of a genotype to a particular one species (Al-Judy, 2004). The value of heterozygosity was varied zero for locus with a mean of (0.1000) because all bands present single (Table 4). The PIC values for the SSR loci ranged were 0.6358, an average of 0.7123. This highest polymorphic information content values provides an estimate of the discriminating power of the marker (Nagy *et al*., 2012). The rise level of polymorphism is due to diverse genotypes and more difference of SSR loci used in the current study.

Figure (3) This primer differed in their capacity in producing polymorphic amplified profiles among studied rice genotypes which no gave was DNA profiles (fingerprints) between genotypes.

Fig. 3 : SSR amplification product with primer RM8085 run on 1.5% agarose, Lanes: 1- Amber 33 , 2-Amber Al-Baraka, 3- Amber Furat, 4- Amber Baghdad , 5- Amber Munatherha, 6-Sumar, 7- Dijlah, 8- Ghadeer, 9- Brnamge-4, 10- Dorfak, 11- Gohar, 12- Khazar, 13- Shiroudi, 14-Neda, 15-Nemat represent rice genotypes. M= 100-10000 bp DNA ladder marker.

The SSR primers are usually scored in expression of presence or absence of a band which can be qualified as a binary variable.

Table 2 : SSRs amplification product with primer RM585: molecular size (M. S) in base pair (bp) of bands and their presence (+), or absence (-). Rice genotypes: 1-Amber 33, 2-Amber Al-Baraka, 3- Amber Furat, 4-Amber Baghdad, 5-Amber Munatherha, 6-Sumar, 7-Dijlah, 8-Ghadeer, 9-Brnamge-4-, 10-Dorfak, 11-Gohar, 12-Khazar, 13-Shiroudi, 14-Neda, 15-Nemat.

M.s of bands in bp	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
224.99	**-**	**-**	**-**	**-**	**-**	**+**	**-**	**-**	**-**	**-**	-	**-**	-	-	-
216.064	-	-	**-**	**-**	**-**	**-**	**-**	**-**	-	**+**	-	**+**	-	**+**	-
200	**+**	-	**-**	**+**	**-**	**-**	-	**+**	**-**	**-**	**-**	**-**	+	-	-
191.094	-	**+**	**+**	**-**	-	**-**	**-**	**-**	**-**	**-**	**-**	**-**	-	**-**	-
182.287	+	-	-	+	**+**	**-**	**-**	**+**	+	**-**	**-**	**-**	-	-	**-**
162.138	-	-	**-**	-	**-**	-	+	**-**	**-**	**-**	+	**-**	-	-	+
No bands	**2**	**1**	**1**	**2**	**1**	**1**	**1**	**2**	**1**	**1**	**1**	**1**	**1**	**1**	**1**

Table 3 : SSRs amplification product with primer RM8085 : molecular size (M. S) in base pair (bp) of bands and their presence (+), or absence (-). Rice genotypes: 1-Amber 33, 2-Amber Al-Baraka, 3-Amber Furat, 4-Amber Baghdad, 5-Amber Munatherha, 6 Sumar, 7-Dijlah, 8-Ghadeer, 9-Brnamge 4-, 10-Dorfak, 11-Gohar, 12-Khazar, 13-Shiroudi, 14-Neda, 15-Nemat.

M.s of bands in bp	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
167.08	-	-	**-**	**-**	**-**	**-**	**-**	-	-	**-**	-	**+**	+	-	-
158.037	-	-	**-**	**-**	**-**	+	+	**-**	**+**	**+**	**+**	**-**	**-**	+	**+**
150	-	-	+	**-**	**+**	**-**	**-**	**+**	**-**	**-**	**-**	**-**	-	-	**-**
142.947	+	+	-	+	-	**-**	**-**	**-**	**-**	**-**	**-**	**-**	-	-	-
No bands	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**

Table 4 : Data of two SSR primers including: fragment size range (bp), no. of (amplified bands, Allele, monomorphic, polymorphic) Major Allele, Major Allele Frequency, Heterozygosity, Gene diversity, Polymorphism information content (PIC), primer efficiency (%).

Primer Efficiency (%)	Polymorphism information content (PIC)	Gene Diversity	Heterozygosity	Polymorphic band	mono-morphic band	Major. Allele. Frequency	Majo. Allel.	Allele No.	Size range (bp)	No. of amplified bands	Marker
54.54%	0.7888	0.8156	0.2000	6	12	0.2333	182.2 87	6	216.064 -162.138	18	RM585
45.45%	0.6358	0.6844	0.0000	0	15	0.4667	158.037	4	167. 08 - 142.947	15	RM8085
	0.7123	0.7500	0.1000			0.3500		5			Mean

Phylogenetic Tree

Phylogenetic tree was constructed to determine the genetic differentiation. The Un weighted pair-group method with arithmetic (UPGMA) was used affecting aconstant rate of development a dendrogram (Figure 4). Analysis of results obtained from Neighbor-joining dendrogram revealed that, separated grouped all the 15 rice genotypes arranged into two main groups at genetic distance with a value of 0.10 : one cluster A and cluster B. The major group A comprised 8 genotypes, while the cluster B comprised seven genotypes. Cluster A which further divided in to two subclusters, the first small one included (8 Ghadeer, 5 Amber Munatherha) and one individual (3 Amber Furat) while the other large one divided in to two subcluster one sub clust. contain 3 genotypes (4 Amber Baghdad,1 Amber 33) Local from Iraq and one individual (2 Amber Al-Baraka) Local from India, while two sub clust consist of 2 genotype (12 Khazar, 13 Shiroudi) Local from Iran. Cluster B Consist of seven which further divided in to equal sub group, included (6 Sumar, 9 Brnamge-4), (10 Dorfak,14 Neda) local from Iran and (7 Dijlah, 11 Gohar, 15 Nemat).

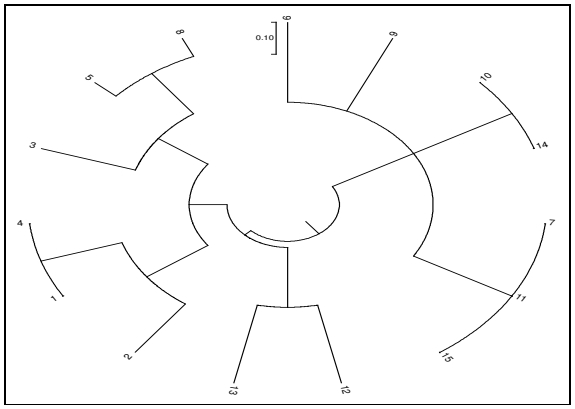

Fig. 4 : Un rooted Neighbor-joining tree illustrating genetic relationship tree among rice genotypes using Two SSR markers. Lanes: 1-Amber 33 , 2-Amber Al-Baraka, 3- Amber Furat, 4- Amber Baghdad , 5- Amber Munatherha, 6-Sumar, 7- Dijlah, 8- Ghadeer, 9-Brnamge-4-, 10- Dorfak, 11- Gohar, 12- Khazar, 13-Shiroudi, 14-Neda, 15-Nemat

Results indicate moderate relationship between genetic divergence and geographic origin of accessions, their ancestor (Mulato *et al.,* 2010). This may due to effect of environment since they came from two different or similar collection sites, which presented that SSR molecular markers force be potential to recognize germplasm in rice genotypes (Kanawapee *et al*., 2011). Who announced relatively high level of similarity between closely linked genotypes.

In conclusion, Genetic variability is the key determinant for any breeding program. Present study detects that PCR based fingerprinting technique, SSR were informational for assessing the range genetic diversity as well as to separate the genetic relationship between various species of *Oryza sativa*. However, the data polymorphism level was sufficiently determined and informational by fingerprints. In such a phenomenon our research doing on significant rice genotypes in genetic diversity detected through two markers. In future our research work is perhaps helpful for inexpensive and better SSR analysis of new genetic species diversity.

In general the current study gives evidence that backing the SSR analysis in separating variability with in a population moreover, it can be pattern for other studies regarding to genetic diversity .The tested RM 8085 and RM585 primers in the rice genotypes could be effectively used to recognize salt and drought tolerant lines and could also be applied in marker support breeding, quantitative trait loci (QTL) mapping.

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