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Molecular Characterization of Wheat (*Triticum* sp.) Genotypes Grown in Humid South Eastern Plain Zone of Rajasthan

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A B S T R A C T

Wheat is qualitatively a vital source of macromolecule, energy and fiber for human nutrition since decades hence, used as a staple food grain for community and as well a major source of fodder for animal feeding. Assessment of genetic diversity using molecular markers is used for characterization of different genotypes with reliable and authentic results. RAPD is a PCR based molecular technique for identification of genetic variability in similar genotypes. It usually preferred for the initiation of this kind of work as this technique is simple, versatile and relatively inexpensive. 35 amplified bands were obtained using 6 RAPD primers, in which (54.29%) were polymorphic and (45.71%) was monomorphic. Total amplified bands varied in between 5 to 7 with an average of 5.83 bands/primer. Average PIC value was 0.143 with ranging from 0.036 to 0.296. The lowest and the highest PIC value were recorded for primer OPA 14 and OPA 05, respectively. UPGMA cluster constructed from RAPD analysis clubbed the 10 wheat genotypes into three major clusters I, II and III and I was further divided in sub clusters IA and IB. The eager knowledge of genetic diversity is used to diagnose genetic programme and beneficial for future crop improvement.

Introduction

Wheat is regarded as the "*King of Cereals*" as it provides more nutrients for human consumption than any other single source of food. Over two dozen species have been characterized as a member of genus "*Triticum*". Of these, generally 2 or 3 are cultivated in the country. Molecular markers are used for characterization in various plant species with reliable and authentic results (Behera *et al.*, 2008; Chandrika and Rai, 2009). The development of different kind of techniques like molecular and biochemical helps not only to detecting genotypes, but also in assessing and exploiting the genetic diversity (Whitkus *et al.*, 1994). In addition, wheat germplasm characterization using molecular markers will contribute the knowledge of genetic relatedness between varieties and hence facilitate the breeding of wheat genotypes according to the commercial purpose and respond to diverse biotic (*e.g.*, disease, insect, pathogens) and abiotic (*e.g.*,

drought, heat, salinity) challenges. Molecular marker is a powerful tool to recognize variability of DNA sequences among genotypes and thus, directly avoid problems related with environmental effects. Knowledge about genetic diversity and relationship among the germplasm and potential merit of genetic diversity would be beneficial for crop improvement. The study of the DNA, obtained by different techniques but the utmost regularly used technique is RAPD (Random Amplified Polymorphic DNA). It's a simple, reliable and effective method for detecting polymorphism in wheat (Vierling and Nguyen, 1992). There are some benefits of RAPD in genetic analysis *viz.* it needs small quantity of DNA, short primers of arbitrary sequences, easy, quick and most beneficial is price effective (Welsh J. and McClelland M., 1990). Significant achievement has been made in some years in the area of molecular markers technique for plant genetic resources characterization and evaluation (Soltis *et al.*, 1992). Nucleic acids estimation is a complex procedure in plants genotypes because polyphenols and secondary metabolites, interfere with DNA isolation and some reactions *i.e.* DNA restriction, cloning and amplification (Sghaier and Mohammed, 2005). Besides this contaminated RNA that precipitates with the DNA may causes various problems including interference with DNA amplification with random primers, *i.e.* RAPD process (Mejjad *et al.,* 1994) and through thermal cycle sequencing improper priming of DNA templates. Thus, an effective protocol for isolation of DNA and for improvement of the PCR process is essentially needed. This type of data is effective for germplasm conservation, individual, population, genotype selection, identification and genetic improvement (Duran *et al.,* 2009). Genetic diversity assessment can increase the effectiveness of breeding programs (Fan *et al.,* 2006).

In the present investigation, a detailed study was performed to evaluate the characterization of wheat genotypes done by genetical assessment through RAPD markers. It's aims to analyze the extent of genetic diversity, using with total 6 RAPD primers, to generate DNA fingerprints of 10 *Triticum* species genotypes with a view to detect polymorphism and access information on diversity among studied genotypes.

Materials and Methods

The experiment was conducted at Dhakarkheri village, Kaithoon road, Kota (Rajasthan) situated in between 25^{o} 11' N latitude and 75^{o} 54' E longitudes at 273 m altitude from mean sea level, during two consecutive *rabi* season 2015-16 and 2016-17. In this study seeds of 2 wheat species 10 genotypes, in which 5 genotypes each of species *Triticum aestivum* L. (Raj 4037, Raj 4238, GW 322, GW 366, HI 1544) and *Triticum durum* desf. (Raj 6560, MPO 1215, HI 8498, HI 8737, HD 4728) was sown in Randomized Block Design with three replications. The genotypes centre of origin is depicted in Table 1.

Genomic DNA isolation and purification (Doyle and Doyle, 1990)

DNA extracted from various wheat genotypes were compared using RAPD techniques. The DNA was extracted from fresh tender young leaves of 2-3 weeks using modified CTAB (Cetyl Trimethyl Ammonium Bromide) extraction method as described by Doyle J. J. and Doyle J. L., (1990). DNA was amplified by using character specific oligonucliotide primer in a DNA thermal cycler (Eppendorf). Later, the amplified samples were electriphoreshed in 1.5% agarose gel. The studies of bands were scored (1 or 0) as their presence or absence.

Reagents

I CTAB extraction buffer (100 ml)

CTAB	:	2 % (w/v)
Tris HCl (pH 8.0)	:	100 mM
Sodium chloride	:	1.4 M
EDTA (disodium, pH 8.0)	:	20 mM
β-mercaptoethanol	:	2 %

(Autoclaved Tris, NaCl and EDTA, CTAB should be added after autoclaving process and extraction buffer should be preheated before utilization)

II TE buffer

Tris- HCl (pH 8.0)	:	10 mM
EDTA (pH 8.0)	:	1 mM

(Dissolved and made up to 100 ml with distilled water, autoclaved and kept at 4^{o}C).

Sodium acetate (3.0 M) pH 5.2 (Adjust p^{H} with glacial acetic acid).
Chloroform: Isoamyl alcohol (24: 1 v/v).
Ice cold Isopropanol and Ethanol 70%.
RNase A 20 mg/ml; Dissolve RNase A in TE and boil it for 15 minutes at 100°C to destroy DNase and store at (–20°C) temperature.

III Protocol

1 gm sample of wheat young leaves (leaf bites) were transferred into a prechilled mortar, frozen using liquid nitrogen, immersed leaves for 30 min and ground to fine powder.
The fine powder was allowed to thaw in the availability of 10 ml of pre-heated extraction buffer and incubated for 30-45 min at 65^{o}C with randomly mixing.
An equal volume of chloroform: isoamyl alcohol mixture (24:1 v/v) was added and mixed by inversion for 1 hrs.
This was followed by centrifugation at 15,000 rpm for 10 min at room temperature.
The aqueous phase was then transferred in another sterile tube and an equal volume of ice cold isopropanol was added softly and mixed gently by inversion and store it in the freezer until DNA is precipitated out.
Using blunt end tips, the precipitated DNA was spooled out into an eppendorf tube.
The spooled DNA was then air dried after removing the supernatant by a brief spin.
500 µl of TE was added to dissolve the DNA followed by addition of 10 µl of RNase and incubated at 37^{o}C for ½ hrs.
Mixture of 500 µl of chloroform: isoamyl alcohol was added and centrifuged for 10 min. The aqueous phase was transferred to another eppendorf tube without disturbing the inner phase.
A 2.5 ml volume of absolute alcohol and $^{1}/_{10}{}^{th}$ volume of sodium acetate was now added and store for incubation overnight.
On next day, the precipitate obtained was centrifuged and the supernatant material was discarded.
Then 500 µl of 70% ethanol were added subsequently to wash the DNA by centrifugation.
The alcohol was discarded and water residue air dried from the DNA pellet.
The DNA pellet was dissolved in 150-250 µl of TE (depending on the pellet size) and stored at 4°C temperature.

IV Purification of DNA

The major contaminants in crude DNA preparation are RNA, protein and polysaccharides. Inclusion of CTAB in DNA extraction buffer helps in elimination of polysaccharides from DNA preparations to an oversized extent. The RNA was removed by treating with DNase free RNase. Protein as well as RNase was removed by treating the sample with phenol: chloroform (1:1) and chloroform: isoamyl alcohol (24:1), subsequently.

The purification process was carried out in the following steps:
RNase (50 µg/ml) was added to crude DNA preparation and incubated at 37°C for 60 min.
After 60 min a mixture of chloroform: isoamyl alcohol in the ratio of 24:1 (v/v) was added and mixed thoroughly for 15 minutes to form an emulsion.
Centrifuged the tubes for 15 min at 15000 rpm and supernatant was collected in another tube by avoiding the whitish layer of interphase.
The DNA was precipitated by mixing double amount of ethanol.
The solution was centrifuged at 10,000 rpm for 15 min. The pellet was settled down and washed with 70% alcohol and dried for overnight.
The DNA was dissolved in 200 µl of TE buffer and stored at (-20ºC) temperature.

Gel analysis

The Integrity of DNA was determining by gel analysis in following steps:
Cast agarose gel (0.8%) 150 ml in 1N TBE (Tris Borate EDTA) buffer containing (0.5 gm/ml) of Ethidium Bromide (EtBr) and load 2 µl of DNA sample.
Load a known amount of uncut Lambda phage DNA as control in the adjacent well and run the gel at 50 V for 60 min and visualize gel under UV light.
Presence of single compact band at the corresponding band of phage DNA indicates high molecular weight of isolated DNA.

Quantification of DNA

DNA quantification was done by observing it at various wavelengths *i.e.* 260 nm and 280 nm by using a spectrophotometer in following steps:
Take a 3 ml TE buffer in a cuevette and calibrate the spectrophotometer at different wavelengths.
Add a 3 µl of DNA, mix properly and record the optical density (OD) at 260 and 280 nm.
Note the quantity of DNA from the ratio of OD value recorded at 260 and 280 nm.

Estimate the DNA concentration with the following formula:

Amount of DNA (g/ l) =

$$\frac{\text{(OD) 260 x 50 x dilution factor}}{1000}$$

Dilution of DNA for PCR

Quantity of DNA was diluted for final concentration of 50 ng/l using TE buffer (10 mM Tris HCl, 1mM EDTA, pH 8.0). The details of PCR reaction mixture is depicted in Table 2.

Details of RAPD primers (Williams *et al.*, 1990)

A total of half dozen decanucleotide RAPD primers were used for the PCR amplification. The sequences of these primers were selected from literature and synthesized from Bangalore Genei Pvt. Ltd., Bangalore (India). The detailed list of primer code sequence and G: C contents are delineated in table 3.

Polymerase Chain Reaction (PCR) amplification

PCR amplification was carried out in programmable thermal cycler from Eppendorf AG, Germany. PCR reaction for RAPD was carried out in 20 µl of reaction mixer containing 25 ng genomic DNA, 2 µl of 10X *Taq* DNA polymerase buffer, 1.5 mM MgCl2, 200 µM each dNTPs, 0.5 µM of primer and 1 unit of *Taq* DNA polymerase in an 200 µl eppendorf. The following procedures were used for PCR amplification: PCR condition

for RAPD analysis included an initial process with a hot start of 94^{o}C for 4 min followed by 40 cycles of denaturation at 94^{o}C for 1 min, annealing process was performed at 40^{o}C for 1 min and final extension was carried out at 72^{o}C for 2 minutes and a hold temperature of 4^{o}C at the end.

Allele scoring (Asif *et al.*, 2008)

After completion of PCR amplification, the PCR products of RAPD were loaded on 1.5% agarose gel. Agarose gel was prepared in 1X TAE buffer containing ethidium bromide (10 mg/ml) concentration of 3µl/100ml. Amplified PCR products were mixed with 2 µl of 6x gels loading dye and loaded in the wells of gel. Electrophoresis was carried out at a constant voltage (5 V/cm of gel) till bromophenol blue/loading dye migrated to other end of the gel. The gel was visualized under UV-transilluminator and take photographed using the gel documentation.

Analysis of amplified profiles

Amplified fragments were scored as '1' for the presence of band and '0' for the absence of band, generating the 0 and 1 matrix and the percent polymorphism was calculated by using the given formula. Only neat and clear bands were scored.

Per cent polymorphism (%) =

$$\frac{\text{Number of polymorphic bands} \times 100}{\text{Total number of bands}}$$

Data generated from molecular analysis (Jaccard, 1908)

The scores (0 or 1) were entered in the form of a rectangular data matrix (qualitative data matrix) for each band acquired from UV photographs.

Pair-wise association for coefficients were may be calculated from the qualitative data matrix with using Jaccard's similarity coefficient. The equation for calculating the Jaccard's similarity coefficients 'F' between two samples A and B is as under:

$f = nxy / (n1 - nz)$

nxy = Number of bands common for sample A and sample B

n1 = Total number of bands available in all samples.

nz = Number of bands absent in sample A or B but present in other samples.

The genetic relatedness for analysis of clusters with using UPGMA, for dendrogram, delineated the relationships of the genotypes with computer programme NTSYS-pc ver. 2.02 (Rohlf, 2000).

2 and 3 dimensional principal component analysis (PCA) was created to provide another means for testing the relationship in studied genotypes with using the developed EIGEN programme (NTSYS-pc).

Polymorphism Information Content (PIC) values calculation (Smith *et al.*, 1997)

To analyze the information of marker system RAPD, the Polymorphic Information Content of marker was calculated according to given formula:

$$PIC = \sum_{i=1}^{n} 1 - pi^2$$

Where,

N = total number of allele find out for a locus of a marker.
Pi = frequency of the 1st allele.

Results and Discussion

DNA isolation, purification and quantification

In this study, the modified method given by (Doyle J. J. and Doyle J. L., 1990) used for DNA isolation. The amount of DNA isolated from various genotypes of *Triticum* species ranged from 1180 to 1880 ng/µl (Table 4). The genotype Raj 4238 yielded the highest amount of DNA (1880 ng/µl) whereas, the lowest amount of DNA (1180 ng/µl) was obtained from genotype MPO 1215.

The ratio of absorbance (A260/A280) ranged from 1.63 to 1.87 revealed that the DNA obtained from sample was free from contaminants like polysaccharides, protein and RNA. Concentrated DNA strongly influences the outcome of the reaction as the quality and quantity of DNA strongly affects the success of PCR (Rahman *et al.,* 2000; Ahmed *et al.,* 2009; Khamassi *et al.,* 2011).

Polymorphism in *Triticum* species using RAPD primers

In the present investigation, 6 RAPD primers having 60% G:C content each were used. A total of 35 amplified bands were obtained of which showed range of (20.00% to 83.33%) polymorphism. The DNA amplicon size and polymorphism generated among various genotypes of *Triticum* species using RAPD primers are presented in Table 5. Total number of bands determined for each primer was noted separately and polymorphic bands checked subsequently.

Total amplified bands varied between 5 (primer OPA-7 and 14) to 7 (primer OPA-03) with a mean of 5.83 bands for each primer. The percent polymorphism was 54.29% for all the studied genotypes. The average PIC value was 0.143 ranging from 0.036 to 0.296. The lowest and the highest PIC value were recorded for primer OPA 14 and OPA 05, respectively.

The UPGMA (Unweighted Pair Group Method with Arithmetic Mean) cluster constructed from RAPD analysis clubbed the 10 wheat cultivars into three major clusters *i.e.* I, II and III (Fig. 1) and similarity indices estimated on the basis of all the 6 primers ranged from 0.74 to 1.00. The Ist big cluster was further bifurcate into two sub-clusters *i.e.* (IA and IB).

The first sub-cluster (IA) was found to be consisted of four genotypes with maximum similarity of (93%) between GW 322 and GW 366, both are *aestivum* genotypes. The second sub-cluster (IB) also included four genotypes, in this maximum similarity of 100 % found between Raj 6560 and HI 8737, both is *durum* genotypes. The second major cluster has only one HI 8498 wheat genotype that showed 79% distances to cluster-I, The third major cluster also have only one MPO 1215 wheat genotype that showed 74% proximal distance with other clusters.

The genetic diversity assessment of accessions is necessary to help the breeders in finding the genetic diversity, germplasm management and protection (Lee, 1995). The genetic diversity in field crops as well as for germplasm is very useful and necessary for promoting of breeding programme.

For proofing of different genotypes, classification of several varieties and diversity estimation in crops like Green Gram, (Karuppanapandian *et al.,* 2006) and Black Gram (Karuppanapandian *et al.,* 2007), RAPD marker technique has been extensively used (Fig. 1–3 and Table 6).

Table.1 Wheat genotypes centre of origin

S. No.	Name of Variety	Centre of Origin	Year of Release	Pedigree	Description of genotypes
1.	Raj 4037	RAU, Durgapura (Rajasthan)	2003	DL788-2/RAJ3717	IR, TS
2.	Raj 4238	RAU, Durgapura (Rajasthan)	2016	HW2021/RAJ3765	IR, LS
3.	GW 322	JAU, Junagarh (Gujarat)	2002	GW173/GW196	IR, TS
4.	GW 366	JAU, Junagarh (Gujarat)	2006	DL802-3/GW232	IR, TS
5.	HI 1544	IARI, Indore (M.P.)	2008	HINDI62/BOBWHITE/CPAN2099	IR, TS
6.	Raj 6560	RAU, Durgapura (Rajasthan)	2005	TOPDY6	IR, TS
7.	MPO 1215	JNKVV, Powarkhera (M.P.)	2009	GW1113/GW1114//HI8381	IR, TS
8.	HI 8498	IARI, Indore (M.P.)	1999	RAJ6070/RAJ911	IR, TS
9.	HI 8737	IARI, Indore (M.P.)	2014	HI8177/HI8158//HI8498	IR, TS
10.	HD 4728	IARI, New Delhi	2016	ALTAR84/STINT//SILVER_45/3/ SOMAT_3.1/4/GREEN_14//YAV _10/AUK	IR, TS

Source: Agricultural Research Station, Ummedganj, Kota (Raj.)

Table.2 PCR reaction mixture content

S. No.	Components	Final Concentration	Single tube (20 µl)
I	DNA templates	50ng	1.00 µl
II	Master mixture		
	1. dNTP mix	200 µM	1.60 µl
	2. Taq DNA polymerase	1U	0.33 µl
	3. Reaction buffer (10X)	1X	2.00 µl
	4. Primer	0.5 µM	2.00 µl
	5. DD H_2O		13.07 µl

Table.3 Details of RAPD primers used in molecular analysis of *Triticum* sp. Genotypes

S. No.	Primer Code*	Sequence (5'- 3')	G:C Content (%)
1.	OPA-03	AGTCAGCCAC	60
2.	OPA-05	AGGGGTCTTG	60
3.	OPA-07	GAAACGGGTG	60
4.	OPA-10	GTGATCGCAG	60
5.	OPA-12	TCGGCGATAG	60
6.	OPA-14	TCTGTGCTGG	60

* Operon series code

Table.4 Quality and quantity of genomic DNA isolated from 10 genotypes of Triticum species

S. No.	Wheat genotypes	Quality (A260 / A280)	Quantity (ng/µl)
1.	Raj 4037	1.87	1680
2.	Raj 4238	1.85	1880
3.	GW 322	1.83	1870
4.	GW 366	1.73	1873
5.	HI 1544	1.84	1789
6.	Raj 6560	1.77	1350
7.	MPO 1215	1.83	1180
8.	HI 8498	1.82	1670
9.	HI 8737	1.63	1355
10.	HD 4728	1.87	1390

Table.5 DNA amplification profile and polymorphism generated in Triticum species by six RAPD primers

S. No.	Primer	Sequence (5'-3')	No. of Scorable bands	No. of polymorphic bands	No. of Monomorphic bands	Polymorphism (%)	PIC* Value
1.	OPA-03	AGTCAGCCAC	7	2	5	28.57%	0.105
2.	OPA-05	AGGGGTCTTG	6	5	1	83.33%	0.296
3.	OPA-07	GAAACGGGTG	5	3	2	60.00%	0.108
4.	OPA-10	GTGATCGCAG	6	3	3	50.00%	0.140
5.	OPA-12	TCGGCGATAG	6	5	1	83.33%	0.173
6.	OPA-14	TCTGTGCTGG	5	1	4	20.00%	0.036
		Total	**35**	**19**	**16**	**54.29±**	**0.143**

*Polymorphic Information Content

Table.6 Jaccards similarity coefficient for RAPD techniques

Genotypes	Raj 4037	Raj 4238	GW 322	GW 366	HI 1544	Raj 6560	MPO 1215	HI 8498	HI 8737	HD 4728
Raj 4037	1.00									
Raj 4238	0.84	1.00								
GW 322	0.90	0.82	1.00							
GW 366	0.90	0.82	0.93	1.00						
HI 1544	0.90	0.76	0.93	0.87	1.00					
Raj 6560	0.84	0.88	0.87	0.87	0.81	1.00				
MPO 1215	0.67	0.71	0.70	0.70	0.64	0.81	1.00			
HI 8498	0.78	0.76	0.75	0.75	0.75	0.87	0.75	1.00		
HI 8737	0.84	0.88	0.87	0.87	0.81	1.00	0.81	0.87	1.00	
HD 4728	0.82	0.85	0.85	0.85	0.79	0.91	0.79	0.79	0.91	1.00

Fig.1 UPGMA dendogram showing the relationships among 10 wheat genotypes

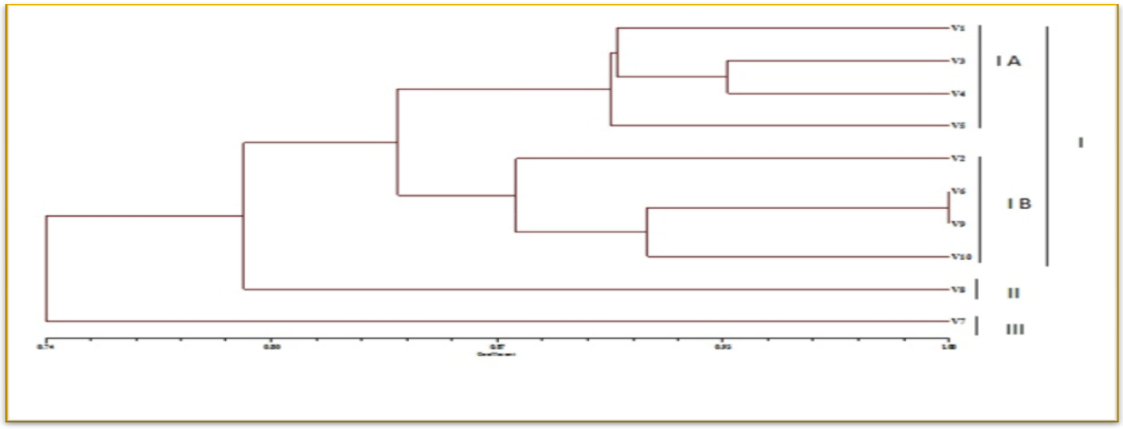


Fig.2 3D Analysis of 10 different wheat genotypes by using RAPD

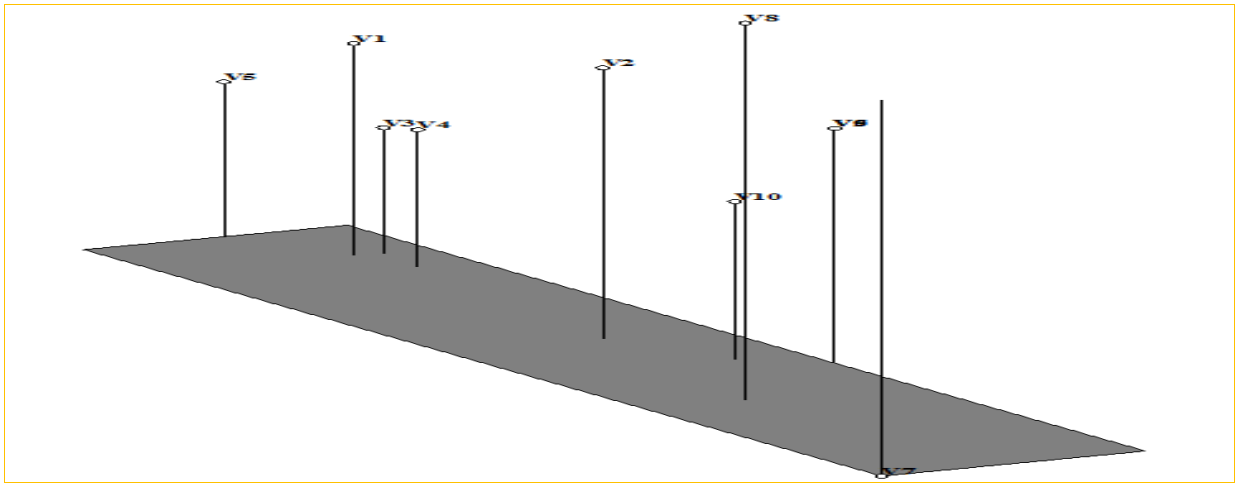
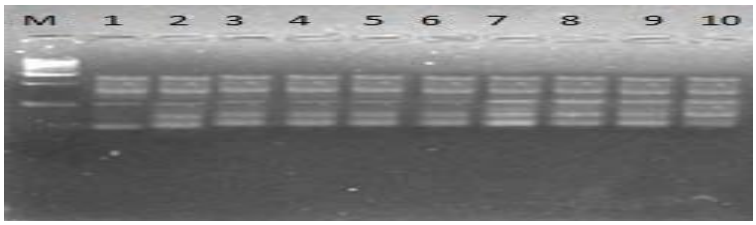


Fig.3 M: Marker, 1- Raj 4037, 2- Raj 4238, 3- GW 322, 4- GW 366, 5- HI 1544, 6- Raj 6560, 7- MPO 1215, 8- HI 8498, 9- HI 8737, 10- HD 4728



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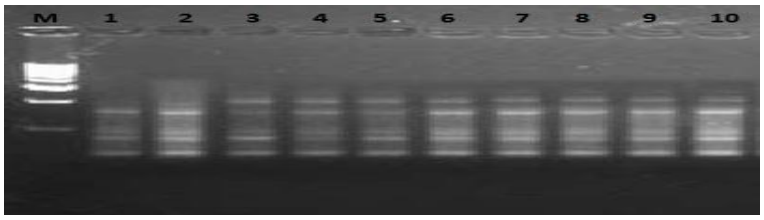

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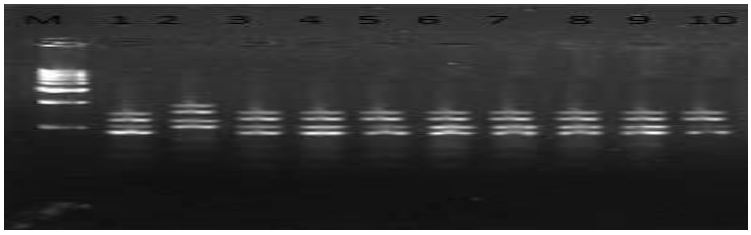

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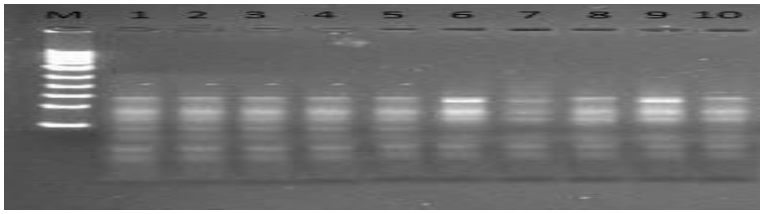
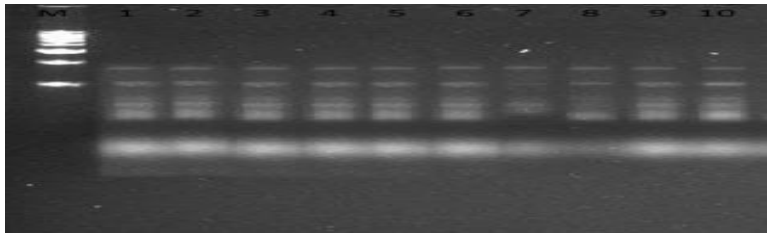
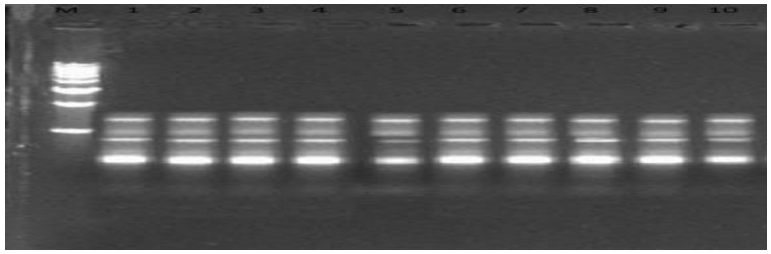


OPA 10



OPA 12



OPA 14

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