International Journal of Applied And Pure Science and Agriculture
www.ijapsa.com

ASSESSMENT OF GENETIC DIVERSITY AMONG MUSTARD (*Brassica juncea* (L.) Czern & Coss) GENOTYPES USING PCR BASED DNA MARKERS

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Abstract

Mustard [Brassica juncea (L.), 2n=36] is the world's third most important source of edible oil. An experiment was conducted to study molecular diversity of mustard Brassica juncea (L.) through ISSR primers. The present investigation was undertaken to explore the diversity among Indian mustard genotypes (varieties) using molecular markers. To determine the discriminatory power of the RAPD primers, polymorphism percentage, PIC were calculated. A total of 109 bands were obtained from the ten ISSR primers, out of which 78 showed polymorphism of 71.56% with an average of 7.8 bands per primer. The PIC ranged from 0.75 to 0.993 and genetic similarity varied from 0.478 to 1.000. The cluster analysis revealed two main clusters with highest 58% similarity percentage. The phylogenetic tree constructed by UPGMA method generated two main clusters. Cluster II contains one solitary genotype (Rohini). While cluster II had rest of the genotypes which grouped together in their respective subcluster. Ten ISSR primers selected in the present study gave 71.56% polymorphism. The highest polymorphism percentage was observed with one primer A17898. Therefore, this ISSR primer can be used further for diversity study in different mustard genotypes.

Key words: Indian mustard; ISSR; Polymorphism; Genetic diversity; PIC

I. Introduction

Brassica juncea commonly known as Indian mustard is an amphidiploid species that originated through the interspecific hybridization of Brassica rapa and Brassica nigra. Oilseed *Brassicas* also referred to as rapeseed-mustard, an important group of oilseed crops in the world, comprise eight cultivated crops of tribe Brassiceae within the family Brassicaceae. They also contain a large number of novel phyto-chemicals, some of which provide protection against carcinogenesis. Hence, *Brassicas* are believed to be useful in the prevention of cancer. The *Brassica* genus comprised of over 150 species and somatic chromosome numbers (2n) of 16,18,20,34, 36 and 48 have been reported in different species. The leaves of young plants are used as green vegetables. Green stem and leaves are a good source of green fodder for cattle. Mustard cake contains about 38 to 42 per cent protein (Nagraj, 1995). In recent years, a number of monographs and reviews (Scarisbrick and Daniels, 1986; Downey and Robblen, 1989) has dealt in detail with many aspect of oilseed improvement.

DNA markers have also been widely used to map agronomically important genes in *Brassica* genomes and to assist mustard breeding and selection procedures. The majority of the work utilizing molecular markers in *Brassica* oilseed breeding has, to date, been based on genetic mapping using various DNA marker systems, in single segregating populations generated for specific investigations

of particular traits of interest. (Hemingway *et al*, 1976) In the present investigation, we analyzed 23 B. juncea L. varieties cultivated in Northern states of India using PCR based markers to examine the efficiency of these techniques viz-a-viz genetic diversity.(Kumar *et al* 2011)

II. Materials and Methods

2.1 Seed materials

The seeds of 20 mustard genotypes used in the present study were collected from the Directoriate of Rapeseed and Mustard, Bharatpur, Rajasthan.

Table 1: List of mustard genotypes used in the present study

No.	Name of genotypes	No.	Name of genotypes
1)	Rohini	11)	Krishna
2)	Sarama	12)	PM 67
3)	Kranti	13)	NDRE 4
4)	NDR 8501	14)	Sanjucta Asech
5)	GM 2	15)	Urvashi
6)	Jagannath	16)	TM 4
7)	PusaAgrani	17)	PBR 97
8)	Saurabh	18)	Varuna
9)	Bhagirathi	19)	RH 30
10)	JM 1	20)	NRCDR 02

2.2 DNA EXTRACTION

Total genomic DNA extraction was carried out by CTAB method as described by Doyle and Doyle (1987) with minor modifications.

Quantification of DNA

The 1.5 µl of DNA sample was loaded into the sample spot of Nanodrop Spectrophotometer (Thermo Scientific, U.S.A.) and the concentration of DNA and absorbance at 260 nm and 280 nm were measured. The A_{260}/A_{280} ratio was automatically calculated by the software. The quantitative DNA was diluted to final concentration of 100 ng/µl in TE buffer.

PCR BASED MOLECULAR MARKERS

For fingerprinting of mustard genotypes, three PCR based molecular marker techniques *viz*., Randomoly Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeat (ISSR) and SimpIe Sequence Repeat (SSR) were used.Chemicals and primers used for molecular studies were received from Merck Bioscience Pvt. Ltd.,Bangalore, Karnataka.

(i) PCR components

The following reagents were used for ISSR -PCR amplification of DNA.

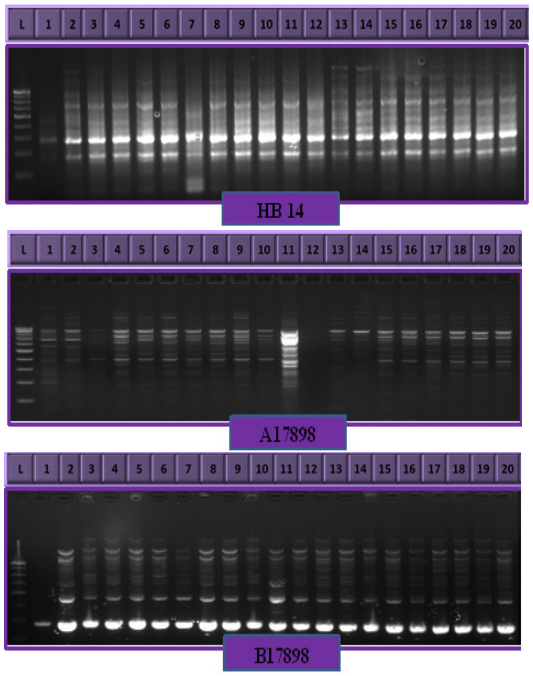
(a) PCR buffer

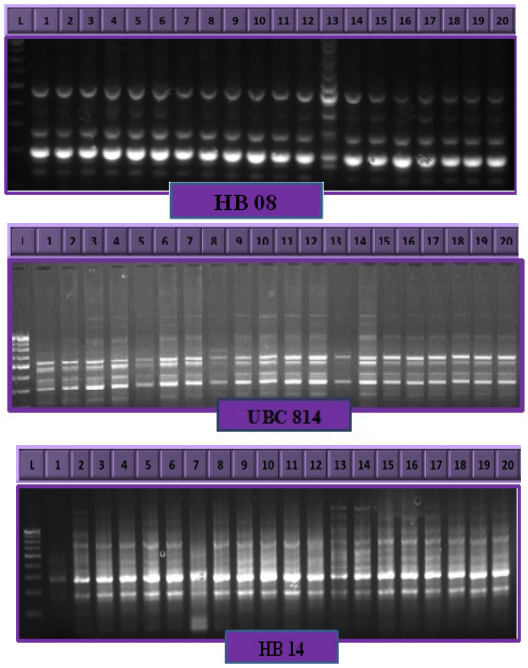
(b) Taq DNA polymerase (3U/µl)
(c) dNTPs mix (dATP, dCTP, dGTP and dTTP)
(d) Primer (25 pMoles/µl)
(e) $MgCl_2$
(f) Template DNA
(g) Millipore sterile distilled water

2.3Electrophoresis of amplified product

The amplified products of ISSR were analyzed using 1.5% agarose gel. The procedure was same as described in RAPD.

Sr. No.	Step	Temperature (ºC)	Duration
1	Initial Denaturation	94	5.0 min
2	Denaturation	94	1 sec
3	Annealing	55	45 sec
4	Extension	72	1.3 min
Repeat the steps 2 to 4 for 36 times			
5	Final Extension	72	7.0 min
6	Hold	4	∞





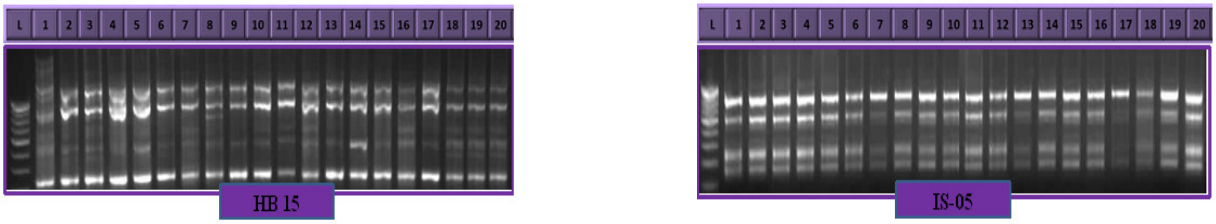


Fig.1: images of band obtain by ISSR marker

2.4 STATISTICAL ANALYSIS

In order to score and preserve banding pattern, photograph of the gel was taken in a Gel Documentation System, under UV trans-illuminator., ISSR bands are designated on the basis of their molecular size (length of polynucleotide amplified). Low range DNA ruler up to 3 kb was also loaded simultaneously with primer products in the gel to estimate the molecular size. The distance run by amplified fragments from the well was translated to molecular size with reference to molecular weight of marker. The presence of each band was scored as '1' and its absence as '0'. Faintly visible bands were not scored.

Clear and distinct bands amplified by ISSR primers are scored for the presence (1) and absence (0) for the corresponding band among the genotypes. The data were entered in to MS-Excel data sheet and subsequently analyzed using NTSYS-pc version 2.02 (Rohlf, 2000).

The data matrix was read by NTSYS-pc version 2.02 (Numerical Taxonomy and Multivariate Analysis System for Personal Computers, Exeter Software) developed by Rohlf (2000) and analyzed by the SIMQUAL (similarity for qualitative data) program with Jaccard's similarity coefficient. The SIMQUAL is a program for computing a variety of similarity and dissimilarity coefficients for qualitative data. The qualitative nature of the absence (0) or presence (1) state of marker was used as the basis for similarity analysis among various mustard genotypes. A matrix of 0 and 1 act as the input and the output is a matrix of similarity or dissimilarity coefficients. The resultant similarity matrix was entered into SAHN (sequential, agglomerative, hierarchical, and nested clustering method) clustering program, a tree matrix was produced and a dendrogram was constructed using UPGMA (unweighted pair-group method with arithmetic averages). The assumption underlying the use of UPGMA clustering is the equal rate of evolution along all the dendrogram branches. The dendrogram of publication quality were produced from the output tree file of SAHN by TREE (tree display) program in graphics mode.

Clustering methods create clusters of the data, no matter whether there are true clusters in the data or not, so a check was made for the existence of true clusters. This was done by using the tree matrix produced by SAHN to calculate the cophenetic values of similarity or dissimilarity by the program COPH (cophenetic values). The cophenetic value matrix was compared with the original tree matrix for goodness of fit of the cluster analysis to the data. This type of cophenetic correlation was done by the MXCOMP (matrix comparison) program (Rohlf, 2000). The program MXCOMP plots the cophenetic value matrix against the original tree matrix, and computes the Cophenetic correlation coefficient (r) and the Mantel test statistic (Z).

2.5 The test criterion of Mantel test

The mantel test statistic (Z) was calculated as follows:

$$Z = \sum_{i<j}^{n} X_{ij} Y_{ij}$$

Where,

X_{ij} = Off-diagonal elements of cophenetic value matrix

Y_{ij} = Off-diagonal elements of original tree matrix

n = Number of elements of the matrixes

As the cophenetic correlation coefficient is positively correlated to the Mantel test statistic and in standardized units, it is easier to use it as a measure of goodness of fit for a cluster analysis than the Mantel test statistic. The degree of fit can be referred as follows (Rohlf, 2000).

Calculation polymorphic information content (PIC)

The PIC value for each locus was calculated on the basis of allele frequency (Anderson *et al.,* 1993) as under:

$$PIC_i = 1 - \sum_{j=1}^{n} P_{ij}^2$$

Where P_{ij} is the frequency of j^{th} allele for i^{th} marker and summation extends over n alleles.

III. Results and Discussions

Similarity index and cluster analysis was done by Jaccard's coefficient and UPGMA, respectively using NTSYSpc-2.02i software. The dendrogram was generated by using Jaccard's coefficient values to estimate the genetic similarity among 20 mustard genotypes. The similarity coefficient values obtained by Jaccard's coefficient were shown in Table 4.5. Jaccard's coefficient of similarity between 20 mustard genotypes ranged from 47.8 % to 100 %. Kumar *et al.* (2011) also estimated Jaccard's similarity coefficients and constructed dendrogram by using UPGMA revealed the presence and extent of genetic similarities among mutants ranged from 0.54 to 0.91. Similarly, Yadav and Rana (2012) recorded that genetic similarity between 30 Indian mustard genotypes was in the range of 0.50 to 1.0, which supported the findings obtained in the present study.

Twenty mustard genotypes were grouped into two main clusters I and II with an average similarity of 55 % The cluster I consisted of solitary genotype of Rohini. The cluster II consisted of nineteen genotypes and was divided into two sub cluster IIA and IIB. The sub cluster IIA consisted of eighteen genotypes and again further divided into two sub clusters IIAi and IIAii. The subcluster IIAi was grouped into seventeen genotype *viz.,* Sarama, NDR 8501, Jagannath, Bhagirathi, Saurabh, JM 1, GM 2, Sanjucta Asech, Urvashi, PBR 97, TM 4, Varuna, NRCDR 02, RH 30, Kranti, Krishna and NDRE 4. Varuna and NRCDR 02 shared similarity of 100 %, Likewise, Urvashi and TM 4 shared similarity of 92 %; Jagannath and Bhagirathi (91 %); NDR 8501 and JM 1 (88 %); Sanjucta Asech and RH 30 (85 %); Sarama and GM 2 (84 %). The subcluster IIAi consisted of single genotype of Pusa Agrani. The subcluster IIB also consisted of single genotype of PM 67. The cluster analysis showed the highest (100 %) similarity between the genotypes Varuna and NRCDR 02. while lowest (47.8 %) similarity between NDRE 4 a

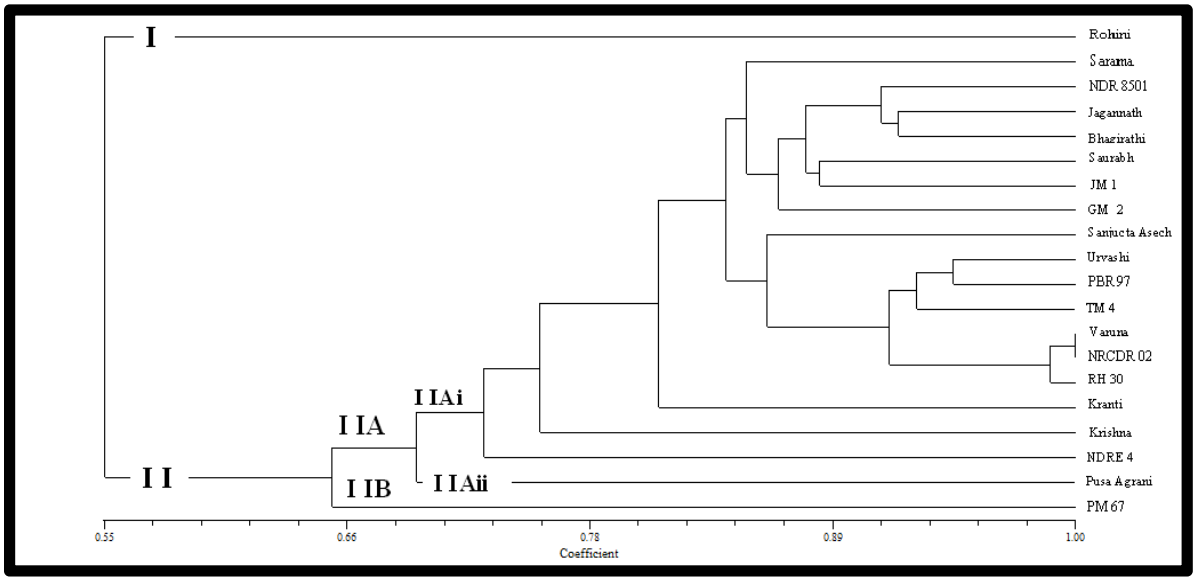


Fig.2: Dendrogram depicting the genetic relationship among 20 mustard genotypes based on the ISSR data

IV. Conclusion

In present study, the genetic diversity observed at molecular level in twenty mustard genotrypes were studied using RAPD, ISSR and SSR markers. Total genomic DNA was isolated from leaf tissues of different mustard genotypes grown in pots. DNA extraction was carried out by CTAB method as described by Doyle and Doyle (1987) with necessary modifications. In order to perform PCR based analysis, the DNA was quantified in NanoDrop Spectrophotometer to determine DNA concentration by calculating the absorbance ratio at A_{260}/A_{280} and it was ranged from 823.80 ng/µl to 4694.00 ng/µl with optical density near about 1.80 to 1.90 indicating that genomic DNA extracted were very pure in all the 20 genotypes.

A total of 109 bands were obtained from the ten ISSR primers out of which 78 bands were polymorphic with an average of 7.8 bands per primer. Total 31 bands were monomorphic, while remaining 15 were unique. The average ISSR polymorphism was 71.56%. The polymorphic information content (PIC) ranged from 0.75 to 0.993 with an average of 0.862 per primer. The phylogenetic tree constructed by UPGMA method generated two main clusters. Cluster II contains one solitary genotype (Rohini). While cluster II had rest of the genotypes which grouped together in their respective subcluster.

Ten ISSR primers selected in the present study gave 71.56% polymorphism. The highest polymorphism percentage was observed with one primer A17898. Therefore, this ISSR primer can be used further for diversity study in different mustard genotypes.

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