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SCoT Markers Analysis for Genetic Diversity in Indian Mustard (*Brassica juncea* L.)

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ABSTRACT

The present study was conducted during *rabi* (December, 2022–April, 2023) at the Integral Institute of Agricultural Science and Technology (IIAST), Integral University, Lucknow, India to assess genetic diversity among ten Indian mustard (*Brassica juncea* L.) genotypes using Start CodonTargeted (SCoT) markers. Ten SCoT primers were screened, and PCR amplification was performed using a programmable thermocycler (Prima-96, Hi-Media, India). The reaction mixture for SSR-PCR included 6 µl of DNA template (15 ng μl^{-1}), 1 µl each of forward and reverse primers, 10 µl of 1X Hi-Chrome master mix, and 2 µl of double-distilled water, totaling 20 µl. High-quality DNA was used for amplification, and primers were selected based on banding patterns for further characterization. Polymorphism information content (PIC) values ranged from 0.26 to 0.37, with an average of 0.31. Primer 8(45) exhibited the highest PIC value (0.37), indicating its effectiveness in detecting genetic diversity, followed by Primer 5(42) and Primer 7(44) (both 0.35). The UPGMA dendrogram, generated using Primer SCoT37, revealed two major clusters: Cluster 1 (PM-31,Urwashi, Maya) and Cluster 2 (NDR8501, Varuna, NDYR-8, RH-749, Pitambari, Rohini, Azad-Mahak). PM-31 and Urwashi showed the closest genetic relationship (distance: 16.0), while Azad-Mahak was the most genetically distinct genotype (distance: 32.2). These findings highlighted the utility of SCoT markers in assessing genetic diversity and relationships among mustard genotypes, providing valuable insights for breeding programs and conservation genetics.

KEYWORDS: Genetic diversity, SCoT, markers, mustard, UPGMA

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Data Availability Statement: Legal restrictions are imposed on the public sharing of raw data. However, author have full right to transfer or share the data in raw form upon request subject to either meeting the conditions of the original consents and the original research study. Further, access of data needs to meet whether the user complies with the ethical and legal obligations as data controllers to allow for secondary use of the data outside of the original study.

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1. INTRODUCTION

Indian mustard (*Brassica juncea* L.), a member of the Brassicaceae family, is one of the most economically important oilseed crops in India, contributing significantly to the country's edible oil production and agricultural economy (Yadava et al., 2011). It is valued for its high oil content (35–45%), nutritional quality, and adaptability to diverse agro-climatic conditions (Chauhan et al., 2012). Despite its importance, the genetic improvement of Indian mustard has been hindered by limited genetic diversity, susceptibility to biotic stresses such as *Alternaria blight* and *Sclerotinia* stem rot, and abiotic stresses like drought and salinity (Prasad et al., 2021). Molecular markers have revolutionized the field ofplant genetics byenabling the precise assessment of genetic diversity at the DNA level, by passing the limitations of phenotypic characterization (Kumar et al., 2016). Genetic diversity analysis provides a foundation for the selection of superior parents, development of hybrids, and conservation of germplasm resources (Singh et al., 2021a). Traditional morphological markers are influenced by environmental factors and often fail to reveal true genetic variation (Shekhawat et al., 2022). Hence, molecular marker systems such as RAPD, SSR, AFLP, and gene-targeted markershave been widely employed for accurate and reliable assessment of genetic diversity in *Brassica juncea* (Sharma et al., 2020). Recent studies have demonstrated significant polymorphism among Indian mustard genotypes using molecular markers, indicating substantial genetic variability that can be utilized in breeding programs (Singh et al., 2022a). Among the available marker systems, Start Codon Targeted (SCoT) markers have gained considerable attention due to their simplicity, reproducibility, and gene-targeted nature (Singh et al., 2021b). These markers are designed based on the conserved regions flanking the ATG start codon in plant genes, thereby targeting functional regions of the genome. SCoT markers are highly effective in detecting polymorphism and have been successfully applied in various crops for genetic diversity, population structure, and phylogenetic studies (Singh et al., 2022b). Start Codon Targeted (SCoT) markers have gained attention due to their simplicity, reproducibility, and ability to target functional gene regions associated with agronomically important traits (Collard and Mackill, 2009). SCoT markers amplify regions near the start codon of genes, which are often linked to conserved functional domains and regulatory elements, making them particularly useful for genetic diversity studies and marker-assisted breeding (Xiong et al., 2011). SCoT markers have been successfully employed in various crops, including wheat, rice, and other Brassica species, due to their high polymorphism and ability to reveal functional genetic variation (Guo et al., 2012). In Indian mustard, SCoT markers have shown promise in identifying genetic diversity and population structure, as well as in distinguishing closely related genotypes. Till date, several type of DNA-based marker systems were used for evaluation of genetic diversity in Indian mustard germplasm accessions including random amplified polymorphic DNA (RAPD) (Khan et al., 2008;), inter simple sequence repeat (ISSR) markers (Huangfu et al., 2009; Yadav and Rana, 2012), restriction fragment length polymorphism (RFLP) (Mir et al., 2015), amplified fragment length polymorphism (AFLP) (Srivastava et al., 2001; Qi et al., 2008). In this context, the present study aimed to analyze the genetic diversity among Indian mustard genotypes using SCoT markers, which can provide valuable insights into genetic relationships, assist in germplasm characterization, and support the development of improved cultivars.

2. MATERIALS AND METHODS

The present study was conducted during *rabi* (December, 2022–April, 2023) at the Integral Institute of Agricultural Science and Technology (IIAST), Integral University, Lucknow, India

2.1. Molecular diversity assessment by SCoT markers

Initially, a total of ten SCoT primers were compiled from the searched literature and were screened. A brief description of SCoT primers used in the present investigation was listed in Table 1.

Table 1: Primer details

Sl. No.	Primer Name	Sequence ('5-3')	Tem-perature (°C)	GC-content
1.	SCoT37	CAATGGCTAC-CACTAGCC	45	55.56%
2.	SCoT38	CAATGGCTAC-CACTAACG	43	50%
3.	SCoT39	CAATGGCTAC-CACTAGCG	45	55.56%
4.	SCoT40	CAATGGCTAC-CACTACAG	43	50%
5.	SCoT42	CAATGGCTAC-CACTGACA	43	50%
6.	SCoT43	CAATGGCTAC-CATTAGCG	43	50%
7.	SCoT44	CAATGGCTAC-CACCGCAG	47	61.11%
8.	SCoT45	CAATGGCTAC-CATTAGCC	43	50%
9.	SCoT46	ACAATGGGTAC-CACTGAC	43	50%
10.	SCoT47	ACAATGGCTAC-CACTGAG	43	50%

2.2. DNA extraction protocolusing XPLORGEN universal kit

First, 1 ml of XUN 1 was added to the beaded vial, and the sample was then introduced into the vial containing XUN 1. The vial was horizontally vortexed at maximum speed for 10 min. Subsequently, 300 µl of XUN 2 solution was added, and the vial was again vortexed horizontally at maximum speed for 7 min. The tube was then centrifuged at 10,000 rpm for 3 min at room temperature. After centrifugation, 800 µl of the supernatant was transferred to a sterile 2 ml vial, and 200 µl of XUN 3 solution was added followed by vortexing for 5 s. The mixture was centrifuged again at 10,000 rpm for 2 min, and 800 µl of the supernatant was transferred to a clean sterile 2 ml vial. Next, 1 ml of XUN 4 solution was added to the supernatant and vortexed for 5 s. Then, 700 µl of lysate was transferred to a spin column (without discarding the residual lysate) and centrifuged at 10,000 rpm for 2 min, after which the flow-through was discarded. This step was repeated to ensure that the entire lysate was processed through the spin column. Subsequently, 600 µl of XUN 5 was added to the spin column and centrifuged at 10,000 rpm for 2 minutes, and the flow-through was discarded. This was followed by the addition of 600 µl of XUN 6 to the spin column and centrifugation at 10,000 rpm for 2 min, after which the flow-through was again discarded. The empty spin column was then centrifuged for 5 min at 10,000 rpm. Afterward, the spin column was placed into a sterile 1.5 ml vial, and 30 µl of XUN 7 (Elution 1) was added to the center of the column and centrifuged for 5 min at 10,000 rpm. Finally, the spin column was placed into a new sterile 1.5 ml vial, discarded, and both elution tubes were stored for further processing.

The gel analysis procedure was carried out by first loading the image into the software, after which the image was edited as necessary by rotating or cropping, and the current view was saved. Lanes were then assigned either automatically or manually for each column, and required parameters were provided where necessary. The "Detect" button was used to identify bands in each column, with options to add or delete bands as needed. The lanes corresponding to weight markers in the gel image were selected, followed by the selection of an existing standard or the definition of a new one, ensuring that bands were entered in decreasing order of molecular weight. The newly defined standard was saved as a marker file in the "standards" directory for future use. The "Compute" button was then used to calculate the molecular weight of all fragments, and the results were saved using the "Export" button and displayed using the "View" button. Subsequently, the "Match" button was used to compute clusters of matched bands, and band matching was adjusted by modifying the "distance" parameter, which determines whether two bands belong to the same cluster. The "Edit" options were used to refine clustering by moving bands between clusters or adding/removing clusters. The resulting matrix was exported in either 1/0 or +/- format. Finally, a suitable method for tree computation was selected, the tree was displayed using the "View" button, distance labels were optionally enabled to show genetic distances on branches, and lane labels were edited using the "Edit Lane Labels" option to rename the analyzed population, with changes confirmed by viewing the updated tree.

2.3. RNase treatment (Purification of DNA)

For the removal of RNA contamination, DNA pellets were treated with4 µl of RNase solution (10 mg ml^{-1}) and incubated in a water bath at 37 °C for1hr. RNase treatment was a widely used method to degrade RNA contaminants in DNA samples, ensuring the purity of the DNA for downstream applications (Green and Sambrook, 2012). After incubation, the tubes were removed, and the pellets were air-dried at room temperature. The pellets were then dissolved in 1XTE elution buffer (100 µl), which helped stabilize the DNA by maintaining a neutral pH and chelating any divalent cations that could degrade the DNA (Sambrook and Russell, 2001). The samples were stored at -20°C until further use, as freezing at this temperature preserves DNA integrity for long-term storage (Ausubel et al., 2003).

2.4. Quantitative estimation of DNA

The quantity and purity of the DNA samples were measured at 260 nm using Nano drop Spectrophotometer (Eppendorf, Germany).For UV spectroscopy, an aliquot of DNA samples was suitably diluted and absorbance (A) was determined at 260 nm and 280 nm wavelength in spectrophotometer. Using the relationship of O.D. unit of

1.0 at 260 nm wavelength equivalent to 50 g DNA ml^{-1} the quantity of DNA was estimated from the following formula: Concentration of DNA (gml^{-1})=A260×50×dilution factor. The DNA concentrations were checked by visual assessment of band intensity in 0.8% agarose gel. The final DNA concentration of each sample was adjusted to 15 ng μl^{-1}.

2.5. Polymerase chain reaction (PCR) optimization and amplification

PCR amplification was carried out in programmable thermo-cycler Prima-96 (Biokart Genomic Lab, India: Reference Number: BKSANG8038). The reaction mixture for SSR-PCR Equipment's Utilized in Molecular Analysis consisted of 6 µl of working solution of DNA template (15 ng μl^{-1}), 1 µl each of forward and reverse primer, 10 µl of 1X Hi-Chrome master mix (containing dNTP, $MgCl_2$, PCR buffer, Taq DNA polymerase) and 2 µl of double distilled water, making total volume of 20 µl to optimize the conditions of the Polymerase Chain Reaction (Table 2, 3 and Figure 1).

Table 2: Polymerase chain reaction (PCR) optimization and amplification

Step	Time	Temperature	Cycle
Initial denaturation	5 min	94°C	45
Denaturation	30 sec	94°C	
Annealing	1 min	50°C	
Extension	1 min 30 sec	72°C	
Final extension	7 min	72°C	

Table: 3: Chemical components required for PCR

Component	Volume
DNA	2 µl
Primer	1 µl
dNTPs (2.5nMeach)	4 µl
10X Taq DNA polymerase assay buffer	7 µl
Taq DNA polymerase enzyme (3U ml^{-1})	1 µl
Water	10 µl
Total reaction volume	

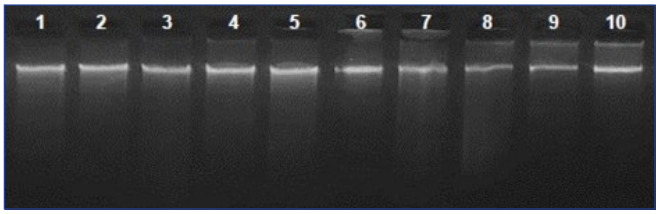


Figure 1: Biokart genomic lab, India: Reference Number: BKSANG8038: Band Images of ten Parents (Genomic DNA)

2.6. Procedure

Amplified DNA fragments along with 100 bp ladder (Hi-Media Laboratories) were observed using submerged horizontal electrophores was in 2.0% (w/v) agarose geland visualized by staining with ethidium bromide. Agarose solution was prepared in 1X TBE (Sambrook and Russell, 2001) by melting in a microwave. Ethidium bromide solution (10 mg ml^{-1}) was added to the gel (at 10 µl 100 ml^{-1} of gel) and mixed gently (Sambrook and Russell, 2001). The gel was poured into a casting plate with an appropriate comb to create wells. DNA samples were loaded using a 20 µl micropipette. Electrophoresis was performed using 1X TBE as running buffer at constant voltage (≈100 $Vcm^{-1}$of gel, 450 mA current) for 50 min (Weising et al., 2005). The amplicons were visualized under UV light and photographed using a gel documentation system (Biokart Genomic Lab, India 2023c). Band profiles were scored visually to assess genetic variation (Peakall and Smouse, 2006). Each amplified product was considered a DNA marker/allele and scored across all samples. Bands were converted in to a binary matrix ('1'forpresence, '0'forabsence). Poorly resolved bands or non-amplified templates were scored as missing data. Molecular weights were estimated using a 100 bp DNA ladder (Hi-Media Laboratories).

2.7. Dendrogram

The matrix of similarity coefficient was subjected to unweighted pair group method for arithmetic mean (UPGMA) to generate a dendrogram using average linkage procedure. The standard data matrix was used to calculate correlations using average among variables. The relation between genetic similarity identified by SCoT and taxonomic distance measured by mean genetic distance was analyzed using Jaccard's similarity index and average taxonomic distance calculated by NTSYS-pc v2.1.

2.8. Polymorphism information content (PIC) Value

PIC value for self-pollinated species suggested by Botstein et al. (1980) and modified by Anderson et al. (1993) was used to refer to the relative value of each marker with respect to the amount of polymorphism exhibited. PIC values for each primer were estimated by using the given formula. PIC=1−Σpi2, Where, pi was the frequency of the i^{th} allele PIC was synonymous with the term 'genediversity' as described by Weir (1990). The PIC takes into account not only the number of alleles that were expressed but also, the relative frequencies of those alleles (Smith et al., 1997). Marker index (MI) signifying the overall utility of the marker system for the diversity study, was calculated using the formula MI=EMR×PIC (Anderson et al., 1993; Varshney et al., 2007). Where, the effective multiplex ratio (EMR) was the product of proportion of polymorphic markers (β) and the total number of polymorphic obtained fragments (n) (Milbourne et al., 1997; Prevost et al., 1999 and Powell et al., 1996). Resolving power (Rp) was the summation of the Band in formativeness (Ib) was given by [Ib=1−[2×|0.5−p|], where p was the proportion of the total genotypes containing the band (Prevost et al., 1999).

3. RESULTS AND DISCUSSION

3.1. Genetic diversity analysis based on SCoT markers

Several marker systems and their combination technologies have been used by various researchers to analyse the genetic diversity in mustard. In the past, most of genetic diversity studies in mustard have been performed only using morphological traits but very limited numbers of reports were available either on the use of molecular markers such as SCoT markers or both morphological and molecular markers for the assessment of genetic diversity in mustard. Now a days DNA based markers were routinely used for analysing genetic diversity in most of crop improvement programmes. SCoT (Start Codon Targeted) markers were valuable tools in molecular diversity studies due to their

unique features and advantages. These markers used a single primer system based on conserved regions around the translation initiation codon, ensuring high reproducibility and ease of use across different species. They were efficient in detecting polymorphisms, offering high polymorphism information content which was essential for assessing genetic diversity. SCoT markers were cost-effective and did not require prior sequence information, making them accessible and suitable for a wide range of plant species, including those with limited genomic data. Additionally, their association with coding sequences provided insights into functional genetic diversity, making them particularly useful in marker-assisted selection and breeding programs aimed at improving specific traits. These attributes collectively make ScoT markers indispensable for accurate, efficient, and comprehensive genetic diversity studies.

3.2. Characterization of SCoT (start codon targeted) markers

In the present study a total of ten SCoT primers were taken initially for screening or random characterization with DNA samples often genotypes of mustard. High quality DNA was used for SSR amplification. Working concentration of 15ngµl^{-1} was used to amplify all the primers. On the basis of banding patterns, primers were selected for characterization in the entire set of ten genotypes/varieties of mustard. A total often SCoT primers which were thoroughly distributed and also in the conformity with the findings of other reported mustard genomes were selected for the advance study.

3.3. Molecular marker analysis

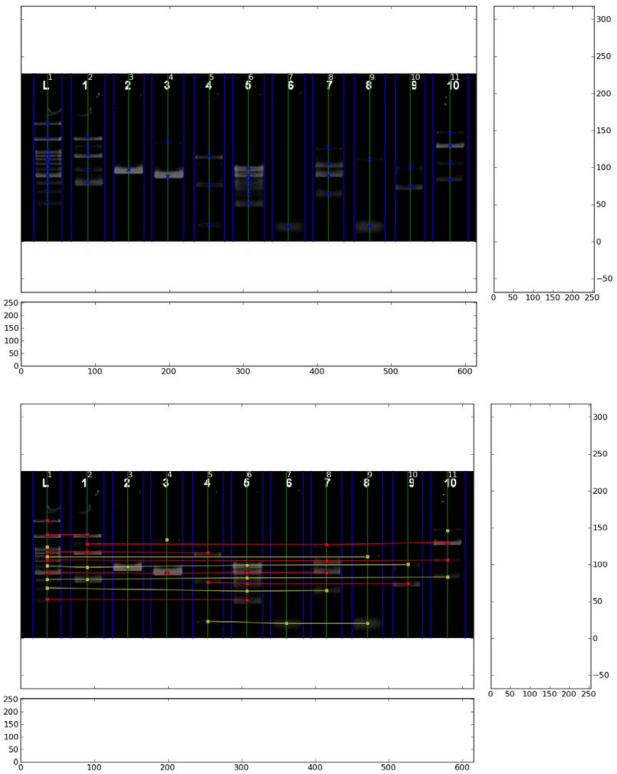
DNA based molecular markers were important tools in breeding programme for crop improvement. The main role of these makers was to detect polymorphism which could be used for qualitative or quantitative traits loci, diversity, pedigree analysis, assess taxonomic, phylogenetic relationships and linkage mapping etc. SSR were polymerase chain reaction (PCR) based markers, helped in the detection of polymorphisms at the molecular level from many individuals or pooled samples at a very fast rate. Good quality DNA free from contaminants, with concentration for all genotypes and optimization of PCR reaction conditions were certain prerequisites which were required for this technique, otherwise could result inerratic amplification. Keeping all this view in mind, the present investigation was undertaken to standardize the DNA isolation methods, optimize the PCR reaction conditions to study polymorphism among the all genotypes of mustard using SSR markers. The polymorphic data was used to find heterozygosity among genotypes. (Shrivastav et al., 2023) assess the genetic diversity of 75 Indian mustard genotypes and results revealed positive amplification for all SSRs, with 21 SSRs exhibiting polymorphic amplicons (Table 4).

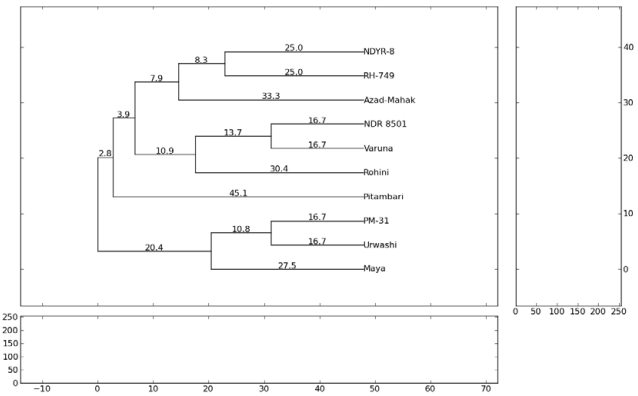
3.4. Polymorphic information content (pic) and statistical analysis for ScoT marker

The level of polymorphism among genotypes was evaluated by calculating allelic number and polymorphic information content (PIC) values for each of the ten evaluated loci (Botstein et al., 1980). The banding patterns of PCR products for different primers were shown in Figure 2. PIC values provided an estimate of the discriminatory power of a locus by accounting for both the number of alleles and their relative frequencies within the population (Liu and Muse, 2005). In this study, PIC values ranged from 0.26 to 0.37, with an average of 0.31. The highest PIC value was recorded for Primer 8(45) (0.37), followed by Primer 5(42) and Primer 7(44)(both 0.35). These findings suggested that Primer 8(45) was the most effective in detecting genetic diversity within the sample population, making it particularly valuable for genetic mapping and population

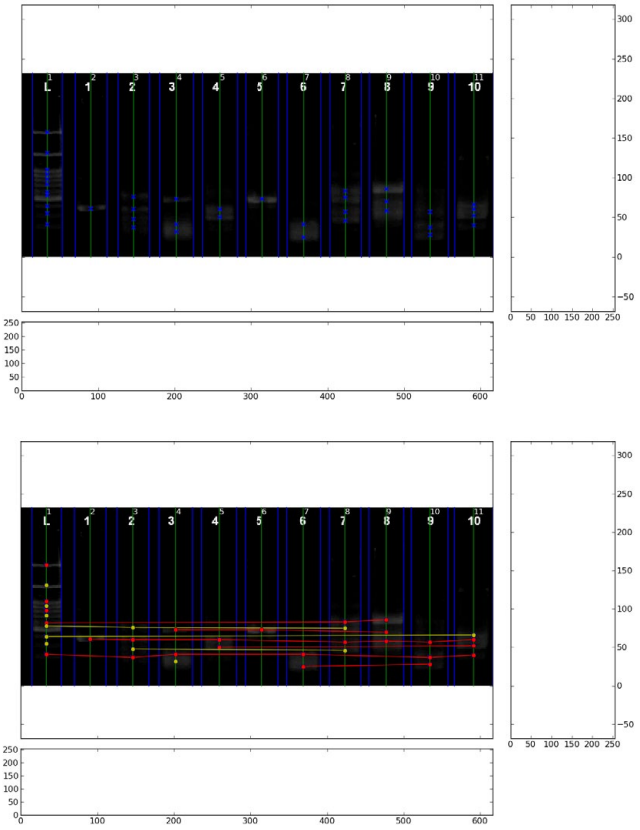
Table 4: Biokart genomic lab, India: Reference number: BKSANG8038: band counts

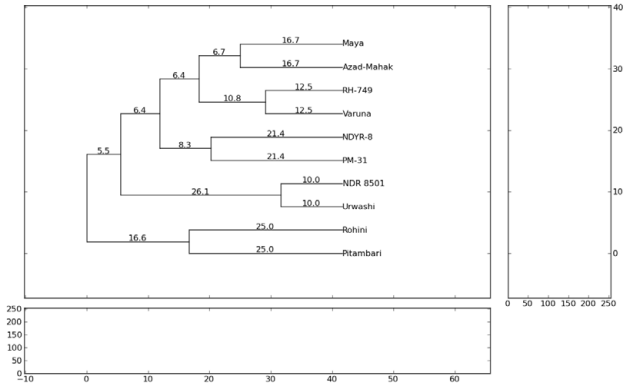
Primer	No. of bands										Total
	Azad-Mahak	Varuna	Pitambari	Maya	Rohini	Urwashi	RH-749	PM-31	NDR 8501	NDYR- 8	
Primer_1(37)	5	1	2	3	6	1	4	2	2	4	30
Primer_2(38)	1	4	3	2	1	2	4	3	3	4	27
Primer_3(39)	4	4	1	5	5	4	4	3	3	4	37
Primer_4(40)	7	1	1	5	3	4	2	4	2	4	33
Primer_5(42)	5	1	4	3	4	4	4	3	4	11	43
Primer_6(43)	8	4	1	5	6	4	6	3	3	4	44
Primer_7(44)	6	3	5	6	5	4	5	4	4	6	48
Primer_8(45)	6	6	2	4	4	4	5	4	5	6	46
Primer_9(46)	3	4	3	4	2	2	3	5	4	3	33
Primer_10(47)	4	2	4	3	4	3	4	4	4	3	35





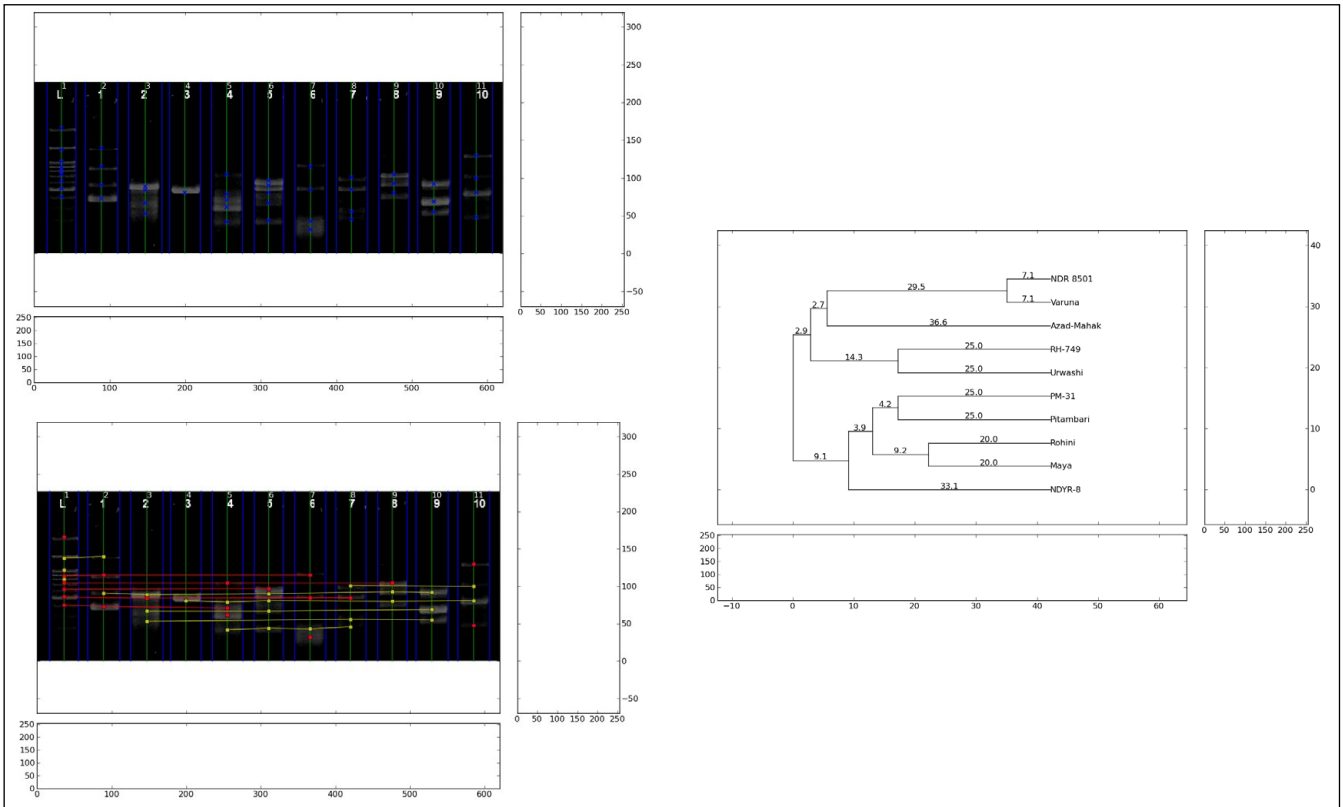
PCR amplified image of sample 1



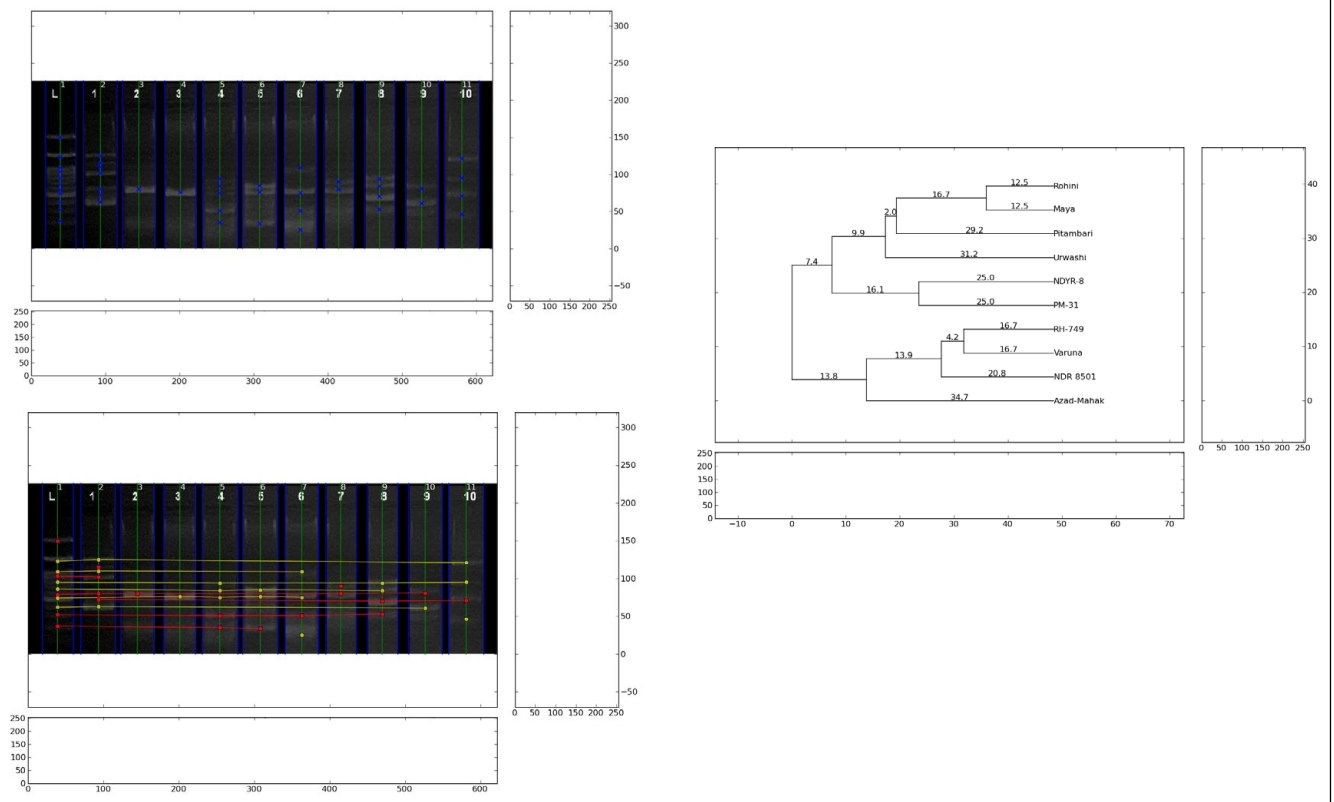


PCR amplified image of sample 2

Figure 2: Continue...

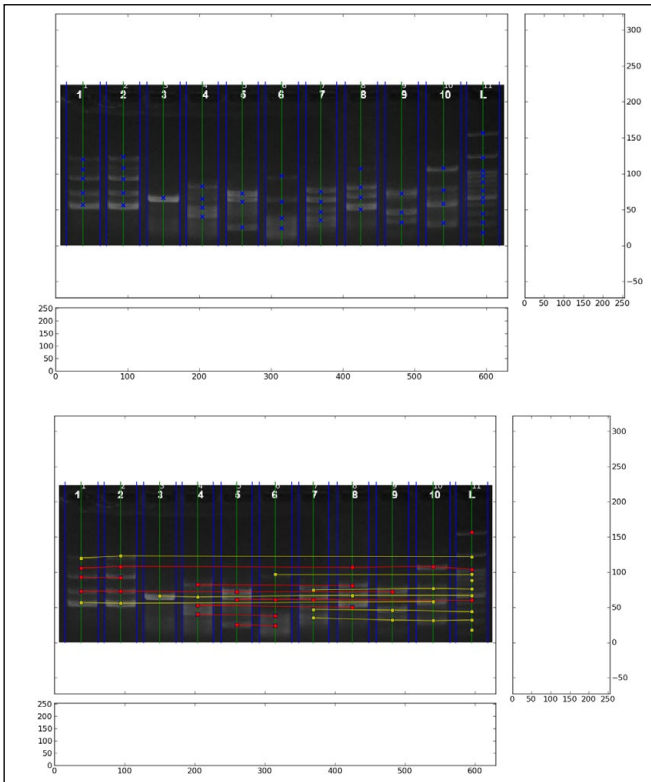


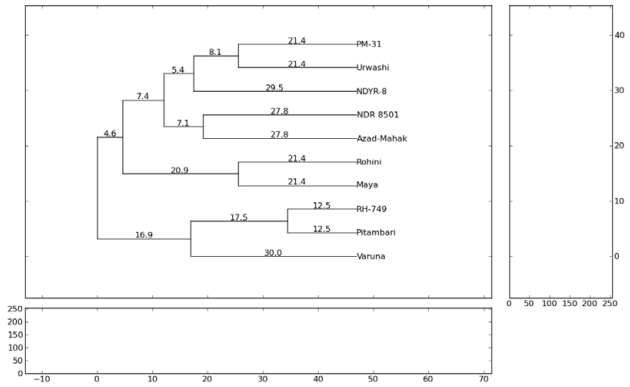
PCR amplified image of sample 3



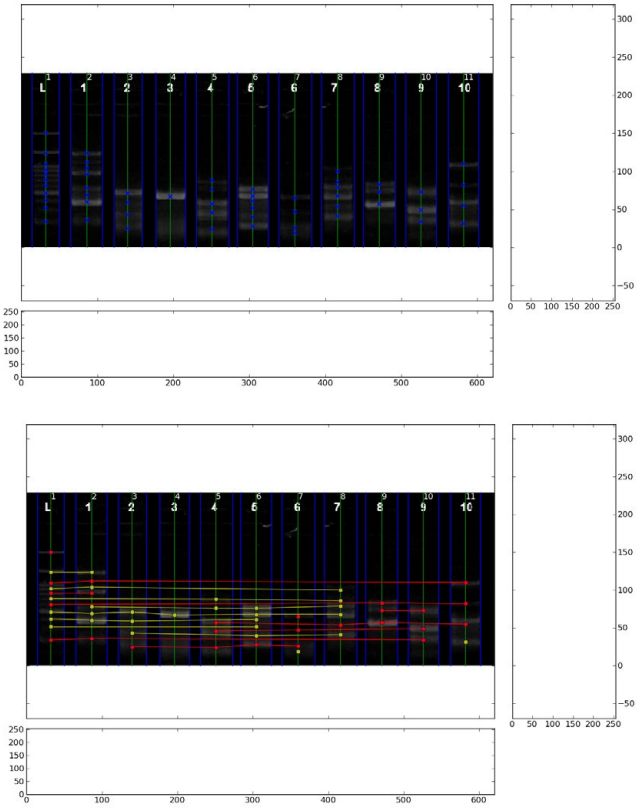
PCR amplified image of sample 4

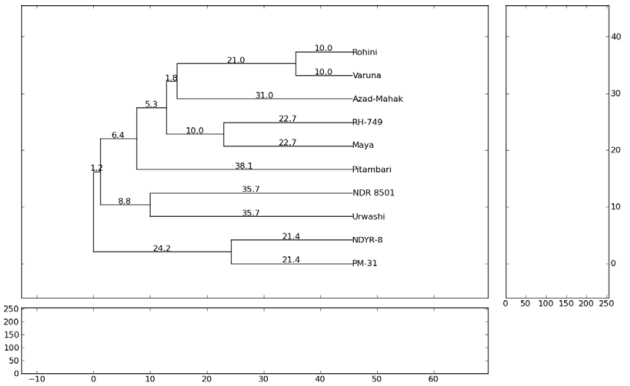
Figure 2: Continue...





PCR amplified image of sample 5





PCR amplified image of sample 6

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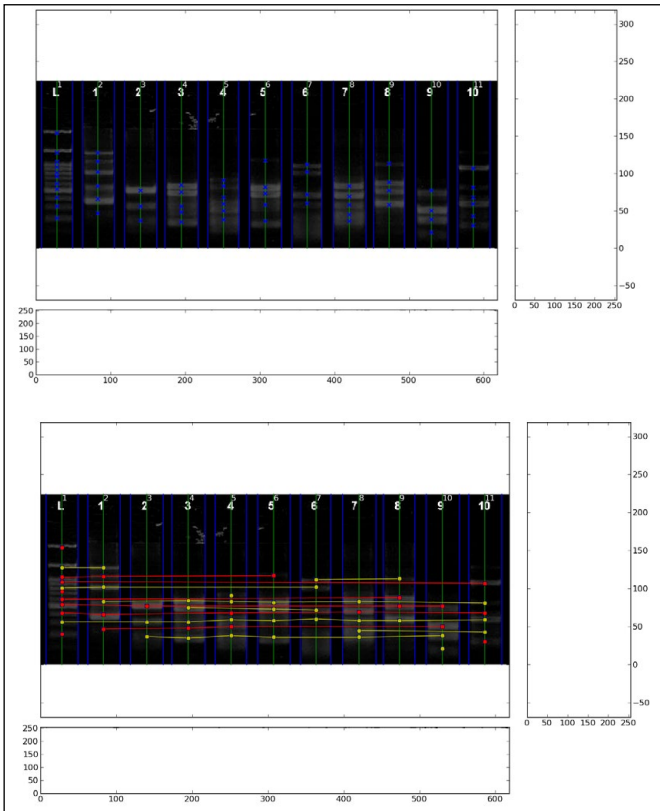
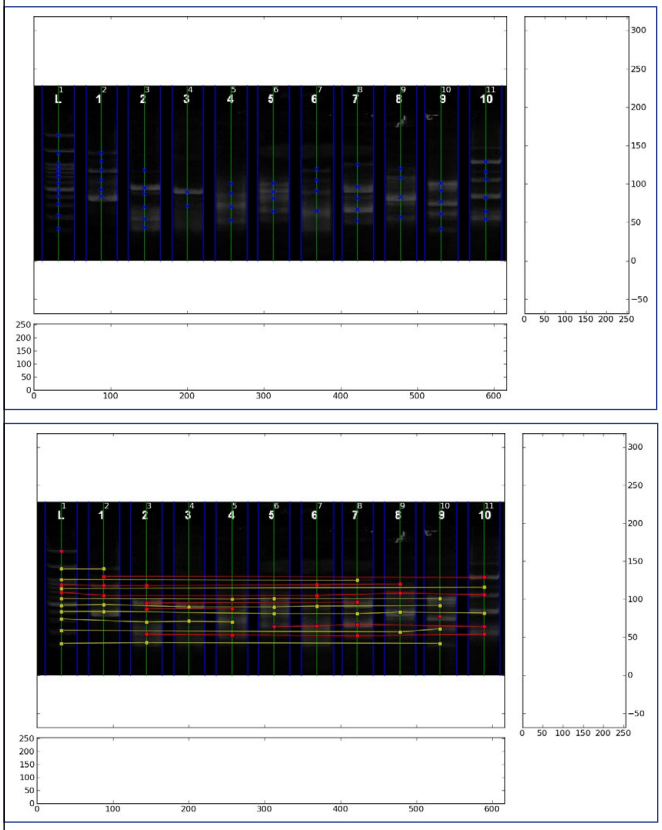
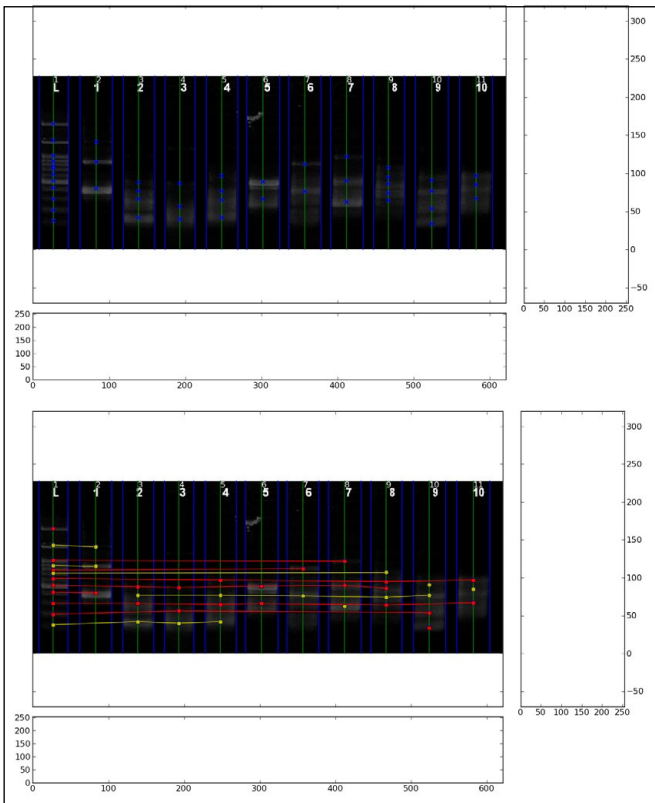


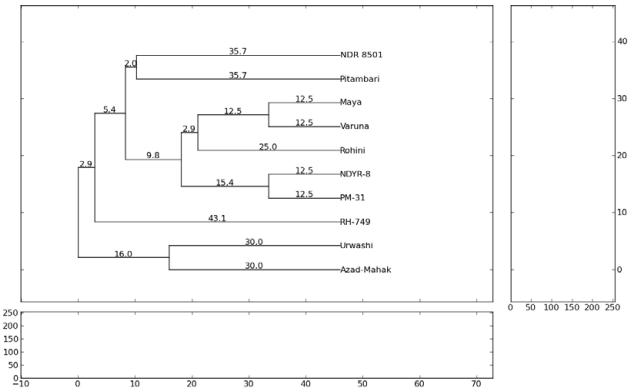
PCR amplified image of sample 7



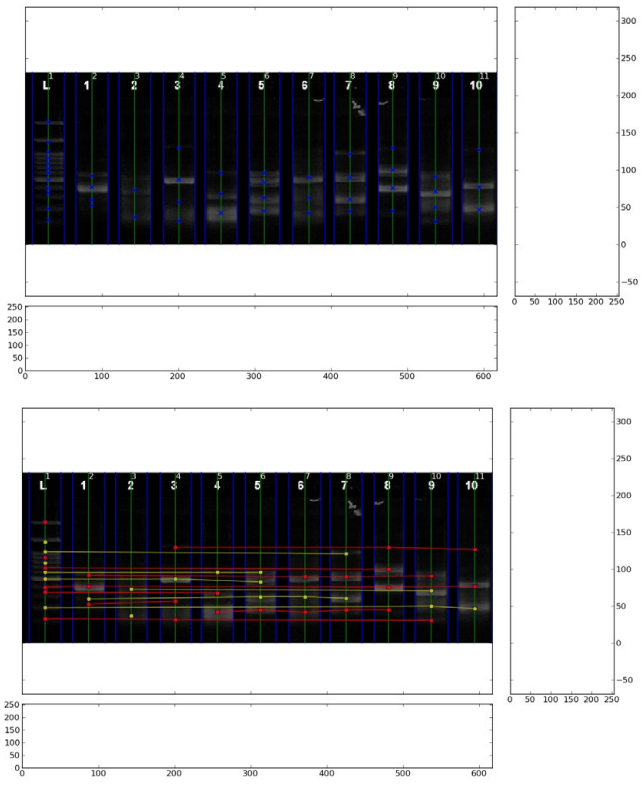
PCR amplified image of sample 8

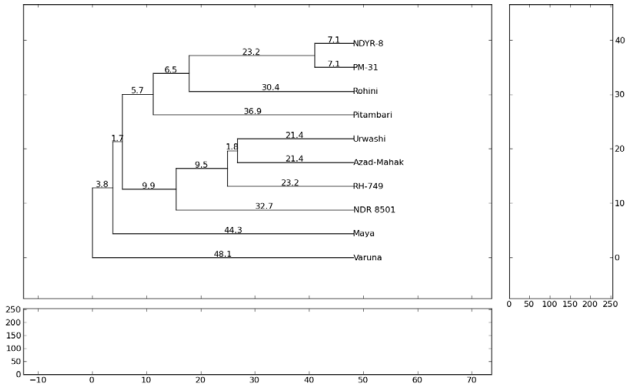
Figure 2: Continue...





PCR amplified image of sample 9





PCR amplified image of sample 10

Figureno 2: Biokart Genomic Lab, India: Reference Number: BKSANG8038: Genetic distance analysis through UPGMA method

genetics studies (Weising et al., 2005).The balanced allele frequencies and high PIC value of these primers indicated their reliability and informativeness for genetic analyses, which was crucial for applications such as breeding programs (Allard, 1999) and conservation genetics (Frankham et al., 2010). (Biokart Genomic Lab, India: Reference Number: BKSANG8038) (Table 5).

3.5. Genetic distance analysis through UPGMA method

3.5.1. Cluster distance analysis from UPGMA tree (Primer 1-SCoT37)

The UPGMA dendrogram generated using Primer SCoT 37 (Collard and Mackill, 2009) revealed the genetic relationships among 10 mustard genotypes, dividing them into two major clusters (Sokal and Michener, 1958). Cluster 1 comprises PM-31, Urwashi, and Maya, while Cluster 2 included NDR 8501, Varuna, NDYR-8, RH-749, Ptambari, Rohini, and Azad-Mahak. Within Cluster 1, PM-31 and Urwashi exhibited the closest genetic relationship, with a cluster distance of 16.0, indicating high similarity (Nei, 1972). PM-3 1and Maya showed moderate genetic divergence, with a distance of 17.3. In Cluster 2, NDR 8501 and Varuna demonstrated a relatively close relationship, with a distance of 12.6. However, RH-749 and Pitambari displayed the largest genetic divergence within this cluster, with a distance of 46.5 (Perrier and Jacquemoud-Collet, 2006). Notably, Azad-Mahak stands out as the most genetically distinct genotype in the group, with a large distance of 32.2, suggesting unique allelic variation (Kumar et al., 2016).

3.1.2. Cluster distance analysis from UPGMA tree (Primer 2-SCoT38)

The UPGMA dendrogram generated using Primer SCoT38 revealed two distinct clusters among the 10 mustard genotypes studied. Cluster 1 comprised Maya, Azad- Mahak, RH-749, and Varuna, while Cluster 2 included NDYR-8, PM-31, NDR 8501, Urwashi, Rohini, and Pitambari (Agarwal A, et al. 2019). Within Cluster 1, Maya and Azad-Mahak exhibited the closest genetic relationship, with a cluster distance of 16.7, indicating highsimilarity. RH-749 and Varuna showed moderate genetic divergence, with a distance of 12.5 (Chhajer et al., 2017). In Cluster 2, PM-31and NDR 8501 demonstrated the closest relationship, with a distance of 10.0, suggesting high genetic similarity. Conversely, Rohini and Ptambari displayed the largest genetic divergence within this cluster, with a distance of 25.0.

3.1.3. Cluster distance analysis from UPGMA tree (Primer 3-SCoT39)

The UPGMA dendrogram generated using Primer SCoT39 revealed three distinct clusters among the 10 mustard genotypes studied (Collard et al., 2009). Cluster 1 comprised NDR 8501 and Varuna, Cluster 2 included Azad-Mahak, RH-749, and Urwashi, while Cluster 3 contained PM-31, Pitambari, Rohini, Maya, and NDYR-8. Within Cluster 1, NDR 8501 and Varuna exhibited an exceptionally close genetic relationship, with a minimal cluster distance of 7.1, indicating high similarity. Incontrast, Cluster 2showed more genetic diversity, with Azad-Mahakand RH-749 displaying a substantial distance of 36.6, suggesting significant genetic dissimilarity. Cluster 3 demonstrated varying degrees of genetic relatedness among its members. PM-31 and Ptambari showed high genetic similarity with a small distance of 4.2, while Rohiniand Maya exhibited moderate divergence with a larger distance of 20.0.

3.5.4. Cluster distance analysis from UPGMA tree (Primer 4–SCoT40)

The UPGMA dendrogram generated using Primer SCoT40 revealed two distinct clusters among the studied mustard

Table 5: Biokart Genomic Lab, India: Reference Number: BKSANG8038: Allele and Polymorphism Information Content (PIC)

Primer	Present	Absent	Present frequency	Absent frequency	Allele frequency	PIC
Primer_1(37)	30	135	0.18	0.82	1	0.30
Primer_2(38)	27	149	0.15	0.85	1	0.26
Primer_3(39)	37	163	0.19	0.82	1	0.30
Primer_4(40)	33	132	0.20	0.80	1	0.32
Primer_5(42)	43	150	0.22	0.78	1	0.35
Primer_6(43)	44	165	0.21	0.79	1	0.33
Primer_7(44)	48	161	0.23	0.77	1	0.35
Primer_8(45)	46	140	0.25	0.75	1	0.37
Primer_9(46)	33	142	0.19	0.81	1	0.31
Primer_10(47)	35	162	0.18	0.82	1	0.29

genotypes (Ghobadi et al., 2021). Cluster 1 comprised Rohini and Maya, while Cluster 2 included Pitambari, Urwashi, NDYR-8, PM-31, and other genotypes.

Within Cluster 1, Rohini and Maya exhibited a close genetic relationship, with aminimal cluster distance of 12.5, indicating high similarity (Singh et al., 2021). In contrast, Cluster 2 showed more genetic diversity, with Pitambari and Urwashi displaying a larger distance of approximately 29.2, suggesting significant divergence within this cluster. Notably, Azad-Mahak stood as the most genetically distinct genotype in this analysis, with the largest distance of 34.7 from other members of the group.

3.1.5. Cluster distance analysis from UPGMA tree (Primer 5–SCoT42)

The UPGMA dendrogram generated using Primer SCoT42 revealed two distinct clusters among the studied mustard genotypes (Iqbal et al., 2015). Cluster 1 comprised PM-31, Urwashi, NDYR-8, and NDR8501, while Cluster 2 included Rohini, Maya, RH-749, Pitambari, and Varuna. Within Cluster 1, PM-31 and Urwashi exhibited the closest genetic relationship,with aminimal cluster distance of 5.4, indicating they shared many alleles. NDYR-8 and NDR 8501 showed moderate similarity, with a distance of 7.1. In Cluster 2, Rohini and Maya demonstrated moderate divergence with a distance of 21.4.RH-749 and Pitambari displayed high genetic similarity, having a smaller distance of 12.5. Notably, Varuna stands out as the most genetically distinct genotype in this analysis, with the largest distance of 30.0 from other members of the group.

3.1.6. Cluster distance analysis from UPGMA tree (Primer 6–SCoT43)

The UPGMA dendrogram generated using Primer SCoT43 revealed distinct clustering patterns among the studied mustard genotypes (Gupta et al., 2019). Rohini and Varuna exhibited the closest genetic relationship, with a minimal distance of 10.0, suggesting a shared breeding history or ancestry. Azad-Mahak showed moderate divergence from this pair, with a genetic distance of 31.0, indicating some shared traits but also distinct differences. RH-749 and Maya form another cluster with a moderate similarity, displaying a distance of 22.7. They were more divergent from the Rohini-Varuna group but still shared some common genetic markers. Pitambaristood as the most genetically distinct variety, with the highest genetic distance of 38.1,suggesting unique traits or a different lineage. NDR 8501 and Urwashi cluster together with identical genetic distances of 35.7, indicating high similarity to each other but significant divergence from other groups (Sudan et al., 2016). NDYR-8 and PM-31 form another cluster with a distance of 21.4, showing moderate similarity to each other but divergence from other clusters.

3.5.7. Cluster distance analysis from UPGMA tree (Primer 7–SCoT44)

The UPGMA dendrogram generated using Primer SCoT44 revealed clustering patterns that were similar yet slightly different compared to those observed with Primer SCoT43. RH-749 and Maya exhibited the closest genetic relationship, with a minimal distance of 13.6, suggesting that they shared significant portions of their genetic material. Interestingly, Pitambaric lusters closer to Rohini in this analysis, with a distance of 6.5, unlike the results from Primer SCoT43, where Pitambari was more divergent (Williams et al., 1990) this indicated that Primer SCoT44 amplified regions where Pitambari shared more similarity with Rohini than previously observed. Varuna, once again, clusters closely with Rohini, displaying a distance of 10.9, confirming its close genetic relationship to Rohini across both primers. Azad-Mahak remained moderately divergent from other varieties, with a distance of 34.8 from NDR 8501, suggesting that it possessed distinct genetic traits.

3.1.8. Cluster distance analysis from UPGMA tree (Primer 8–SCoT45)

The UPGMA tree constructed using Primer SCoT 45 revealed distinct clustering patterns among the rice varieties analyzed. Maya and Varuna formed a close cluster with identical genetic distances of 20.0, indicating their genetic similarity while remaining distinct from other groups (Thakur et al., 2021). Pitambari demonstrated significant divergence from the Maya-Varunacluster, with a genetic distance of 35.4, which aligned with its previously observed highly divergent nature. NDR 8501 emerged as the most genetically distinct variety in this dataset, exhibiting the highest genetic distance of 38.5. This analysis highlighted the genetic relationships and variations among these rice varieties, with Maya and Varuna showing close genetic similarity, Pitambari maintaining its divergent characteristics, and NDR 8501 standing out as the most genetically unique variety in the group.

3.1.9. Cluster distance analysis from UPGMA tree (Primer 9–SCoT46)

The UPGMA tree generated using Primer SCoT46 provided further insights into the genetic relationships among the rice varieties studied (Sharma et al., 2018). NDR 8501 and Pitambari exhibited identical genetic distances of 35.7, indicating a close genetic similarity between these two varieties while also highlighting their significant divergence from other groups in the analysis. Maya and Varuna form a tight cluster with identical distances of 12.5, suggesting that these two varieties share substantial genetic traits and likely have a close evolutionary relationship. RH-749 emerged as the most divergent variety in this analysis, displaying the largest genetic distance of 43.1. This high level of divergence suggested that RH-749 might possessed unique genetic

characteristics not shared by the other samples, potentially making it a valuable source of novel traits for breeding programs.

3.5.10. Cluster distance analysis from UPGMA tree (Primer 10–SCoT47)

The UPGMA tree constructed using Primer SCoT47 revealed key genetic relationships among the rice varieties analyzed. NDYR-8 and PM-31 demonstrated a remarkably close genetic relationship, with a minimal distance of 7.1, suggesting they shared a substantial portion of their genetic material. This similarity might be attributed to common ancestry or parallel breeding programs. Rohini showed a moderate clustering with NDYR-8 and PM-31, exhibiting a distance of 6.5, which indicated some shared genetic traits but also a degree of divergence from this pair (Park et al., 2021). Pitambari clusters significantly further from Rohini, with a distance of 30.4, highlighting its considerable genetic divergence from the Rohini group. Notably, Varuna stood as the most genetically distinct variety in this analysis, displaying the highest genetic distance of 48.1. This UPGMA clustering provided valuable insights into the genetic diversity and relationships among these mustard varieties, potentially informing future breeding strategies and genetic studies.

4. CONCLUSION

The study showed moderate to high polymorphism in Indian mustard, with PIC values of 0.26–0.37, confirming the effectiveness of SCoT markers. Primer 8(45) was most informative (PIC=0.37). UPGMA analysis grouped genotypes into two clusters: Cluster 1 (PM-31, Urwashi, Maya) and Cluster 2 (others). PM-31 and Urwashi showed close similarity, while Azad-Mahak was most divergent, making it useful for breeding.

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